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Spraying compound probiotics improves growth performance and immunity and modulates gut microbiota and blood metabolites of suckling piglets

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One factor that shapes the establishment of early neonatal intestinal microbiota is environmental microbial exposure, and probiotic application has been shown to promote health and growth of piglets. Thus, this study hypothesized that environmental probiotic application in early days of life would be beneficial to newborn piglets. This study aimed to investigate the effect of spraying a compound probiotic fermented liquid (CPFL) into the living environment of piglets on their early growth performance and immunity. This work included 68 piglets, which were randomized into probiotic and control groups. Blood and fecal samples were collected at 0, 3, 7, 14, and 21 days of age. Spraying CPFL significantly reshaped the microbiota composition of the delivery room environment, increased piglets' daily weight gain and weaning weight (P<0.001), and modulated piglets' serum cytokine levels (increases in IgA, IgG, and IL-10; decrease in IFN-γ; P<0.05 in each case) in piglets. Additionally, spraying CPFL during early days of life modified piglets' gut microbiota structure and diversity, increased the abundance of some potentially beneficial bacteria (such as *Bacteroides uniformis*, *Butyricimonas virosa*, *Parabacteroides distasonis*, and *Phascolarctobacterium succinatutens*) and decreased the abundance of *Escherichia coli* (P<0.05). Interestingly, CPFL application also significantly enhanced the gut microbial bioactive potential and levels of several serum metabolites involved in the metabolism of vitamins (B2, B3, B6, and E), medium/long-chain fatty acids (caproic, tetradecanoic, and peptadecanoic acids), and dicarboxylic acids (azelaic and sebacic acids). Our study demonstrated that spraying CPFL significantly could improve piglets' growth performance and immunity, and the beneficial effects are associated with changes in the gut microbiota and host metabolism. Our study has provided novel data for future development of probiotic-based health-promoting strategies and expanded our knowledge of probiotic application in animal husbandry.

compound probiotic fermented liquid, gut microbiota, immunity, piglets, serum metabolites

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INTRODUCTION

The structure and function of the gastrointestinal tract of piglets adapt to the transition from parenteral nutrition (via placenta) to enteral nutrition (colostrum/milk) quickly after birth (Pluske, 2016). This process is accompanied by dynamic colonization of microbes originated from the living environment and the vagina, skin, and feces of the mother sow (Cheng et al., 2019). Neonatal gut microbiome establishment has critical influence on piglets' growth and physical health, as it influences the development and maturity of the gastrointestinal tract, metabolic homeostasis, and immune defense via multiple host-microbe interactions (Wang et al., 2021b; Xiang et al., 2020). The gut microbiota of newborn piglets is less stable and is vulnerable to environmental stressors and various pathogens, causing intestinal dysfunction (Hu et al., 2019; Ren et al., 2022). Therefore, routine early probiotic intervention (e.g., with *Lactobacillus* spp. and *Bifidobacterium* spp.) during very early days of life might improve gut microbial colonization, as well as growth, development, and disease resistance of neonatal mammals (Xiang et al., 2020).

Previous studies have shown that probiotics can improve the growth performance, reduce the diarrhea rate, enhance immune function, and prevent diseases in farm animals (Deng et al., 2013; Gao et al., 2017). These beneficial effects are likely achieved through improving the integrity of the intestinal barrier and modulating the host gut microbiota promoting desirable bacteria and inhibiting the harmful ones (Cao et al., 2019; Dowarah et al., 2017). Changes in the gut microbiota composition also affect the contents of colonic metabolites, exerting biological effects on the host (Li et al., 2021b; Wang et al., 2019b). Several studies reported that probiotic supplementation beneficially modulated n-3 polyunsaturated fatty acids, fecal short-chain fatty acids (SCFAs), and serum low-chain fatty acids levels to promote piglet metabolism (Sopková et al., 2017; Zhang et al., 2019). Although many researchers have reported positive effects of using probiotics in pig farming, inferences drawn from different studies are largely inconsistent with rather low reproducibility (Barba-Vidal et al., 2018). More importantly, the underlying probiotic mechanism is not well understood, particularly the role of intestinal microbiota and metabolites in improving piglets' growth performance and immunity remains to be established. In addition, probiotic effects are known to be treatment-specific, largely depending on the applied probiotic strains and dose. On the other hand, host-related physiological and environmental factors may also influence the health-promoting effects of probiotic supplementation (Bosi and Trevisi, 2010). It is therefore necessary to evaluate the specific effect of candidate probiotics in animal husbandry.

Remarkably, supplementing multiple probiotic strains has been shown to exert synergistic beneficial effects on host health, immunity, pathogen defense, and other biological functions compared with single-strain supplementation (Chapman et al., 2011). Liu et al. (2017) found that the supplementation of two probiotic strains (*Lactobacillus casei* and/or *Enterococcus faecalis*) to piglets significantly increased the average daily gain compared with the control group. Moreover, the diarrhea rate of piglets was significantly reduced, while the protease activity in the stomach and colon of piglets increased (Liu et al., 2017). Another study found that complex probiotic formulation (*Lactobacillus reuteri* KT260178 and *Lactobacillus salivarius* MH 517354) was more effective than single-strain supplementation in promoting organic acid secretion and optimizing piglets' gut bacterial composition, enhancing symbiosis among gut microbes and applied probiotic strains (Yang et al., 2020). The probiotic strains, *Lactobacillus casei* Zhang (LCZ), *Lactobacillus plantarum* P-8 (P-8), and *Lactobacillus rhamnosus* Probio-M9 (Probio-M9), were originally isolated from koumiss, naturally fermented yoghurt, and human breast milk of a healthy woman, respectively. These strains have previously been reported to prevent *Escherichia coli* K88-induced jejunal epithelial damage to early-weaned piglets (Wang et al., 2019c), improve antioxidant capacity and reduce antibiotic-induced gut microbial dysbiosis in rats (Yao et al., 2021; Zhang et al., 2010), and accelerate gut microbiota maturation in broilers (Gao et al., 2017). In addition, they increased the contents of milk immunoglobulins and lactoferrin (Xu et al., 2017), enhanced the efficacy of mouse tumor immunotherapy (Gao et al., 2021), and suppressed colonic tumor formation by ameliorating inflammation (Xu et al., 2021). A complex probiotic mix containing these three strains has been used in multiple clinical trials, showing beneficial effects in maintaining the intestinal species diversity of long-term voyagers (Zhang et al., 2020a) and synergizing with conventional drug as adjuvant treatment of ulcerative colitis and irritable bowel syndrome (Chen et al., 2020; Xu et al., 2021).

This study hypothesized that very early environmental exposure of a mixed probiotic formulation to newborn piglets would enhance their growth performance and immune function through modulating the gut microbiota and blood metabolites. Thus, this study evaluated the health-promoting effects (piglet growth performance and immune function) of direct spraying of a compound probiotic fermented liquid (CPFL; containing LCZ, P-8, and Probio-M9) into the living environment of the mother sows and their newborn piglets. The second objective of this study was to analyze probiotic-driven changes in piglets' microbiomes and blood metabolite biomarkers along with animal health improvement. The present study has provided comprehensive reference information of probiotic application on the improvement of growth and development, disease resistance, and immune

maturity of suckling piglets, expanding our knowledge of managing newborn animals with probiotics-based therapy.

RESULTS

Spraying probiotic fermented liquid enhanced piglet growth performance

Firstly, the effects of spraying CPFL on sow reproductive performance were analyzed. No significant difference was detected in the healthy litter piglet rate, weak litter piglet rate, stillbirth rate, lactation survival rate, and mortality between probiotic and control groups (P>0.05; Table S1 in Supporting Information), suggesting that spraying CPFL had no observable effect on the litter characteristics. The daily weight gain and weaning weight were significantly higher in the probiotic group than the control group (P<0.001; Figure 1B), although there was no significant difference in the birth weight between the two groups. The diarrhea rate of piglets of the probiotic group was non-significantly (P=0.73, Table S1 in Supporting Information) lower than that of the control group. These results demonstrated that spraying CPFL in early life can improve piglet growth performance.

Spraying probiotic fermented liquid reshaped the environmental microbiota composition in delivery rooms

Metagenomic sequencing was performed on 33 samples collected in a total of 11 delivery rooms (five and six of the probiotic and control groups, respectively) at 0, 7, and 14 days of age. The overall microbiota comprised 14 genera and 22 species (each comprised >1% relative abundance; Figure S1A and B in Supporting Information). Significantly fewer *Clostridium perfringens* (P<0.05) and *Streptococcus suis* (P<0.03) were detected in the living environment of the probiotic group compared with the control group at all three time points, while an opposite trend was observed in *Lactobacillus plantarum* (P>0.05 at all three time points). The relative abundance of *Escherichia coli* was also significantly lower in the probiotic group compared with the control group at 0 days of age (P=0.01). Interestingly, significantly more *Lactobacillus rhamnosus* was found in the probiotic group than the control group at 7 and 14 days of age (Figure S1C in Supporting Information). These changes indicated that spraying CPFL in the delivery room is conducive to improving the microbial composition of the living environment of sows and piglets.

Spraying probiotic fermented liquid regulated piglet gut microbiota composition

To evaluate the impact of spraying CPFL on the intestinal microbiome of piglets, changes in the alpha diversity (represented by the Shannon diversity index) and beta diversity (principal coordinate analysis and Adonis test) were analyzed. As the suckling phase started, the Shannon index of the gut microbiota of all piglets significantly increased (P<0.001). However, at 7 days of age, the Shannon index of the gut microbiota of the probiotic group was significantly higher than that of the control group (P<0.05; Figure 2A). Interestingly, significant differences in the gut microbiota structure were only observed between the two groups at 3 (R^2=0.079, P=0.003), 7 (R^2=0.057, P=0.002), 14 (R^2=0.077, P=0.001), and 21 (R^2=0.063, P=0.001) days of age, but not at birth (R^2=0.043, P=0.064; Figure 2B), suggesting that the host gut microbiota modulatory effect of spraying CPFL occurs soon before farrowing and until after birth.

To explore how spraying CPFL affected the piglet gut microbiota, the species-level gut microbiota composition (12 major species of over 1% relative abundance) of the probiotic and control groups was compared (Figure 2C). To pinpoint specific changes in the gut microbiota of preweaning piglets at a finer level, responsive species-level genome bins (SGBs) were identified and tracked (Figure 2D). Responsive SGBs were defined as those showing no significant difference in abundance between two groups at 0 days of age but became differentially abundant afterwards. They were identified at all time points, but the smallest and largest number of responsive SGBs were found at 3 days of age (two responsive SGBs) and 14 days of age (20 responsive SGBs), respectively. Significantly more Firmicutes bacterium and *Phascolarctobacterium succinatutens* were detected in the probiotic group at 7, 14, and 21 days of age compared with the control group, while the abundance of *Escherichia coli* decreased significantly at 7 and 14 days of age (P<0.05). In addition, several taxa in the probiotic group, including *Bacteroides uniformis*, *Butyricimonas virosa*, *Parabacteroides distasonis*, *Eubacterium* sp., and *Gemmiger* sp., were significantly enriched at 7 days of age, while other taxa, including *Enterocloster aldensis*, *Enterocloster clostridioformis*, *Faecalicatena orotica*, and *Faecalimonas umbilicata*, were significantly depleted at 14 days of age, compared with the control group (P<0.05; Figure 2D). Significant differences were also observed in the carbohydrate-active enzyme (CAZy) gene profile between the probiotic and control groups (Figure S2A in Supporting Information), characterized by significantly more CAZy genes in the probiotic group than the control group at 7 days of age (Figure S2B in Supporting Information). These results showed that spraying CPFL could reshape the species-level composition of piglets' gut microbiota and its metabolic potential modestly by promoting some butyrate acid-producing bacteria, inhibiting potentially harmful bacteria like *Escherichia coli*, *Enterocloster aldensis*, and *Enterocloster clostridioformis*, and modulating the overall gut microbiota-encoded carbohydrate metabolic potential.

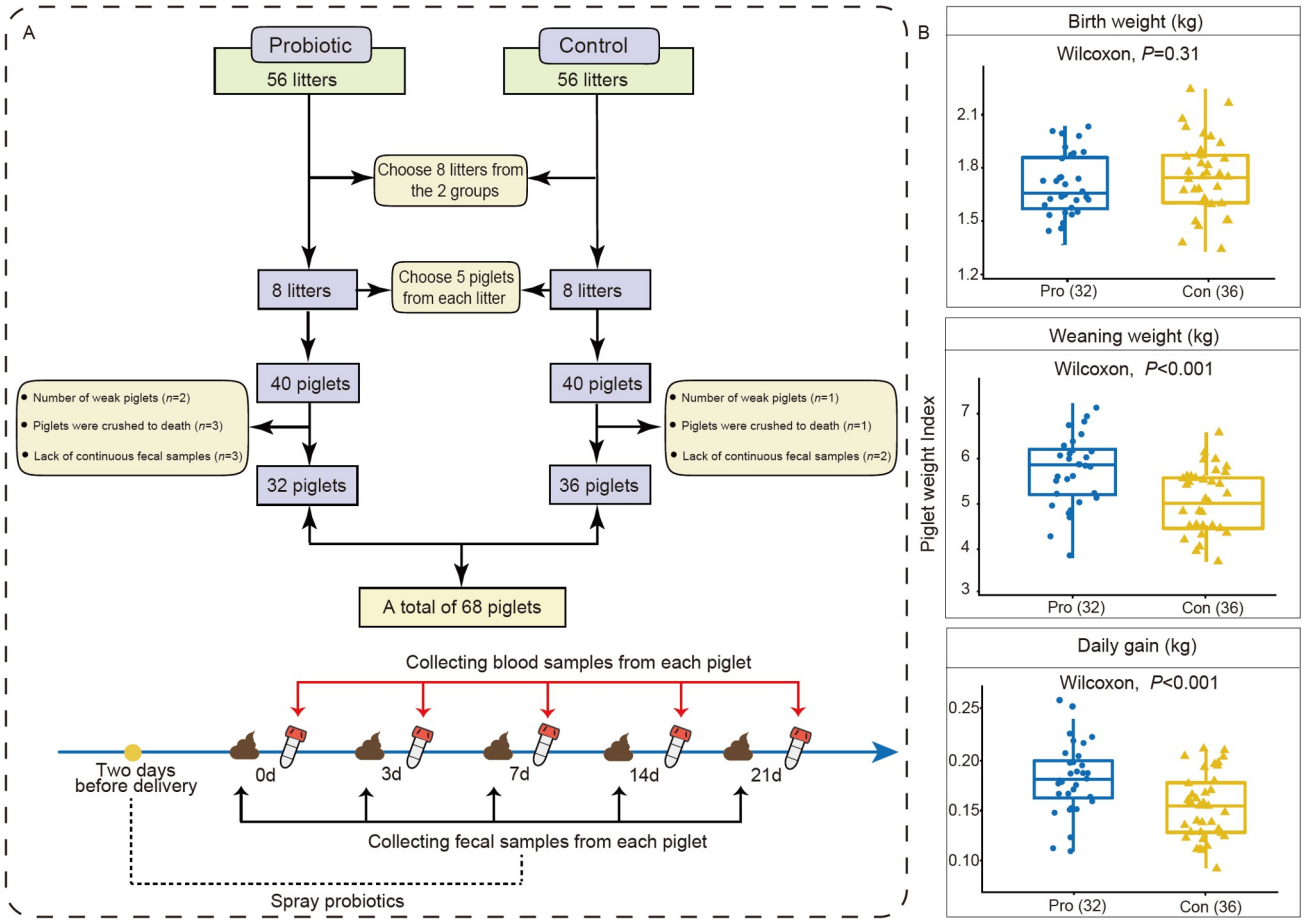

Figure 1 Experimental design and piglet growth performance. A, Piglet screening process, spraying of compound probiotic fermented liquid, and sample collection. A total of 68 piglets (n=32, 36 in the probiotic and control groups, respectively, after excluding accidentally died and weak piglets and those with missing samples). B, Comparison of birth weight, weaning weight, and daily weight gain between probiotic (Pro) and control (Con) groups by Wilcoxon test.

In addition, shotgun sequencing enabled us to track the supplemented probiotic strains, i.e., LCZ, P-8, and Probio-M9, in the fecal samples by mapping the clean reads of the metagenomic dataset to the reference genome of each strain. There was a gradual increase in genome abundance of these three probiotic strains in the probiotic group but not the control group at 3 and 7 days of age, suggesting that the ingested probiotic bacteria could easily pass through the upper gastrointestinal tract and be detected in piglets' intestine (Figure S2C in Supporting Information).

Spraying probiotic fermented liquid modulated piglets' serum immunoglobulin and cytokine levels

To reveal the effects of spraying CPFL on piglets' immunity, the serum immunoglobulin A (IgA), immunoglobulin G (IgG), interleukin-10 (IL-10), interleukin-6 (IL-6), tumoral necrosis factor-alpha (TNF-α), and interferon-γ (IFN-γ) levels were monitored. Compared with the control group, significantly more IgG (at most time points), IgA (at 14 and 21 days of age), and IL-10 (at 21 days of age) were detected in the sera of piglets in the probiotic group (P<0.05; Figure 3). In contrast, significantly less IFN-γ was detected in the probiotic group compared with the control group at 7, 14, and 21 days of age (P<0.05; Figure 3), showing a decreasing trend over time. In addition, the serum IL-6 level of piglets increased (especially at 7, 14, and 21 days of age versus 0 days of age; P<0.05) in the control group but not the probiotic group. The piglets' serum TFN-α level significantly decreased after CPFL treatment at 14 and 21 days of age compared with 3 days of age (P<0.05; Table S2 in Supporting Information). These results highlighted the effect of CPFL application on piglets' immunity.

Spraying probiotic fermented liquid modulated piglets' gut metabolites

A genome-centric metabolic reconstruction was conducted to identify changes in the gut metabolites encoded in the 387 SGBs along the trial using the MetaCyc and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. In the functional annotation, modules related with vitamins,

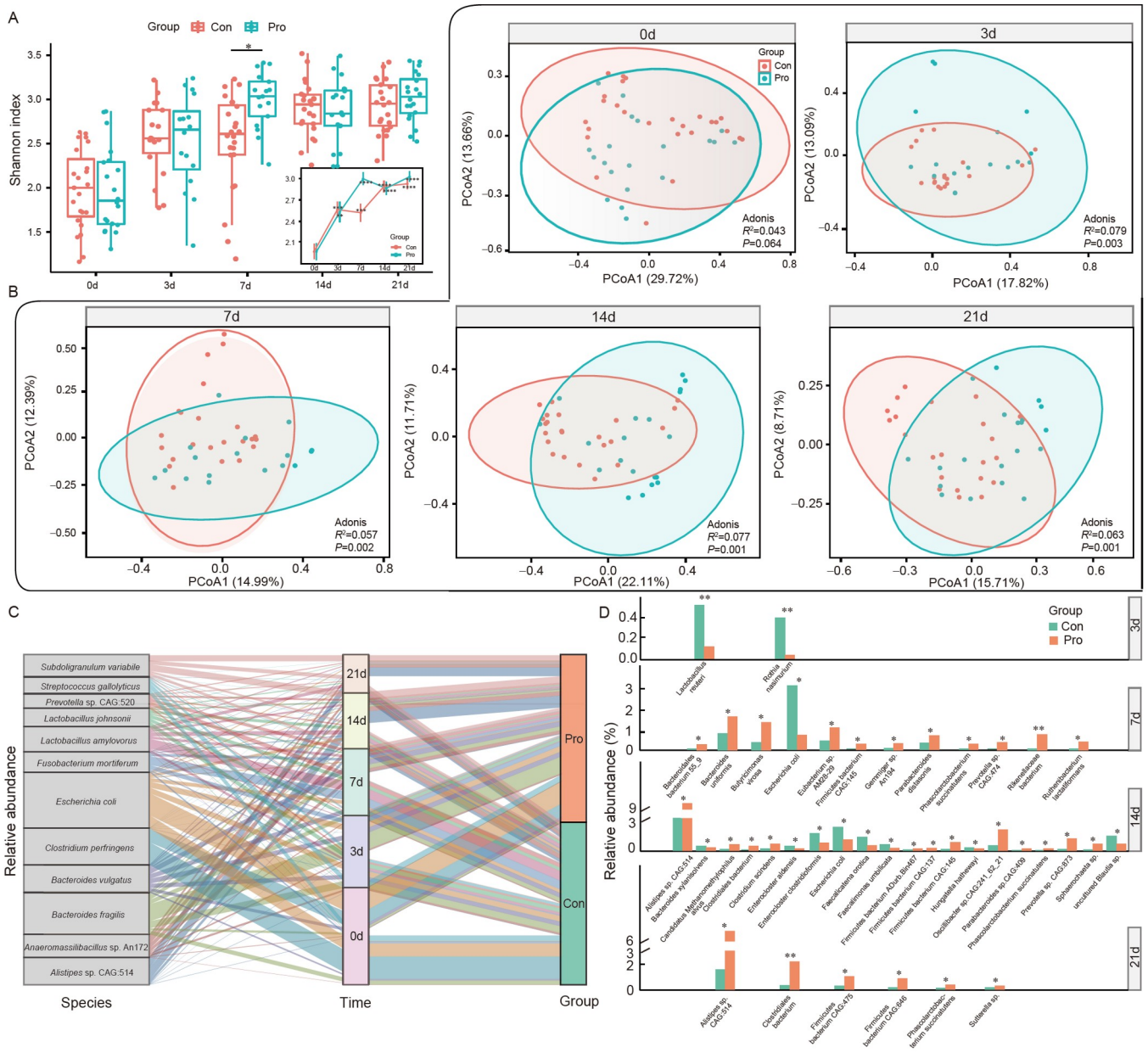

Figure 2 Microbial diversity and SGBs features of piglets' fecal metagenome dataset. A and B, Shannon diversity index (A) and principal coordinate analysis (PCoA) score plots of probiotic (Pro) and control (Con) groups (B) at 0, 3, 7, 14, and 21 days of age. Symbols representing samples of the two groups are shown in different colors. C, Distribution of 12 major SGBs (each of relative abundance>1%) across the piglets' fecal metagenomes of probiotic and control groups at different time points. Different colors represent changes in the abundance of 12 major SGBs with time. D, Responsive SGBs showing significant differential abundance between probiotic and control groups at different time points. Light green represents the control group, and orange represents the probiotic group. Wilcoxon test was used to evaluate statistical differences; *P* values were corrected by the Benjamini-Hochberg procedure; *, $P<0.05$; **, $P<0.01$.

neurotransmitters, amino acids, and short-chain fatty acid were focused. The identified gut metabolic modules belonged to 13 different phyla, mainly Firmicutes (57.10%), Bacteroidetes (21.96%), and Proteobacteria (8.53%). The three most ubiquitous modules (prevalence>80% SGBs) were acetate synthesis I (94.57%), thiamine biosynthesis III (82.43%), and glutamate synthesis I (80.36%; Table S3 in Supporting Information). Interestingly, the gut microbiome of the probiotic group was enriched in modules of propionate synthesis III, butyrate synthesis I, vitamin B6 biosynthesis I, and vitamin B6 biosynthesis I, while the gut microbiome of the control group was highly represented with the module of cysteine degradation I (Table S4 in Supporting Information).

The gut metabolites of piglets' fecal samples were predicted using MelonnPan. A total of 80 metabolites were identified. Firstly, good cooperativity was observed between the gut microbiome and metabolome (correlation=0.6933, $P=0.001$, Procrustes analysis; Figure S3A in Supporting

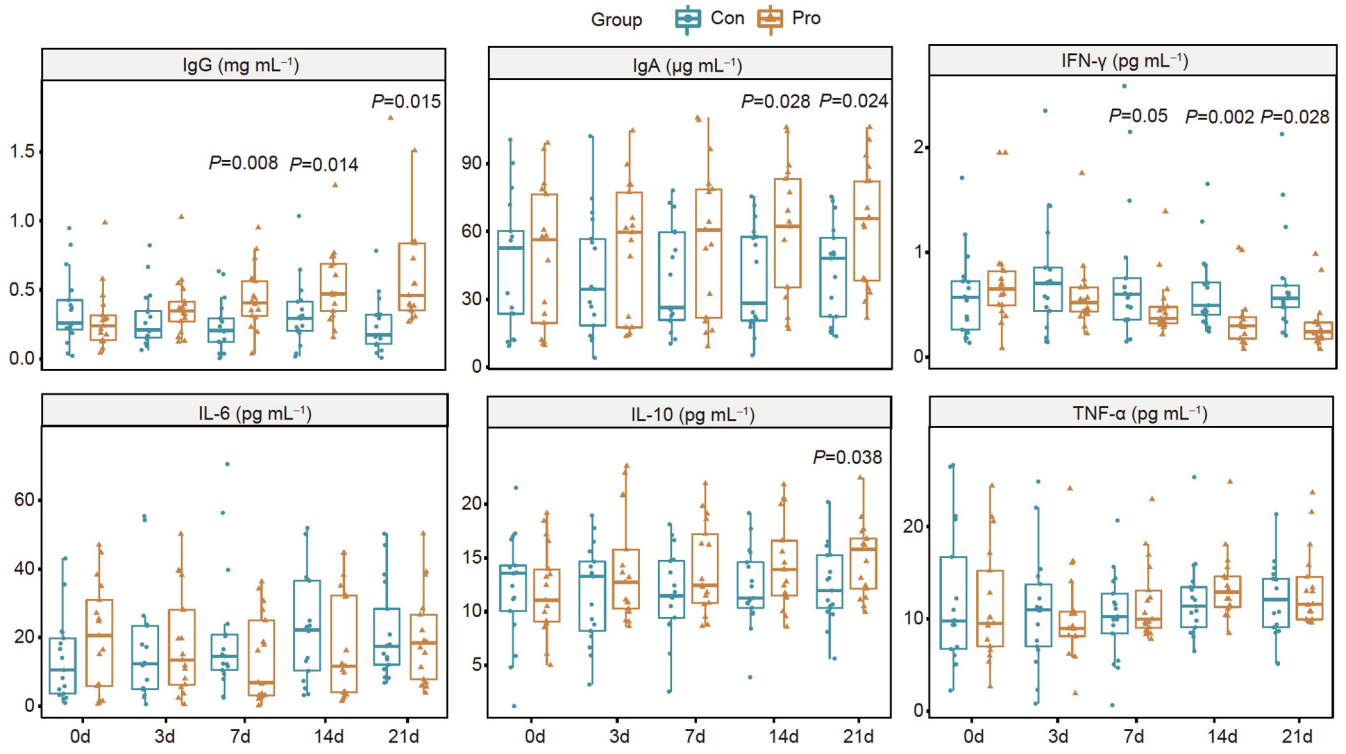

Figure 3 Changes in serum immunoglobulin and cytokine levels in the probiotic (Pro) and control (Con) groups at different time points. Differences in the serum concentrations of IgG, IgA, IFN-γ, IL-6, IL-10, and TNF-α between groups were evaluated using Wilcoxon test.

Information), revealing cohesive interactive changes between the predicted intestinal metabolites and SGBs during the intervention. These predicted gut metabolites showed obvious changes as the piglets aged (Figure 4A), especially at 7 and 14 days of age (Figure 4B and C), exhibiting significant differences in the gut metabolite profile between the probiotic and control groups (P<0.02, Adonis test; Figure 4D). Metabolite-level analysis predicted 25 responsive metabolites (three and 22 differential metabolites at 3 and 7 days of age, respectively) that only showed significant differences in abundance between the two groups at later time points but not at 0 days of age (Table S5 in Supporting Information). The levels of some of these predicted metabolites, including nicotinic acid, pyridoxamine, caproic acid, azelaic acid, sebacic acid, bilirubin, N-acetylglutamate, glutamate, adrenic acid, uracil, cholestenone, and C20:4 carnitine, were significantly higher in the probiotic group compared with the control group (P<0.05; Figure 4E).

Spraying probiotic fermented liquid modulated piglet's serum metabolome

To further reveal the physiological responses of piglet's after CPFL treatment, changes in their serum metabolome were monitored at two time points (at birth and weaning). Symbols representing the quality control (QC) samples clustered closely on the PCA plot, indicating that the liquid chromatography-mass spectrophotometry (LC-MS) system and conditions for measuring serum metabolites were highly stable, and that the generated data were reliable for follow-up analysis (Figure S3B in Supporting Information). Symbols representing serum metabolomes of the two groups clustered closely at birth, but clear group-based clustering trends were found at weaning (Figure 5A; P<0.001), suggesting that there were significant probiotics-driven changes in the serum metabolome at weaning. Eighteen differential abundant serum metabolites were identified between the two groups in a partial least squares-discriminant analysis (PLS-DA) model (cut-off variable importance in projection (VIP)>2; Figure 5B). The identified features were compared against the blood exposome database, annotating eleven metabolites. Interestingly, several metabolites (e.g., riboflavin, vitamin E, tetradecanoic acid, and heptadecanoic acid) were enriched in the probiotic group compared with control at weaning (P<0.03; Figure 5C). The significant changes in piglets' serum metabolome at weaning between the two groups implicated that CPFL spraying before and until after birth could exert specific physiological effects on the piglets.

Multi-omics revealed that spraying probiotic fermented liquid improved piglet growth performance and immunity development

Firstly, an association analysis was performed to explore the

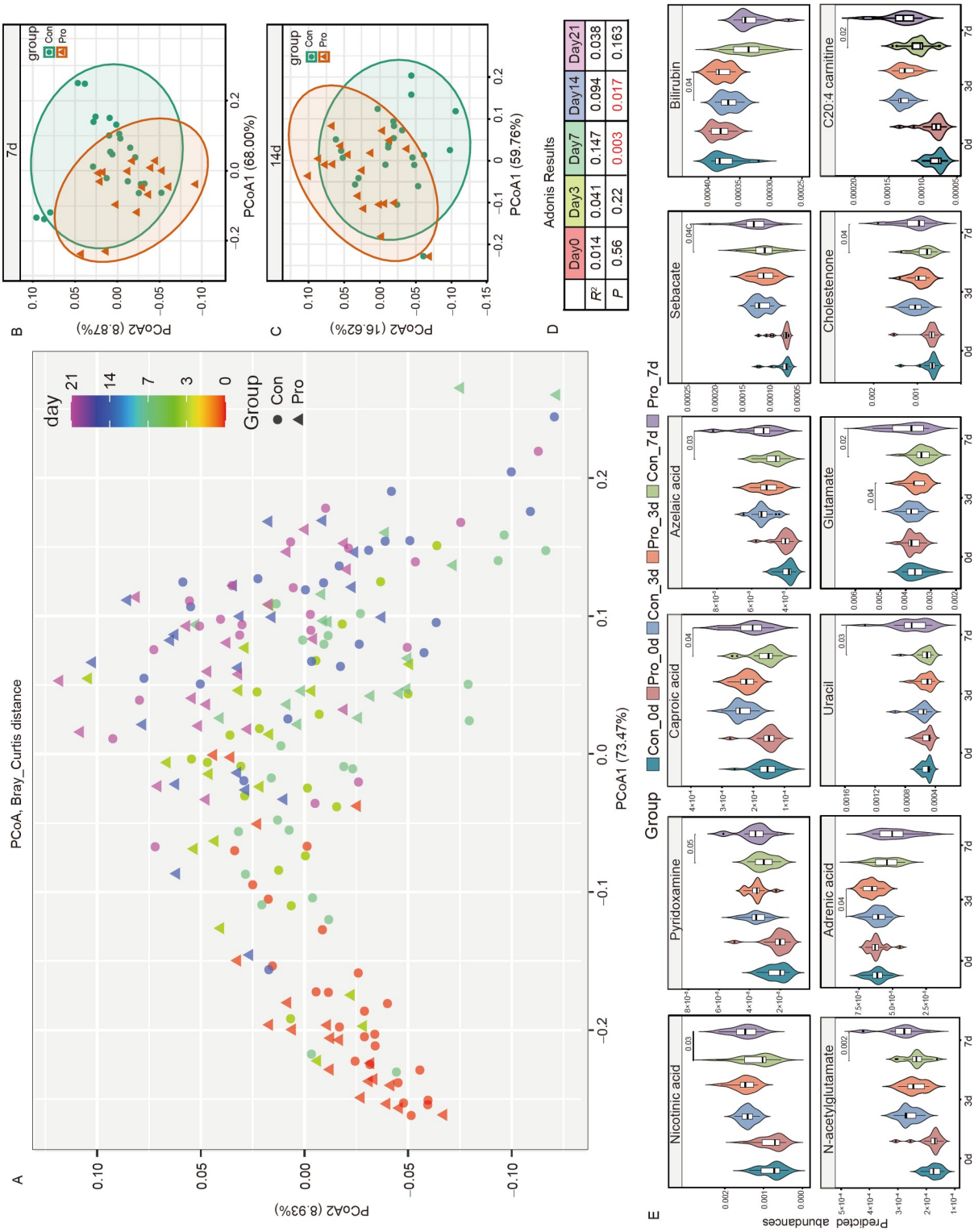

Figure 4 Changes in the predicted gut metabolome in probiotic (Pro) and control (Con) groups during the trial. Principal coordinate analysis (PCoA) score plot showing changes in the predicted gut metabolome in two groups of piglets at (A) all five time points, (B) 7 days and (C) 14 days of age. D, Evaluation of statistical differences in piglet gut metabolome between probiotic and control groups at different time points by Adonis test. E, Violin plots comparing the abundances of predicted differential gut metabolites that were responsive to the probiotic treatment. Wilcoxon test *P* values were corrected with the Benjamini-Hochberg procedure, and a corrected $P<0.05$ was considered statistically significant.

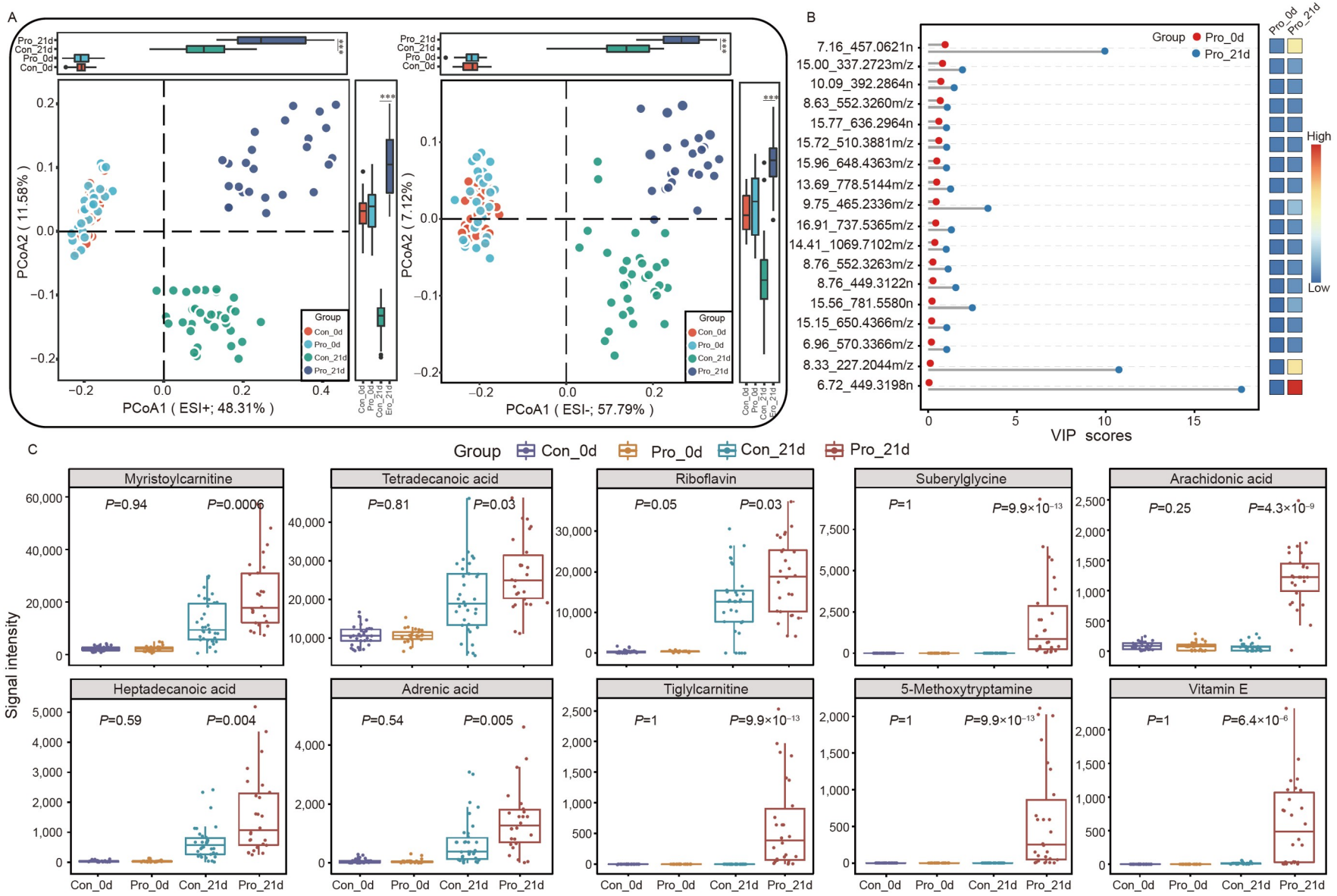

Figure 5 Comparison between serum metabolomes of the probiotic (Pro) and control (Con) groups at 0 and 21 days of age. A, Principal coordinate analysis (PCoA) score plots of serum metabolomes of Pro and Con groups at 0 and 21 days of age. Positive and negative ion modes are abbreviated as ESI^+ and ESI^-, respectively. *** $P<0.001$, Wilcoxon test. B, Significant differential metabolites between probiotic and control groups at 0 and 21 days of age, detected by LC-MS. These metabolites showed significant differences only at 21 days of age but not at 0 days of age (cut-off VIP score of >2; $P<0.05$). The heatmap shows the relative abundance of these metabolites. C, Boxplots comparing the serum levels of probiotic responsive metabolites. Wilcoxon test P values were corrected with the Benjamini-Hochberg procedure, and corrected $P<0.05$ was considered statistically significant.

relationship between piglet growth performance, immune factors, gut microbiota, and gut metabolites (Table S6 in Supporting Information). The results showed that *Clostridium scindens*, *Sutterella* sp., and *Phascolarctobacterium succinatutens* correlated positively with piglet weight (all $r>0.4$), while *Faecalimonas umbilicata* and *Escherichia coli* exhibited negative correlation ($r<-0.40$). *Sutterella* sp. correlated positively with IgG ($r=0.42$), while nicotinic acid ($r=-0.41$) and pyridoxamine ($r=-0.42$) correlated negatively with *Escherichia coli*. In addition, several gut metabolites, such as azelaic acid, sebacic acid, and glutamate, correlated positively with piglet weight (all $r>0.40$; Figure S3C in Supporting Information).

Secondly, to further reveal the association between piglet serum metabolites with gut microbes and immune factors, correlation analyses were performed at two time points (birth and weaning). There was significant positive correlation between *Phascolarctobacterium succinatutens* and vitamin E ($r=0.48$); *Alistipes* sp. CAG 514 and adrenic acid ($r=0.69$); and Firmicutes bacterium CAG 475 and tetradecanoic acid ($r=0.70$). Moreover, the serum IgA and IgG levels correlated significantly and positively with *Alistipes* sp. CAG 514, heptadecanoic acid, and tetradecanoic acid ($r>0.45$), while the riboflavin and vitamin E levels showed significantly negative correlation with IFN-γ ($r<-0.44$; Figure S3D in Supporting Information).

DISCUSSION

Accumulated evidence has demonstrated that early exposure to desirable microbiota and at an appropriate time window would be critical for gut health and immunity development in neonatal mammals (Torow and Hornef, 2017). Previous studies have reported that administering probiotics could increase growth performance, reduce infectious diarrhea, and improve mortality of piglets (Cao et al., 2019; Gebert et al., 2011; Liu et al., 2017). Thus, this study investigated the beneficial effects of spraying a specifically formulated CPFL (applied before delivery until early days of life) on the health of piglets from birth to weaning. Meanwhile, changes in multiple parameters accompanying health improvement were monitored using multi-omics techniques. Our data supported that the probiotic treatment could exert significant growth- and health-promoting impacts to suckling piglets, and these desirable changes were accompanied by gut microbiome and serum metabolome modulation (Figure 6).

The gut microbiota plays a crucial role in the development of the innate immune system (Wang et al., 2021a; Wang et al., 2021c). Our studies monitored several indicators, including IgG, IgA, IL-10, IL-6, TFN-α, and IFN-γ, to evaluate the beneficial effect of CPFL application on piglet immunity. IgA and IgG are major serum immunoglobulins that protect the host against microbes and reduce the risk of intestinal damage and systemic infection (Zeng et al., 2016). Increases in protective antibodies like IgA and IgG in CPFL-treated piglets but not the non-treated ones might implicate probiotic-specific enhancement in the humoral immunity in these animals. The increase in the humoral immunity in the probiotic-treated piglets was accompanied by the decreases in some classical pro-inflammatory cytokines, including serum IFN-γ and TNF-α. Meanwhile, the serum IL-10 concentration was significantly higher in the probiotic group compared with the control group at 21 days of age, and an increasing trend was shown throughout the course of our study. A recent study also reported that the application of *Bacillus subtilis* decreased piglets' serum concentrations of IFN-γ and TNF-α, while increased the serum IL-10 level (Li et al., 2021a). These results indicated that spraying CPFL could strengthen the humoral immunity and reduce the inflammatory state in piglets.

The alpha diversity of piglets' gut microbiota increases as they age (Chen et al., 2017; Guevarra et al., 2019). Consistently, our study observed a significant increasing trend in the Shannon index in all piglets from birth to weaning. A high diversity of gut microbiota is generally considered beneficial to host health, and it is regarded as a sign of gut microbiota maturity until establishing a relatively stable adult-like microbial community (Chen et al., 2017; Le Chatelier et al., 2013). Interestingly, at 7 days of age, the Shannon index was significantly higher in the probiotic group compared with the control group, indicating that early probiotic intervention may accelerate gut microbiota maturation. Moreover, the probiotic and control piglets showed significant differences in the beta diversity of their gut microbiota, indicating that the CPFL treatment modulated the microbial community structure of piglets in their first few days of life. Our observations are consistent with two recent studies reporting the effectiveness of probiotics supplementation in regulating and reshaping the intestinal microbiota structure and composition of piglets (Wang et al., 2021b; Wang et al., 2019a). Although CPFL treatment did not drastically restructure the piglets' gut microbiome in our study, probiotic-driven responses were detected, which were reflected by significant changes in the abundance of several SGBs together with their encoded metabolic modules. For example, propionate synthesis modules were dominated in the probiotic group but not control group. Two SGBs ascribed to *Phascolarctobacterium succinatutens* were involved in propionate synthesis III, and their abundances were significantly higher in the probiotic group than the control group at 7, 14, and 21 days of age. Propionate is an important metabolic precursor for gluconeogenesis, liponeogenesis, and protein synthesis in the host (Schippa and Conte, 2014), and bacteria mediated such metabolic processes may be related to piglets' pre-weaned weight gain (Ding et al., 2019).

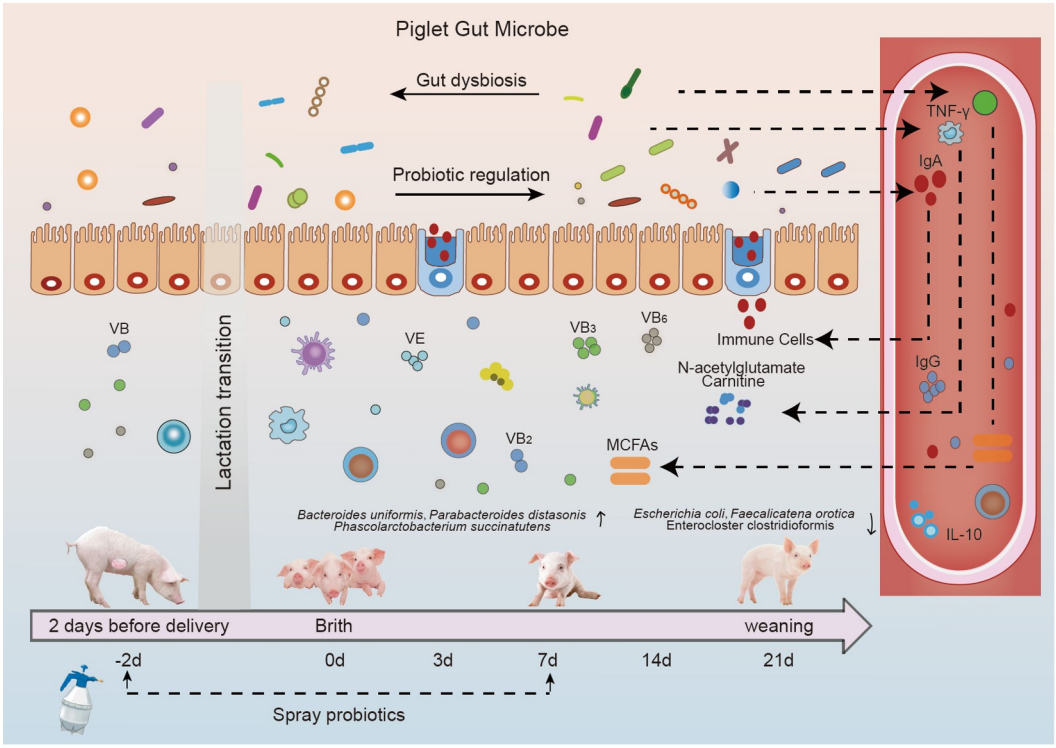

Figure 6 A proposed model of the effects of probiotic fermented liquid in improving the growth performance and immune function of piglets. The beneficial effects were accompanied by changes in specific microbial species and blood metabolites. VB2, VB3, VB6, VE, and MCFAs represent vitamin B2, vitamin B3, vitamin B6, vitamin E, and medium-chain fatty acids, respectively.

In our study, piglet weight gain correlated positively with *Phascolarctobacterium succinatutens* but negatively with *Escherichia coli*. Being the most common gut microbial resident, *Escherichia coli* is closely linked to diarrhea in newborn piglets, and it could weaken the intestinal barrier integrity (Bednorz et al., 2013; Chiang et al., 2015). Several studies have reported that probiotics administration significantly reduced the abundance of *Escherichia coli* in the intestine of piglets (Wang et al., 2020a; Yang et al., 2020), which is consistent with our results. At 7 days of age, some other potentially beneficial and SCFAs-producing bacteria, e.g., *Bacteroides uniformis*, *Butyricimonas virosa*, *Parabacteroides distasonis*, *Eubacterium* sp., and *Gemmiger* sp., were enriched in the probiotic group. These results together suggested that CPFL treatment is associated with beneficial changes in piglets' gut microbiota community.

Another interesting finding of our study was that spraying CPFL enhanced the microbial bioactive potential, which was confirmed by the observed changes in piglets' serum metabolome. The treatment with CPFL significantly increased the contents of B vitamins (nicotinic acid (vitamin B3) and pyridoxamine (vitamin B6)), medium-chain fatty acids (MCFAs, e.g., caproic acid), and medium-/long-chain dicarboxylic acids (azelaic acid and sebacic acid) in piglets' feces. Vitamin B3 and B6 are beneficial for gut health, and vitamin B3 treatment could accelerate mucosal healing in patients with moderately active ulcerative colitis (Li et al., 2017). Vitamin B6 is an anti-inflammatory trace element, and its supplementation could reduce colonic inflammation in IL-10$^{-/-}$ knock-out mice and TNF-α production in HT-29 colonic epithelial cells (Allen et al., 2019). On the other hand, *Escherichia coli* produces lipopolysaccharides with high endotoxin activity, which can trigger intestinal inflammation and lead to endotoxin translocation when the intestinal barrier is damaged (Reisinger et al., 2020; Wallace et al., 2016). The significant reduction in the abundance of *Escherichia coli* and increase in vitamin B6 in the gut could both contribute to a less inflammatory state. A recent work found that supplementing a probiotic strain of *Bacillus* could significantly increase the levels of vitamin B3 and B6 in animal feces (Choi et al., 2021). Our results of genome-centric metabolic reconstruction revealed that vitamin-producing genes were present mainly in members of *Lactobacillus*, *Prevotella* sp., *Parabacteroides* sp., and *Bacteroides* sp.; and the abundances of these bacteria (such as *Prevotella* sp. CAG:873, *Parabacteroides* sp. CAG:409, *Parabacteroides distasonis*, *Bacteroidales* bacterium 55_9 and *Bacteroides uniformis*) increased significantly after probiotic supplementation.

Apart from vitamins B3 and B6, spraying CPFL significantly enhanced piglets' serum concentrations of riboflavin (vitamin B2) and vitamin E at weaning age. Vitamin

B2 is a coenzyme in a variety of enzymatic reactions, participating in key metabolic functions, e.g., biological oxidation-reduction associated with energy production, antioxidation, and homocysteine metabolism (Thakur et al., 2017). Supplementing sulfur-containing amino acids to gilt pigs from late pregnancy to lactation could modulate the gut microbiome and serum metabolome (increase in vitamin B2 concentration) of offspring piglets, thereby promoting their health (Azad et al., 2020; Wang et al., 2020b). An adequate level of vitamin E could protect piglets from enteritis and improve immune function (Habeanu et al., 2015). Our study found that the piglets' serum levels of riboflavin and vitamin E showed significant positive correlation with IgA and IgG, but were negatively correlated with IFN-γ. The serum vitamin E concentration of piglets decreased dramatically at weaning, accompanied by a decreased growth rate and increased susceptibility to disease after weaning (Orengo et al., 2021). Buchet et al. (2017) observed that low average daily gain piglets had significantly lower pre-weaning vitamin E levels. Moreover, piglets with the highest serum vitamin E level (7.80 to 21.1 μmol L^{-1}) showed better post-weaning growth compared with those having a low level of serum vitamin E (0.17 to 7.79 μmol L^{-1}), suggesting that a large pre-weaning vitamin E pool is crucial for the post-weaning health and growth of piglets (Buchet et al., 2017).

The increase in the bioactive potential for MCFAs is also desirable. These molecules are used directly by the enterocytes of piglets for energy production (Hanczakowska, 2017) and as energy sources for supporting growth and promoting weight gain (Hanczakowska et al., 2016). Apart from serving as energy sources, MCFAs could regulate the gut microbiota composition and indirectly inhibit potential pathogens like *Salmonella* and coliforms, exert immunemodulation effects, and maintain the integrity of intestinal epithelial tissue in piglets (Zentek et al., 2011). Several studies found that caproic acid was produced as a result of gut bacteria metabolism from taxa such as *Clostridium* sp. and *Ruminococcaceae* bacterium (Dan et al., 2020; Zhu et al., 2017). Thus, the higher level of caproic acid detected in the probiotic group in this work might have been related to the significant increases in Clostridiales bacterium and *Clostridium scindens*. The supplementation of CPFL also increased the bioactive potential for azelaic acid and sebacic acid, which are saturated dicarboxylic acids. In addition, the serum concentrations of long-chain fatty acids (tetradecanoic acid and peptadecanoic acid) in CPFL-treated piglets increased, and tetradecanoic acid correlated positively with Firmicutes bacterium CAG 475. These results implicated that CPFL administration enhanced fatty acid oxidation in piglets. Moreover, the fecal contents of glutamate, N-acetylglutamate, and C20:4 carnitine of CPFL-treated piglets increased significantly. Glutamate has multiple metabolic functions in the small intestine, e.g., increasing immune function, promoting transamination, participating in the tricarboxylic acid cycle, and stimulating the intestinal biosynthesis of citrulline and arginine through the formation of N-acetylglutamate (Jiao et al., 2015). Similarly, adding carnitine to feed improved birth weight and growth performance of piglets (Blavi et al., 2021).

In this study, probiotics were supplied to piglets via direct spraying of CPFL into the living environment. Indeed, adding probiotics into feed and oral gavage are two other common means of exogenous provision of probiotics to farm animals. Adding probiotics into feed is a convenient strategy for supplementing probiotics to pigs. However, it may not be an ideal method for piglets in their very early days (the first week) of life because piglets are fed gradually seven to ten days after birth. Oral gavage is an alternative way of supplementing probiotics to newborn piglets, but this method is time-consuming when implementing in commercial pig farms with a high number of newborn piglets (Barba-Vidal et al., 2018). Moreover, oral gavage could easily cause gavage stress and organ damage, exerting stress to animals unnecessarily, especially for neonates. Environmental spraying of probiotics is thus another option. Although spraying probiotics to the environment might not be as direct as oral gavage, it is easy to implement and is a gentler way to the neonatal piglets. One disadvantage of environmental spraying of probiotics is that it is more wasteful than direct feeding. The other potential problem with direct spraying of probiotics is that routine flushing and disinfection of pig houses during feeding and management would affect the concentration, viability, and thus the effectiveness of the probiotic intervention. Therefore, here, an ample dose (1×10^9 CFU mL^{-1}) of probiotics was sprayed and the spraying was started in the sow delivery room two days before until seven days after delivery. During the course of probiotic application, the level of viable probiotics in the delivery rooms (delivery beds) was monitored and maintained at around 0.5×10^8 to 1×10^8 CFU mL^{-1}, which was not lower than similar studies (Chen et al., 2005; Zhang et al., 2020b). The spraying of probiotics should also be accompanied by meticulous environmental impact assessment to ensure that no harmful effect is exerted to the surrounding environmental microbiome.

In conclusion, this study demonstrated that early-life intervention by spraying compound probiotics can effectively reshape the microbiota composition of the delivery room environment and significantly improve the growth performance and immune function of piglets. The beneficial effects are associated with changes in the gut microbiota, bioactive potential, and metabolism of piglets. Our findings provide interesting and practical information to both the academic community and the pig industry, expanding the knowledge and application of probiotics in animal husbandry.

MATERIALS AND METHODS

Preparation of compound probiotic fermented liquid

The three different bacterial strains used were LCZ, P-8, and Probio-M9, which were maintained and cryopreserved in the Lactic Acid Bacteria Culture Collection of the Inner Mongolia Agricultural University.

Considering the fact that the number of sprayed probiotic cells accessible to the piglets would likely be lower than direct feeding, the CPFL was produced by optimized fermentation procedures aiming to generate a high cell density probiotic liquid. The specific preparation steps were as follows: firstly, the cryopreserved LCZ, P-8, and Probio-M9 cells were inoculated into MRS liquid medium, cultured at 37°C for 18–24 h, and subcultured for one to two times to obtain fully active bacteria. Secondly, a fermentation medium optimized previously by our laboratory for mixed growth of these three strains was prepared by dissolving: glucose (4%), yeast powder (1.5%), soy peptone (1.5%), K_2HPO_4 (0.318%), anhydrous sodium acetate (0.8%), sodium citrate (0.212%), Tween-80 (0.088%), $MgSO_4$ (0.088%), and $MnSO_4$ (0.0047%) in distilled water. The medium was adjusted to pH 6.5 and sterilized for 15 min at 121°C. Then, the activated bacteria were inoculated into the sterilized medium and were cultivated at 37°C for 8–10 h for preparing a seed fermentation culture. Afterward, the seed fermentation culture was inoculated into the prepared medium for larger scale fermentation for another 8–15 h. The pH change was monitored during the fermentation process. The fermentation was terminated when the acid production stopped. The fermented liquid containing a high-density of composite probiotic bacteria, which was sprayed after appropriate dilution.

We sampled the prepared fermented liquid multiple times to evaluate the number of viable probiotic bacteria by plate count method, and the results showed that the average cell density at the fermentation end point was 1×10^9 CFU mL^{-1}. The number of probiotics in the environmental samples of delivery beds collected by sterile cotton swabs was about 0.5×10^8 to 1×10^8 CFU mL^{-1} after spraying.

Experimental design and animal subjects

Two groups of parturient sows of the same breed, similar weight, and same batch were selected from the delivery room. The farrowing beds and piglets were given combinatory codes for recognition. “C” and “P” represented control and probiotic groups, respectively, while the number signified a specific farrowing bed or an individually ear-tagged piglet.

Each group had 56 litters, and 8 litters among which were randomly chosen and divided into the probiotic and control groups. After parturition, 5 piglets were randomly selected from each litter for subsequent evaluation for growth performance and collection of feces and blood samples. However, only 32 and 36 piglets remained in the probiotic and control groups, respectively (Figure 1A), after excluding accidentally died and weak piglets unsuitable for further study and those failed in complete sample collection for all five time points. All piglets were offspring of crosses between Landrace×Large White hybrid sows and Duroc boars.

Both probiotic and control groups were exposed to the same feeding and farrowing management procedures. Procedures of castration, health care, and immunization of suckling piglets were carried out as usual. Piglets were weaned at 21 days of age. The farrowing beds of the sows and piglet activity areas (bed floor and fence) of the probiotic group but not the control group were sprayed with CPFL premixed with two volumes of sterile water. The diluted CPFL was sprayed twice daily at 9:00 am and 3:00 pm (100 mL per nest in each spraying). The spraying was implemented from two days before to seven days after delivery.

Feeding management and ethics approval

The field experiment was performed at the pig breeding site under the Zhengye Project of Inner Mongolia Zhengda Food Co., Ltd. Stable environment was maintained in the farrowing room (room temperature of 24–27°C and relative humidity of 50%–70%). Each farrowing bed was equipped with a heating lamp to keep the piglet activity area at about 30°C. The lactating sows were raised in a 2.2 m×1.8 m delivery bed on plastic slatted floor, equipped with stainless steel adjustable troughs and nipple drinking fountains for sows. Piglets were breast-fed by the sows after farrowing and in the first few days of their life until seven to ten days after birth, when creep feed was gradually offered. Piglets were dewormed and immunized according to routine management procedures.

Collection of environmental samples in the delivery rooms

Samples were collected from the delivery rooms after spraying CPFL by using the five-point sampling method, which sampled at the four corners and the center of the delivery bed. To minimize the disturbance to the sows and piglets, only a total of 11 delivery rooms (five and six for probiotic and control groups, respectively) were sampled at three different time points (i.e., 0, 7, and 14 days of age) using sterile cotton swabs. The collected environmental samples were stored at −80°C for microbiome analyses.

Piglet growth performance, feces and blood collection

The total number of piglets, and the number of healthy, weak,

and stillbirth piglets of each sow was recorded. Piglets were vaginally delivered, and their birth weight, daily weight gain, weaning weight, and diarrhea rate were monitored. The health status and feces morphology of piglets were observed and recorded daily by professional workers since birth.

Blood and fecal samples of piglets were collected at 0, 3, 7, 14, and 21 days of age. Blood samples were collected via the precaval vein of each piglet. The collected blood was immediately centrifuged to obtain serum, which was then stored at −20°C. A cotton swab infiltrated with sterile phosphate buffered saline was gently inserted into the anus of piglets to collect feces, and the collected fecal samples were stored at −80°C for microbiome and metabolite analyses. All samples were collected by experienced veterinary personnel to avoid frightening or causing any physical harm to the piglets.

Determination of serum levels of immune markers

The serum immune markers of piglets were detected at 0, 3, 7, 14, and 21 days of age after spraying the probiotic liquid. The serum concentrations of IgA, IgG, IL-10, IL-6, TNF-α, and IFN-γ were determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the porcine IgA, IgG, IL-10, IL-6, TNF-α, and IFN-γ kits (Meimian Biotechnology, Jiangsu, China), according to the manufacturer's protocols.

Sample sequencing, contig binning, genome dereplication

Metagenomic DNA was extracted from fecal and environmental samples with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) and Omega Soil DNA Kit (Omega Biotek, USA). Metagenomic sequencing was executed on all samples by using an Illumina HiSeq X Ten instrument. Libraries were constructed using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina to generate DNA fragments of ~150 bp length. The generated metagenomic sequences were processed with the KneadData quality control pipeline (http://huttenhower.sph.harvard.edu/kneaddata), which used Trimmomatic tool and Bowtie2 to filter out low-quality reads shorter than 60 nucleotides and pig contaminating reads, respectively (Langmead and Salzberg, 2012). Species-level profiles of the environmental samples were derived through MetaPhlAn3 (https://huttenhower.sph.harvard.edu/metaphlan/). Reads of fecal samples were assembled into contigs using MEGAHIT (Li et al., 2015), and contigs greater than 2,000 bp were chosen for binning using MetaBAT2 (Kang et al., 2019), VAMB, and DAS Tool with default options (Sieber et al., 2018). Then, all bins were combined to gain metagenome-assembled genomes (MAGs) using in-house scripts.

The completeness and contamination of MAGs were evaluated through CheckM, and these MAGs were categorized as high-quality (completeness≥80%, contamination ≤5%), medium-quality (completeness≥70%, contamination ≤5%), and partial-quality (completeness≥50%, contamination≤5%). The high-quality genomes were clustered, and the most representative genomes of each replicate set were selected by dRep to obtain SGBs, using the options "-pa 0.95" and "-sa 0.95".

Annotation of SGBs, gut metabolites prediction

The SGBs were annotated by Kraken2 and NCBI nonredundant Nucleotide Sequence Database (retrieved in 2020.11), and the relative abundance of each SGB was calculated through coverM using the option "–min-read-percent-identity 0.95 –min-covered-fraction 0.4" (https://github.com/wwood/CoverM). Probiotic strains were evaluated by mapping sample reads to contigs through BBMap (https://github.com/BioInfoTools/BBMap) using the parameters "minid=0.95 idfilter=0.95", and the pileup.sh tool was used to calculate the coverage of contigs.

For each SGB, the predicted open reading frames were compared against the KEGG Orthologies (KOs) database to identify key metabolic modules (such as vitamins, neurotransmitters, amino acids, and short-chain fatty acid modules). Profiles of SGBs encoding synthesis or degradation-related modules were identified by Omixer-RPM using the parameter "-c 0.66" (Valles-Colomer et al., 2019). Profiles of gut metabolites were predicted according to the methods described in our previous work (Liu et al., 2021). Briefly, one million reads per sample were extracted using seqtk (https://github.com/lh3/seqtk); the gene abundance was calculated by DIAMON comparison; and the MelonnPan-predict pipeline was used to generate predicted gut metabolites profiles (Mallick et al., 2019).

Determination of serum metabolites by LC-MS

Detailed procedures and conditions of serum metabolite extraction were adopted from our previous study (Liu et al., 2021). Piglets' serum metabolites were measured by mass spectrometry using Agilent 6545A Q/TOF (Agilent Technologies, USA) in both positive and negative ion modes. The QC sample was prepared by mixing the same amount (10 μL) of each sample, and the QC sample was injected five times before the actual analysis to evaluate the stability of the system. Obtained raw data were filtered through the Progenesis QI platform and preprocessed by MetaboAnalyst (https://www.metaboanalyst.ca/), and features with missing values of more than 50% were deleted. In addition, baseline noise and near-constant values were excluded by using mean, and low repeatability features (based on comparison

with QC samples) with a peak area relative standard deviation (RSD)>20% were also excluded from the analysis.

Metabolomic data were analyzed by PLS-DA, and differential abundant biomarkers were manually inspected based on peak shape and signal-to-noise ratio. Differential abundant features were compared against the blood exposure database (https://bloodexposome.org) to determine the best annotation results.

Statistical analyses

All statistical analyses and data visualization were performed using the R software (v.4.0.2) and Adobe Illustrator environment. Microbial species diversity analysis, principal coordinate analysis, PLS-DA, Adonis test, and procrustes analysis were executed by using different R packages, namely vegan (v.2.5.6), optparse (v.1.7.1), mixOmics (v.6.10.9), and ggpubr (v.0.4.0). Considering the skewed distribution and non-parametric nature of our data, the Mann-Whitney U test was used to evaluate inter-group (probiotic group versus control group at the same time point) and intra-group (between data of the same group at different time points) differences, and *P* values were corrected using the Benjamini-Hochberg procedure. Correlation analyses (cut-off: $r>0.4$ or <-0.4) of piglet growth performance, immune factors, differential abundant species, predicted gut metabolites and serum metabolites were performed using Pearson rank correlation coefficient.

Data availability statement

The raw data supporting the conclusions of this article are made available by the authors without undue reservation (https://dataview.ncbi.nlm.nih.gov/object/PRJNA779106).

Compliance and ethics *The author(s) declare that they have no conflict of interest. This study was approved by the Special Committee on Scientific Research and Academic Ethics of Inner Mongolia Agricultural University.*

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