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RESEARCH ARTICLE

Effects of Dietary Intake of Heat-Inactivated *Limosilactobacillus reuteri* PSC102 on the Growth Performance, Immune Response, and Gut Microbiota in Weaned Piglets

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ABSTRACT

This study aimed to investigate whether dietary supplementation with heat-inactivated *Limosilactobacillus reuteri* PSC102 (LR) enhances immunological responses and growth performance in weaned pigs. Twenty-five weaned piglets were randomly allocated to five groups. Each treatment group had separate pens with five pigs each. The pigs were fed a diet supplemented with heat-inactivated LR at a concentration of 1×10^9 CFU/g for 28 days. Throughout the study period, regular measurements, including body weight, food consumption rate, and diarrhoeal frequency, were conducted. At the end of the experiment, blood samples were collected to assess cytokine and haptoglobin levels. Faecal samples, rectal swabs, and nasal swabs were collected for analysis of pathogenic bacterial growth using selective media. Additionally, faecal samples were analysed to determine the composition of the microbiota. Heat-inactivated LR enhances growth rate and feed efficiency while decreasing diarrheal frequency in weaned piglets. Compared to pigs fed a control diet, diets containing heat-inactivated LR exhibited increased serum levels of cytokines and decreased haptoglobin levels, demonstrating a beneficial effect on the presence of pathogenic microorganisms. Heat-inactivated LR diets alter the microbiota of piglets by enhancing alpha diversity and increasing the composition of beneficial bacteria. Overall, dietary heat-inactivated LR enhanced growth rates, regulated immunological responses in weaned pigs, and reduced post-weaning diarrhoea. Therefore, this suggests its potential as a health functional ingredient for humans and animals.

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INTRODUCTION

Probiotics are beneficial microorganisms when consumed in appropriate quantities (Roobab *et al*., 2020). Supplementing pig diets with probiotics enhances nutrient utilization, growth rates, and immunity (Cho *et al*., 2011; Pospíšková *et al*., 2013; Patil *et al*., 2015). Furthermore, probiotics promote microbiota balance, disease resistance, and gut health in pigs (Ali *et al*., 2023a). Consistent probiotic intake enhances gut barrier integrity in pigs affected by enterotoxigenic *E. coli* (ETEC) issues (Zhou *et al*., 2014). Additionally, oral administration of the probiotic *Lactobacillus rhamnosus* GG stimulates the production of active metabolites and enhances immune responses in weaned piglets (Geng *et al*., 2021).

Adding probiotics to animal feed may lead to adverse effects, such as bacterial toxicity, increasing the risk of infection (Anadón *et al*., 2021). This risk is attributed to the strong adherence of bacteria in the gastrointestinal tract (Adherence *et al*., 2023b). Gene transfer can occur through horizontal and clonal gene mechanisms (Daniali *et al*., 2020). Ensuring the stability of the metabolic properties of probiotics over time is crucial (Gueimonde and Sanchez, 2012). Recent studies suggest that inactivated probiotics provide benefits such as immune modulation and metabolite delivery (Berlanga and Miñana-Galbis, 2019; Almada *et al*., 2021).

Appropriate methods for inactivating probiotics can mitigate potential risks (Taverniti & Guglielmetti, 2011). Heat treatment is widely used for this purpose, alongside

other methods (Liao *et al*., 2016). Heat-inactivated probiotics release immunoregulatory components such as exopolysaccharides (EPS), peptidoglycans, and lipoteichoic acids (Wu *et al*., 2015; Liu *et al*., 2017; Zhang *et al*., 2023). Studies indicate that heat-inactivated lactic acid probiotic bacteria enhance growth rates, feed efficiency, and immune regulation in preweaning pigs (Piqué, Berlanga, & Miñana-Galbis, 2019).

A previous study shows that heat-inactivated *Limosilactobacillus reuteri* PSC102 (LR) enhances immune responses in mice (Ali *et al*., 2022) and rats (Ali *et al*., 2024). However, studies investigating immunoactive and antimicrobial activities in pigs remain lacking. Therefore, this study aims to investigate, for the first time, how LR supplementation affects the immune response, growth, and gut microbiota in weaned piglets.

MATERIALS AND METHODS

Sample Preparation: The *Limosilactobacillus reuteri* PSC102 strain (Accession number given by the International depositary authority: KCCM12927P and Genebank: MZ127631.1) was cultured in de Man Rogosa and Sharpe (MRS) media overnight at 37°C. The heat-inactivation procedure of LR was achieved by heating for 15 min at 80°C (Ali *et al*., 2024). The viability of the resulting flowable powder was assessed by incubating it overnight at 37°C in an MRS agar plate.

Experimental design: The experimental protocols were approved by the Animal Care and Use Committee of Kyungpook National University, South Korea (KNU-2022-0014). Twenty-five weaned piglets (6.75 ± 1.88 kg, 30 ± 1.4 days old) were randomly allocated into five groups (Table 1). Each treatment group was housed separately in pens containing five pigs each and fed with heat-inactivated LR for 28 days (Table 1). The pigs were maintained under standard laboratory conditions with *ad libitum* access to water and feed.

Growth performance analysis: Growth performance was determined by measuring the average daily weight gain and feed efficiency, calculated from the weight of each pig and pen feeder. Daily visual diarrhoea scores for individual pigs were recorded over four weeks, using a scale from 1–5 (1: hard and dry normal faeces, 2: well-formed faeces, 3: moist faeces, 4: diarrheal faeces, and 5: watery diarrheal faeces). The frequency of diarrhoea was determined and expressed as a percentage (%) by calculating the average number of diarrhoea incidents with a score of > 4 relative to the number of pens each day (Kang *et al.*, 2021).

Determination of cytokines and haptoglobin in weaned piglets: After 28 days of treatment, whole blood was collected and subsequently centrifuged at 3,000 × g for 15 min to separate the serum. The test was conducted using an ELISA kit (ELK Biotechnology, Wuhan, China) following the instructions of the manufacturer. Subsequently, the absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, VT, USA). The concentration of cytokines was determined by creating a standard curve and multiplying the dilution factor expressed in pg/ml. The haptoglobin concentration was measured following the instructions of the manufacturer (Immunology Consultants Laboratory, Inc., Oregon, USA). The microplate was read at 450 nm within 30 min. Haptoglobin concentration was calculated from the standard curves and expressed in ng/ml.

Determination of immune blood cells in weaned piglets: Blood samples collected from pigs were kept in EDTA-coated tubes. An automatic blood analyser (URIT Medical Electronic Co., Ltd., Guangxi, China) was used to count different blood immune cells, including white blood cells, granulocytes, and lymphocytes.

Determination of pathogenic bacteria in weaned piglets: After 28 days of treatment, various pathogenic bacteria were measured using different samples (faeces, rectal swabs, and nasal swabs). Specific selective media were utilised: *Salmonella Shigella* (SS) agar for *Salmonella* spp., MacConkey agar for *E. coli*, MRS agar for *Lactobacillus* spp., manitol salt agar (MSA) for

Table 1: Formula, ingredients, and chemical composition of the experimental diets.

Groups	T1	T2	T3	T4	T5
Ingredients (%)	Glucose	EST	hLR + EST	hLR	BCL
Yellow corn	44.92	44.92	44.92	44.92	44.92
SBM-46	25.32	25.32	25.32	25.32	25.32
Soy protein K[a]	10.0	10.0	10.0	10.0	10.0
Prototype	0.1	0.1	0.1	0.1	0.1
DSM[b]	0.97	0.97	0.97	0.97	0.97
Lactose	16.2	16.2	16.2	16.2	16.2
MCP	1.26	1.26	1.26	1.26	1.26
Limestone	0.53	0.53	0.53	0.53	0.53
Lysine, 98%	0.05	0.05	0.05	0.05	0.05
Methionine, 98%	0.09	0.09	0.09	0.09	0.09
Choline chloride, 50%	0.05	0.05	0.05	0.05	0.05
Antioxidant	0.2	0.2	0.2	0.2	0.2
Vitamin premix[c]	0.1	0.1	0.1	0.1	0.1
Mineral premix[d]	0.1	0.1	0.1	0.1	0.1
Salt	0.2	0.2	0.2	0.2	0.2
Total	100	100	100	100	100

Note: [a]Soy protein concentration manufactured by ADM: Europoort BV.; [b]Dry skimmed milk, [c]Provided the following per kilogram of diet: vitamin A, 8,000 IU; vitamin D3, 1,600 IU; vitamin E, 32 IU; d-biotin, 64 g; riboflavin, 3.2 mg; calcium pantothenic acid, 8 mg; niacin, 16 mg; vitamin B12, 12 g; vitamin K, 2.4 mg; [d]Provided the following per kilogram of diet: Se, 0.1 mg; I, 0.3 mg; Mn, 24.8 mg; $CuSO_4$ 54.1 mg; Fe, 127.3 mg; Zn, 84.7 mg; Co, 0.3 mg. Control diet (glucose/kg); Enrofloxacin 20 g, sulfamethoxazole 50 g, trimethoprim 10 g (EST); Heat-inactivated *Limosilactobacillus reuteri* PSC102 (10^9 CFU) (hLR + EST/kg); Heat-inactivated *Limosilactobacillus reuteri* PSC102 (10^9 CFU); (hLR); *Bacillus subtilis* (10^9 CFU), *Cellulomonas cellulans* (10^9 CFU), *Lactobacillus fermentum* (10^9 CFU)/kg (BCL mixture).

Staphylococcus aureus, and tyrptone sulphite neomycin (TSN) agar for *Clostridium perfringens.* Samples weighing 0.1 g were aseptically collected and homogenised in 900 µl of peptone water. The homogenates were serially diluted 10-fold onto their respective agar plates and incubated at 37°C for 24 h. Bacterial colonies grown on the selective media were counted and expressed as colony-forming units per millilitre (CFU/ml).

Gut microbiota analysis: To determine the composition of gut microbiota, three representative groups (T1, T2, and T4) were selected. Microbial DNA was extracted from faecal samples, ensuring a DNA concentration of ≥ 15ng/µl and an A260/A280 ratio of ≥ 1.8. Microbiome profiling was performed using 16S rRNA gene sequencing with the universal bacterial primers 27F and 1492R by Pacific Bioscience (ChunLab, Seoul, Korea), with the EzBioCloud workflow and 16S database (Yoon *et al.*, 2017). Low-quality reads were filtered out using a 97% similarity cutoff, and alpha diversity was measured by determining Chao1, ACE, Shannon and Simpson indices.

Statistical analysis: Data were analysed using GraphPad Prism software version 8 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between groups were performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. The chi-square test was used to examine the frequency of diarrhoea. The results were presented as mean ± standard error of the mean (SEM). A P<0.05 was considered statistically significant.

RESULTS

Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on growth performance in weaned piglets: The dietary supplementation of heat-inactivated LR used in this study significantly improved the growth rate and feed efficiency in weaned pigs (P<0.05; Fig. 1A, B). This improvement in growth rate was accompanied by an increase in feed efficiency. Concurrently, weaned pigs in the heat-inactivated LR group exhibited a lower frequency of post-weaning diarrhoea than those in the control group (P<0.05; Fig. 1C).

Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on cytokine, haptoglobin, and immune cell levels in weaned piglets: The administration of dietary supplements containing heat-inactivated LR significantly modulated the expression levels of cytokines and haptoglobin. Serum cytokine levels, including TNF-α, IL-1α, IL-1β, IL-18, IL-10, IL-1ra, IL-4, IL-6, IL-2, IL-12, IFN-γ, GM-CSF, and IL-8 in weaned pigs were significantly higher in the heat-inactivated LR group than in the control group (P<0.05; Table 2). However, serum haptoglobin levels in weaned piglets were lower in the heat-inactivated LR treatment group than in the control

Table 2: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on cytokine levels and haptoglobin in weaned piglets

Parameters	T1	T2	T3	T4	T5
TNF-α (pg/ml)	35.22±1.52	61.47±3.46***	72.26±1.25***	62.86±1.08***	71.15±2.72***
IL-1α (pg/ml)	35.53±0.84	44.20±2.50*	68.14±2.21***	61.47±2.16***	42.30±2.66
IL-1β (pg/ml)	51.03±1.96	63.72±2.75**	63.05±1.70*	65.05±4.10**	64.22±2.19**
IL-18 (pg/ml)	17.06±0.65	22.66±1.08**	25.46±1.24***	20.66±1.28	19.86±0.45
IL-10 (pg/ml)	19.16±1.95	37.86±0.74***	55.73±2.91***	38.30±0.77***	43.86±0.66***
IL-1ra (pg/ml)	29.25±2.74	42.47±3.45	56.18±4.09***	72.96±3.22***	46.77±2.74*
IL-4 (pg/ml)	37.17±2.37	47.65±2.12	59.39±2.45	53.04±2.45	56.23±4.25
IL-6 (pg/ml)	57.33±1.58	79.17±1.76***	82.23±1.15***	76.47±2.03***	73.41±2.61**
IL-2 (pg/ml)	98.66±2.94	134.33±8.26***	149.02±2.36***	163.95±2.95***	119.26±2.35*
IL-12 (pg/ml)	671.59±26.32	821.15±12.77***	846.07±11.31***	805.69±31.67**	770.06±15.90*
IFN-γ (pg/ml)	53.96±3.22	76.11±2.20***	86.92±1.73***	73.56±2.43***	65.95±3.43*
GM-CSF (pg/ml)	17.59±0.47	24.70±1.21***	32.78±1.00***	23.23±0.90**	24.19±0.80***
IL-8 (pg/ml)	668.13±28.29	1052.99±19.04***	1084.42±10.60***	867.11±9.69***	1269.30±25.20***
Haptoglobin (ng/ml)	125.33±11.34	63.63±7.89***	88.84±4.39*	65.57±9.65***	50.30±7.54***

Note: Data are presented as mean ± SEM (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 vs. T1 group.

Table 3: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on pathogenic bacterial counts in faeces, rectal swabs, and nasal swabs in weaned piglets.

Group	T1	T2	T3	T4	T5
Bacteria (Log CFU/ml)			Faeces		
Lactobacillus spp.	7.12±0.23	8.76±0.02**	9.55±0.26***	9.04±0.32**	8.53±0.11*
E. coli	7.35±0.18	5.39±0.20*	6.72±0.62	6.98±0.06	6.92±0.19
Salmonella spp.	5.73±0.44	4.63±0.27	5.23±0.22	5.19±0.10	5.43±0.16
Staphylococcus aureus	6.26±0.19	5.59±0.01**	5.49±0.03**	5.58±0.04**	5.52±0.03**
Clostridium perfringens	5.68±0.20	4.89±0.05	5.11±0.15	5.30±0.19	5.50±0.16
			Rectal swab		
Lactobacillus spp.	5.37±0.15	6.35±0.06*	5.87±0.16	7.81±0.17**	6.74±0.40*
E. coli	6.96±0.73	3.02±0.32**	5.18±0.69*	3.09±0.34*	5.41±0.54*
Salmonella spp.	6.15±0.41	2.93±0.16***	5.36±0.09*	2.56±0.21***	5.13±0.56*
Staphyloccus aureus	6.25±0.01	4.98±0.31	4.99±0.38	4.30±0.48*	6.11±0.33
Clostridium perfringens	5.86±0.38	4.35±0.26	5.14±0.53	4.20±0.29	5.75±0.22
			Nasal swab		
Lactobacillus spp.	5.44±0.30	6.58±0.61	6.59±0.28*	5.98±0.27	6.08±0.17
E. coli	5.66±0.02	4.16±0.17*	4.61±0.17*	4.74±0.24	5.04±0.44
Salmonella spp.	4.73±0.07	4.08±0.19*	4.29±0.11*	3.75±0.20	4.02±0.21
Staphyloccus aureus	5.77±0.25	5.68±0.14	4.58±0.12*	4.79±0.33*	5.47±0.08
Clostridium perfringens	6.43±0.27	5.57±0.15*	5.19±0.40*	4.59±0.35**	4.34±0.12**

Data are presented as mean ± SEM (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001 vs. T1 group.

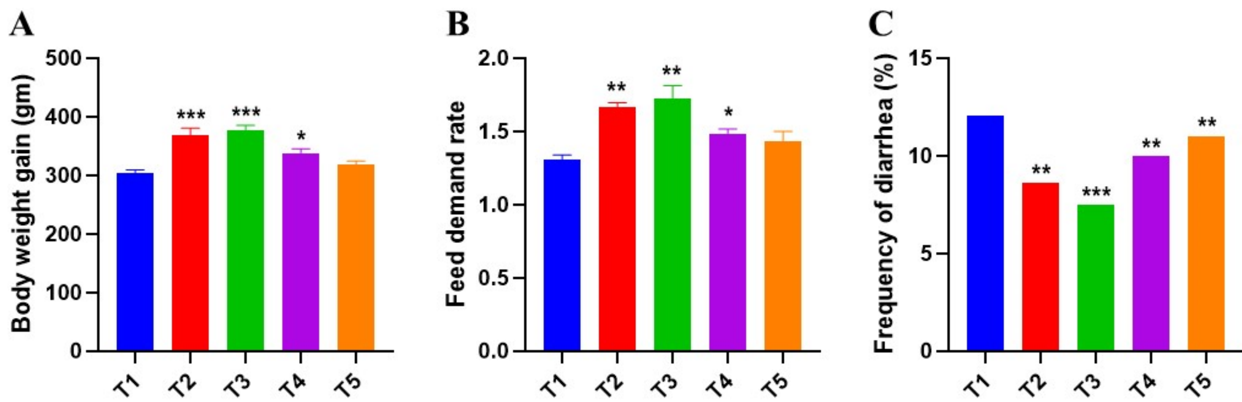

Fig. 1: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on growth performance and diarrhoea frequency in weaned piglets. (A) Average daily body weight gain, (B) Feed demand rate, and (C) Diarrheal frequency in weaned piglets. Data are presented as mean ± SEM (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. T1 group.

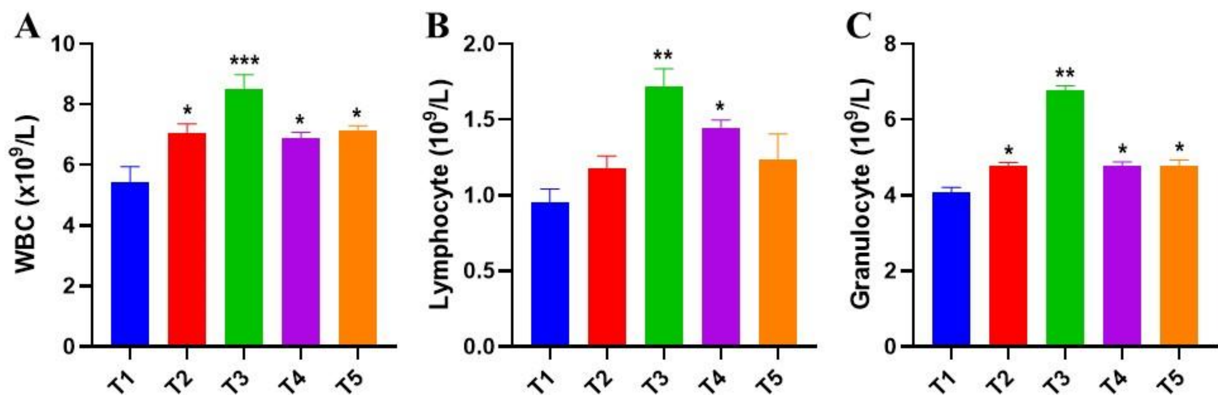

Fig. 2: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on immune blood cell counts in weaned piglets. (A) White blood cells (WBC), (B) Lymphocytes, and (C) Granulocytes. Data are presented as mean ± SEM (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. T1 group.

group (P<0.05; Table 2). In addition, the dietary heat-inactivated LR group showed a significant rise in the number of immune blood cells, such as white blood cells (WBCs), lymphocytes, and granulocytes, suggesting that heat-inactivated LR activates the hematopoietic system in pigs (P<0.05; Fig. 2).

Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on pathogenic bacteria in weaned piglets: The probiotic mixture demonstrated a favourable effect on the experimental group that was fed the heat-inactivated LR supplement compared to the control group. The probiotic bacterial (*Lactobacillus* spp.) counts were significantly elevated in the faeces, rectal, and nasal swabs of treatment groups (P<0.05; Table 3). For pathogenic bacteria such as *E. coli*, *Salmonella* spp., *Clostridium perfringens*, and *Staphylococcus aureus*, the heat-inactivated LR supplement significantly decreased their counts (P<0.05; Table 3). The ability to reduce pathogenic bacteria and increase beneficial bacteria was greater in the mixture of heat-inactivated LR with antibiotics than in the heat-inactivated LR group. Consequently, the application of probiotic and antibiotic combinations had a significant effect on reducing harmful microorganisms.

Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on weaned piglet microbiota: The potential effects of dietary heat-inactivated LR on the microbiota of weaned piglets were evaluated by analysing the alpha diversity and taxonomic composition at the phylum, family, and genus levels. The alpha diversity of gut microbiota was assessed using Chao1, ACE, Shannon, and Simpson indices, indicators of bacterial diversity and community richness. The results showed a consistent trend of increasing these indices (Chao1, ACE, Shannon, and Simpson) (Fig. 3). Moreover, at the phylum level, Firmicutes, Bacteriodetes, and Proteobacteria constituted the predominant phyla, accounting for >98% of all microorganisms (Fig. 4A, B). However, following oral administration of a heat-inactivated LR diet, a higher relative abundance of Bacteriodetes was observed, with lower levels of Firmicutes. Nevertheless, these changes were not statistically significant across the groups. The composition of other bacteria at the phylum level was low, potentially not affecting the outcomes. At the family level, three major families were detected (Fig. 4C, D). The relative abundances of the families *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were higher in the heat-inactivated LR group than in the control group. However, similar to the phylum level, the composition of other bacteria, such as *Muribaculaceae*, *Peptostreptococcaceae*, *Acidaminococcaceae*, and *Selenomonadaceae*, was below 5% of the total composition. At the genus level, Prevotella and Roseburia constituted the majority of the composition (Fig. 4E, F). The composition of other bacteria, such as *Clostridium*, *Faecalibacterium*, *Oscillibacter*, *Agathobacter*, *Terrisporobacter*, *Alloprevotella*, *Anaerovibrio*, and *Lactobacillus,* were also less abundant.

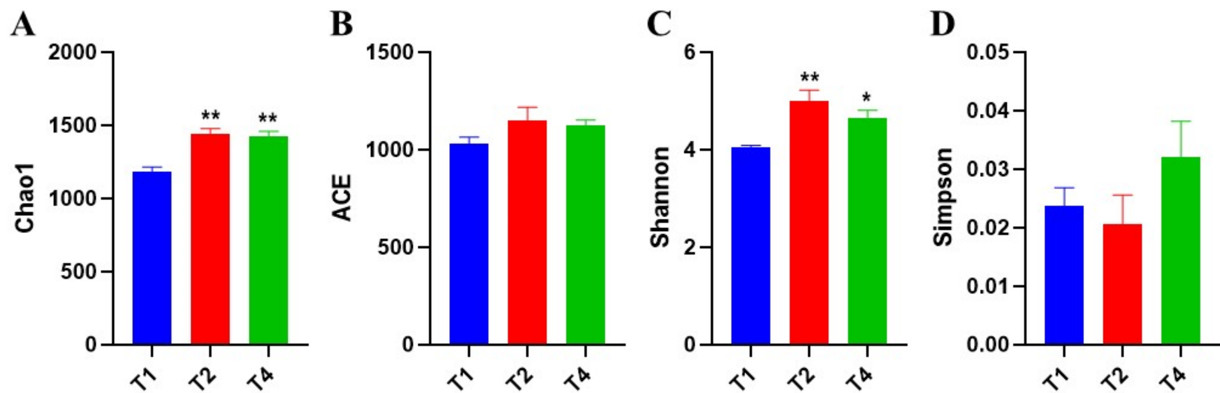

Fig. 3: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on the faecal microbiota in weaned piglets. Alpha diversity was evaluated by measuring (A) Chao1, (B) ACE, (C) Shannon, and (D) Simpson indices. Data are presented as mean ± SEM (n = 5). *$p < 0.05$ and **$p < 0.01$ vs. T1 group.

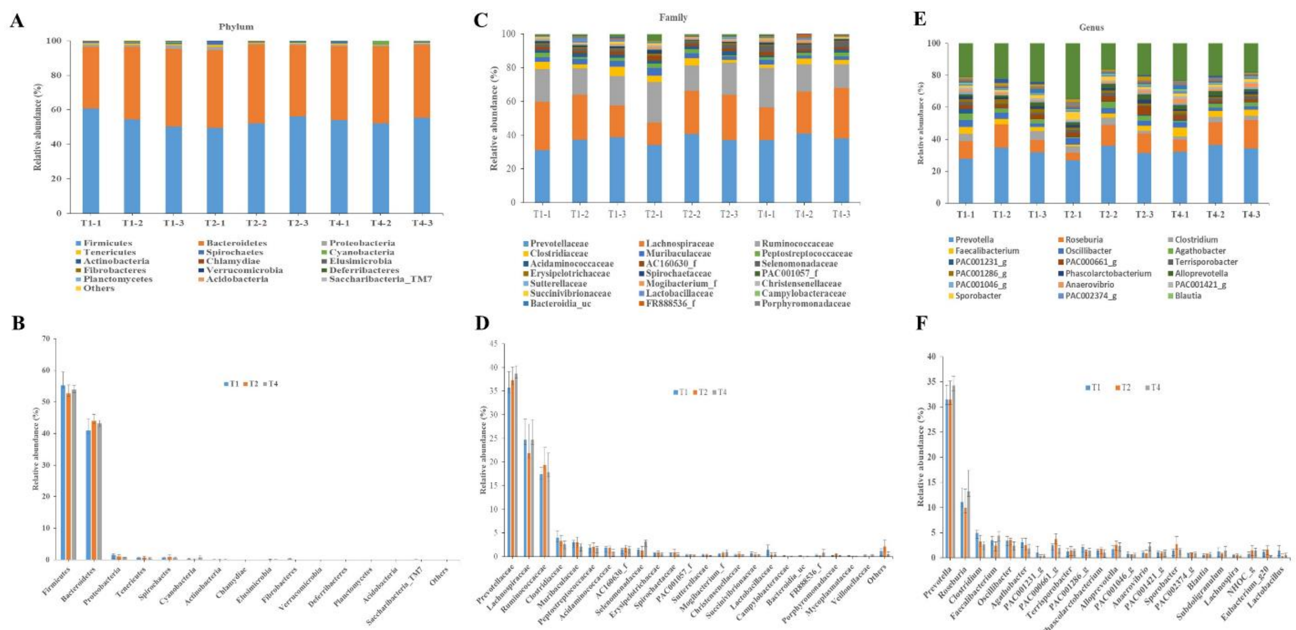

Fig. 4: Effects of heat-inactivated *Limosilactobacillus reuteri* PSC102 on the faecal microbiota in weaned piglets. Microbiota taxon composition at the (A, B) Phylum, (C, D) Family, and (E, F) Genus levels. Data are presented as the mean ± SEM (n = 3).

DISCUSSION

Weaning of the piglets to the maturing immune system is a significant challenge as it adjusts to colonizing gastrointestinal microbes and the transition from milk to solid feed. The swine industry has long routinely used antibiotics to mitigate stress during the weaning period, enhance growth, and prevent infections (Cromwell, 2002). However, due to side effects, such as antimicrobial resistance, many countries have banned antimicrobials in swine production, while probiotics are used as an alternative to antibiotics (Arsène *et al.*, 2021). Nevertheless, the live probiotics can potentially infect and transfer antibiotic resistance to the host (Daniali *et al.*, 2020). Therefore, the use of inactivated probiotics as feed in the swine industry has been increasing day by day.

We found that dietary intake of heat-inactivated LR significantly enhanced the growth rate and feed efficiency and reduced the diarrheal frequency in the weaned piglets. This finding is consistent with that of previous studies demonstrating that live and heat-inactivated *Lactobacillus* species promote weight gain in mice (Ali *et al.*, 2022), rats (Ali *et al.*, 2024), fish (Sewaka *et al.*, 2019), and pigs (Busanello *et al.*, 2015), while alleviating diarrhoea in humans and other animals (Li *et al.*, 2012; Xavier-Santos *et al.*, 2022). ETEC infection induces post-weaning diarrhoea in pigs by increasing the luminal volume of electrolytes and fluids while decreasing ETEC absorption into intestinal cells (Tang *et al.*, 2024). Additionally, the ETEC infection increases paracellular permeability, allowing pathogens and toxins to enter the lumen, elevate inflammatory responses, and inhibit animal growth. The findings of this study could be attributed to the ability of live and inactive probiotics to inhibit pathogen adhesion to intestinal barriers and regulate the immune systems (de Almada *et al.*, 2016).

In this study, feeding dietary supplements containing heat-inactivated LR markedly elevated the levels of all tested cytokines but reduced haptoglobin expression in piglets. Previous investigations showed that live and heat-inactivated *Lactobacillus* elevated cytokine levels in experimental mice (Li *et al.*, 2009; Ou *et al.*, 2011; Ali *et al.*, 2022) but decreased haptoglobin levels in pigs (Pereira *et al.*, 2022). Acute phase proteins, such as

haptoglobin, increase in concentration following trauma, infections, and inflammation. During tissue damage and haemolytic anaemia, haptoglobin levels increase to prevent haemoglobin loss. In this study, haptoglobin levels were significantly reduced in all treatment groups, including the heat-inactivated LR group, compared to that in the control group. This suggests that heat-inactivated LR can help recover haemoglobin loss in diarrheal piglets. Additionally, the number of immune blood cells significantly increased in the dietary heat-inactivated LR group, indicating that heat-inactivated LR stimulates the hematopoietic system in pigs. A previous report indicates that oral treatment with heat-inactivated LR increases immune blood cell counts in mice (Ali *et al.*, 2022). Weaned pigs frequently experience social, physical, and physiological challenges that escalate intestinal permeability and suppress their immune systems (Xiao *et al.*, 2016). EPS produced by *Lactobacillus* was found to regulate immune responses by controlling cytokine release and preserving sticky surface polymers to prevent infections from attaching to the intestinal barrier (Castro-Bravo *et al.*, 2018).

The administration of heat-inactivated LR supplements elevated the number of beneficial bacteria but decreased the pathogenic bacteria in the feces, rectal, and nasal swab samples of weaned piglets. It was revealed that pathogenic bacteria significantly affect the prevalence of diarrheal illnesses in weaned piglets (Franz *et al.*, 2011). Moreover, several studies show that probiotics influence the composition of gut microflora by increasing the number of beneficial microorganisms and decreasing the number of harmful ones. This potential effect of heat-inactivated LR was confirmed in this study, aligning with the findings of previous research (Ali *et al.*, 2024). Probiotics can be used to treat intestinal infections, diarrhoea, and constipation and enhance the resistance of an organism (Pospíšková *et al.*, 2013). This study showed an increase in the probiotic strain *Lactobacillus* spp. counts and a reduction in the growth of potentially harmful pathogens such as *E. coli*, *Salmonella* spp., *Clostridium perfringens,* and *Staphylococcus aureus* in the treatment groups. These bacteria can cause various illnesses and diarrheal disorders, which are highly undesirable in pig farming (Canibe *et al.*, 2022).

We found that the administration of heat-inactivated LR intervenes in the gut microbiota in weaned piglets by modulating the taxonomic composition at different levels, including phylum, family, and genus. Gut microbiota influences gut homeostasis and the immune system by inducing immunological responses and preserving epithelial barrier function. Additionally, studies show that cytokines can influence intestinal barrier functions, which are associated with the relative abundance of *Prevotellaceae* (Million *et al.*, 2018). Toll-like receptor 4 (TLR4) can recognize Bacteroidetes, a genus of Gram-negative bacteria primarily composed of lipopolysaccharides (LPS). Furthermore, TLR4 can activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). The increased p65 expression indicates that this NF-kB pathway was activated, promoting the release of cytokines such as TNF-α and IL-1β (Yu *et al.*, 2020). Reports suggest that Bacteroidetes can improve immunological and metabolic diseases in individuals with obesity (Liebana-Garcia *et al.*, 2021). Bacteroidetes may enhance immune defence by reversing declines in TNF-α expression caused by a high-fat diet and restoring the ability of dendritic cells to facilitate T-cell proliferation (Scheithauer *et al.*, 2020).

Conclusions: In conclusion, the dietary heat-inactivated LR enhanced growth rate and feed efficiency and reduced post-weaning diarrhea in piglets. Moreover, heat-inactivated LR supplements improved immune responses by elevating cytokine levels and immune blood cells. In addition, dietary feeding of heat-inactivated LR supplements increased the beneficial bacteria and decreased pathogenic bacterial counts, modulating the gut microbiota composition in weaned piglets. Therefore, these findings suggest that heat-inactivated LR can be used as an effective immunomodulating agent in pigs and other animals.

Authors contributions: SCP and MSA developed the concept. HYC, GYL, and MSA performed the experiments, analyzed the data, and wrote the manuscript. SCP reviewed the manuscript and acquired fund.

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