



■ Author(s)

Chen F¹
Zhu L¹
Qiu H^{II}

¹ Institute of Animal Nutritional and Metabolic Disorders, College of Animal Science and Veterinary Medicine, Qingdao Agricultural University, Qingdao, 266109, China

^{II} Haidu College, Qingdao Agricultural University, Laiyang, 265200, China

■ Mail Address

Corresponding author e-mail address
Huiling Qiu
Haidu College, Qingdao Agricultural University, Laiyang, 265200, China
Tel: +86-535-2922076
Email: hlqiu_njau@163.com

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Isolation and Probiotic Potential of *Lactobacillus salivarius* and *Pediococcus pentosaceus* in Specific Pathogen Free Chickens

ABSTRACT

This study was conducted to isolate *Lactobacillus salivarius* and *Pediococcus pentosaceus* strains from cecal content and investigate their probiotic potential in specific pathogen free (SPF) chickens. *L. salivarius* and *P. pentosaceus* strains were isolated from the cecal content of SPF chickens and identified by 16s rDNA sequence analysis by BLAST analysis at the National Center for Biotechnology Information and phylogenetic analysis using DNASTar software. In an *in vivo* experiment, 180 7-day-old SPF chickens were randomly assigned into three groups. Group 1 served as a control that was fed a basal diet without probiotic supplementation, and groups 2 and 3 were fed the basal diets supplemented with *L. salivarius* and *P. pentosaceus* at 2×10^8 CFU/g, respectively. Body weight (BW), average daily gain (ADG), feed conversion ratio (FCR), dressing percentage (DP), and the apparent digestibility of crude protein (AD-CP) were calculated. We also determined meat color, fat content, shear force, water content and pH value of breast and thigh muscles; ammonia, urea nitrogen and uric acid content in plasma; fecal ammonia emission level and pH value; and *Lactobacillus* and *Escherichia coli* in ceca. Compared with the control group, *L. salivarius* and *P. pentosaceus* supplementation significantly increased BW, ADG, DP, AD-CP, fat content of meat, and the number of *Lactobacillus* in ceca ($p < 0.05$), and decreased FCR, plasma ammonia content, fecal ammonia emission, and pH value and the number of *E. coli* in ceca ($p < 0.05$). In the *in vitro* experiment, *L. salivarius* and *P. pentosaceus* treatments significantly decreased the ammonia content in medium compared with the control group without probiotic treatment ($p < 0.05$). These results suggest that *P. pentosaceus* and *L. salivarius* strains show promising probiotic properties for improving growth, meat quality and microenvironment in chickens and decreasing ammonia content in the medium.

INTRODUCTION

Probiotics such as *Lactobacillus*, *Pediococcus*, and *Bifidobacterium* have been defined as "a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance" and widely distributed in nature, vegetables, dairy products, and the gastrointestinal tract of humans and animals (Carraro *et al.*, 2011; Vidhyasagar *et al.*, 2013). Increasing evidence indicates that *Lactobacillus* and *Pediococcus* strains exert beneficial effects by a variety of complementary mechanisms, including an ability to modulate host immune function, high adhesion capacity, cancer-preventing attributes, reduction of serum cholesterol levels, high antimicrobial activity against pathogens, and normalization of the microbiota composition (Okamoto *et al.*, 2007; Noujaim *et al.*, 2009;



Meng *et al.*, 2010; Zhang *et al.*, 2011; Kiran *et al.*, 2015; Xie *et al.*, 2015; Zhang *et al.*, 2015). Promising probiotic strains should have some superior biological properties, especially improving growth performance, intestinal microbiota balance, and meat quality, and decreasing noxious gas emission (Meng *et al.*, 2010; Fan *et al.*, 2013; Jeong *et al.*, 2014; Peng *et al.*, 2016). These have become essential indicators for evaluating the potential of food biopreservatives or probiotic additives in humans and animals (Kabir *et al.*, 2004; Biagi *et al.*, 2007; Nagpal *et al.*, 2012; Mookiah *et al.*, 2014; Altaher *et al.*, 2015; Peng *et al.*, 2016; Bai *et al.*, 2017). Therefore, there is growing interest in the isolation of potentially successful probiotic strains and determination of their functional characteristics.

Pediococcus pentosaceus and *Lactobacillus salivarius* are most frequently isolated from the duodenal mucosa of piglets (Zhang *et al.*, 2011; Zhang *et al.*, 2015) and human breast milk (Osmanagaoglu *et al.*, 2010; Kiran *et al.*, 2015), but little information is available about the isolated strains from animals and their bioavailability. Therefore, the objectives of this study were to isolate and identify *P. pentosaceus* and *L. salivarius* strains from cecal content and investigate their potential probiotic effects on growth, meat quality and microenvironment in specific pathogen free (SPF) chickens, and ammonia treatment *in vitro*.

MATERIAL AND METHODS

Identification of *L. salivarius* and *P. pentosaceus* strains

The cecal content from 20-day old SPF chickens was used for isolation of lactic acid bacteria. The cecal content was serially diluted 10-fold with phosphate-buffered saline (PBS, pH 7.4), plated on MRS agar, and incubated for 48 h at 37°C in an anaerobic incubator. Colonies were spread into MRS broth medium and incubated for 24 h at 37°C. Genomic DNA was extracted using the DNA extraction kit (TaKaRa Biotechnology, Dalian, China) and 16s rDNA gene was amplified using the primers: 5'-agagtttgatcctggctcag-3' and 5'-ggttacctgttacctgactt-3' and PCR kit (TaKaRa Biotechnology). PCR products were sequenced with an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at TaKaRa Biotechnology. Nucleotide sequences were analyzed by BLAST analysis at the National Center for Biotechnology Information (Bethesda, MD, USA), and phylogenetic tree and homology analyses were performed using the SeqEd and Cluster V sequence

alignment programs in the DNASTar software package (DNASTAR Inc., Wisconsin, USA). *L. salivarius* (QDLS-CF-01–QDLS-CF-02) and *P. pentosaceus* (QDPP-CF-01–QDPP-CF-04) isolates were selected and propagated at 37°C for 48 h. The freeze-dried bacterial powder containing 10¹⁰ CFU/g was prepared and stored in sterile tubes at 4°C.

Experimental animals and diets

One hundred and eighty 7-day-old SPF male chickens with an initial body weight (BW) of 43.5 ± 2.2 g were purchased from Institute of Animal Husbandry and Veterinary Medicine, Shandong Academy of Agricultural Sciences and randomly allocated to three experimental treatments with three replicate pens of 20 chickens. Group 1 was fed a basal diet without probiotic supplementation, and groups 2 and 3 were fed the basal diet mixed with *L. salivarius* or *P. pentosaceus* at 2 × 10⁷ CFU/g, respectively. The basal diet was a corn–soybean meal-based diet, which was formulated to approximately meet National Research Council-recommended nutrient requirements for chickens (1994). The basal diet formulation and approximate composition are shown in Table 1. The feeding experiment lasted for 24 days and was carried out at Laboratory Animal Centre, Qingdao Agricultural University. Over the entire experimental period, water and feed were provided *ad libitum*, and the residual feed was collected from the cages daily.

Table 1 – Effects of *Lactobacillus salivarius* and *Pediococcus pentosaceus* supplementation on growth performance of chickens

Group/treatment	<i>L. salivarius</i>	<i>P. pentosaceus</i>	Control
BW (d 12, g)	125.0±5.10 ^a	129.7±2.05 ^a	107.3±3.77 ^b
BW (d 24, g)	239.8±5.68 ^a	256.3±10.5 ^a	209.9±12.1 ^b
ADG (d 1–12, g/d)	7.42±0.37 ^a	7.73±0.15 ^a	5.89±0.28 ^b
ADG (d 13–24, g/d)	9.57±0.14 ^b	10.55±0.71 ^a	8.64±0.49 ^c
ADG (d 1–24, g/d)	8.54±0.19 ^b	9.20±0.44 ^a	7.23±0.53 ^c
FCR (d 1–12)	1.67±0.09 ^b	1.60±0.03 ^b	2.10±0.10 ^a
FCR (d 13–24)	2.93±0.02 ^b	2.70±0.18 ^b	3.29±0.18 ^a
FCR (d 1–24)	2.42±0.06 ^b	2.25±0.11 ^b	2.87±0.19 ^a
AD-CP	46.7±0.89 ^a	45.2±0.67 ^a	41.5±0.71 ^b
DP (%)	92.3±0.87 ^a	92.0±1.62 ^a	86.4±0.36 ^b
HEYP (%)	84.8±0.57 ^a	81.4±1.57 ^b	79.9±0.89 ^b
EYP (%)	60.6±1.07 ^a	58.9±1.40 ^{ab}	56.9±1.09 ^b
TMP (%)	6.03±0.58	6.09±0.48	5.85±0.12
BMP (%)	4.70±0.35	4.61±0.20	4.20±0.37

Means in the same line with different letters were significantly different ($p < 0.05$). Data are presented as means ± SD.



Evaluation of growth performance

On days 12 and 24, BW and feed were recorded, and average daily gain (ADG) and feed conversion ratio (FCR) were calculated. The crude protein in feed and feces was analyzed and the apparent digestibility of crude protein (AD-CP) was calculated. On day 24, all chickens were killed with dimethyl ether. Dressing percentage (DP), the percentages of eviscerated yield (EYP) and the half eviscerated yield (HEYP), and the percentages of breast (BMP) and thigh muscle (TMP) were calculated.

Evaluation of microenvironment

On days 12 and 24, the ammonia emission level was detected by Nessler's reagent colorimetry. On day 24, anticoagulant blood samples were drawn from the heart and centrifuged at $3,000 \times g$ for 15 min, and plasma was collected for analysis of ammonia, uric acid and urea nitrogen (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

On day 24, 10 chickens in each replicate were randomly selected, and the ceca were removed for measuring pH value by using a digital pH meter with a 1.5-mm microelectrode (PHS-3C; Shanghai REX Instrument Factory, China). For the measurement of cecal *E. coli* and *Lactobacillus*, the cecal contents of each chicken were thoroughly mixed in a 50 mL plastic centrifuge tube and diluted 10^6 – 10^7 folds with PBS (pH 7.4) by the multiple dilution method. Then, 1.0 mL aliquots were spread into Eosin Methylene Blue Agar or *Lactobacillus* Selection Agar plates, and incubated for 24–48 h at 37°C. Colonies were counted, and the results were expressed as absolute numbers of CFU. The experiments were performed in triplicate, and the average values were calculated.

Evaluation of meat quality

On day 24, breast and thigh muscles were collected and used for detecting meat color, tenderness, fat content, water content and pH value within 24 h. Meat color was evaluated by measuring total pigment content; pH value of muscle was measured by a digital pH meter (PH-STAR; Matthaus, Eckelsheim, Germany); tenderness was represented by shear force, which was measured by a digital muscle tenderness meter (C-LM3; Tenovo International, Beijing, China); water content was measured by the drying method; and fat content was measured by Soxhlet extractor method (Thiex *et al.*, 2003).

Evaluation of ammonia treatment *in vitro*

Sixty tubes were randomly allocated to three experimental treatments, each containing 20 tubes,

and each tube contained 3 mL PBS (pH 7.4) with 0.5 $\mu\text{mol/L}$ ammonium chloride. Group 1 was treated without probiotic supplementation, and groups 2 and 3 were treated with *L. salivarius* or *P. pentosaceus* at 108 CFU/mL, respectively. The tubes were incubated in the air rocking incubator (Thermo Fisher Scientific Inc., Massachusetts, USA) at 37°C for 48 h. Five tubes in each treatment were randomly chosen at 12, 24, 36 and 48 h to determine the ammonia content in medium (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis

All data were expressed as means \pm standard deviation (SD) and SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) was used to perform an analysis of variance test. $p < 0.05$ was considered significant.

RESULTS

Identification of *L. salivarius* and *P. pentosaceus* strains

Using the BLAST sequence analysis services of NCBI, QDPP-CF-01–QDPP-CF-04 were most similar to the sequences of *P. pentosaceus* (KJ477391 and KX262845) with 97%–98% sequence identity. QDLS-CF-01–QDLS-CF-02 were most similar to the sequences of *L. salivarius* (AF089108 and AY112743) with 98%–99% sequence identity. Phylogenetic tree and sequence homology analyses (Figure 1) showed that QDPP-CF-01–QDPP-CF-04 were placed in the *P. pentosaceus* branch with 97.8%–98.1% sequence homology and belonged to the species *P. pentosaceus*. QDLS-CF-01–QDLS-CF-02 were placed in the *L. salivarius* branch with 97%–98% sequence homology and belonged to the species *L. salivarius*.

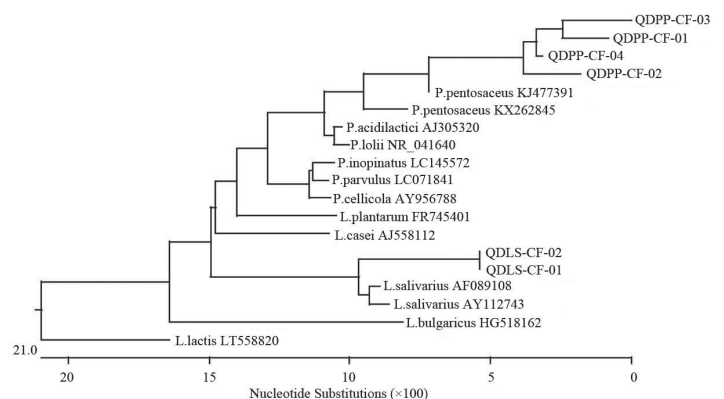


Figure 1 – Phylogenetic relationships among lactic acid bacteria species inferred by a neighbor-joining analysis of a fragment of the partial 16s rRNA sequence. QDLS-CF-01 ~ QDLS-CF-02 and QDPP-CF-01 ~ QDPP-CF-04 are the isolates of lactic acid bacteria from SPF chickens.



Effects of *L. salivarius* and *P. pentosaceus* supplementation on growth performance of chickens

Compared with the control group, *L. salivarius* and *P. pentosaceus* supplementation significantly increased BW, ADG, AD-CP, DP and HEYP and decreased FCR ($p < 0.05$) (Table 1), and *L. salivarius* supplementation significantly increased EYP ($p < 0.05$). ADG (d 13–24) and ADG (d 1–24) of *P. pentosaceus* supplementation

were significantly higher than that of *L. salivarius* supplementation ($p < 0.05$).

Effects of *L. salivarius* and *P. pentosaceus* supplementation on meat quality of chickens

Compared with the control group, *L. salivarius* and *P. pentosaceus* supplementation significantly increased fat content in meat ($p < 0.05$), and fat content of *P. pentosaceus* supplementation was significantly higher than that of *L. salivarius* supplementation ($p < 0.05$) (Table 2).

Table 2 – Effects of *Lactobacillus salivarius* and *Pediococcus pentosaceus* supplementation on meat quality of chickens

Group/treatment		<i>L. salivarius</i>	<i>P. pentosaceus</i>	Control
Thigh muscle	Color	0.56±0.04	0.50±0.03	0.42±0.12
	Shear force (N)	13.0±0.24	13.6±0.33	13.3±0.38
	Fat content (%)	15.3±0.35 ^b	17.7±0.48 ^a	12.4±0.31 ^c
	Water content (%)	64.9±3.43	63.5±2.15	65.2±3.13
	pH value	6.33±0.09	6.17±0.08	6.15±0.09
Breast muscle	Color	0.13±0.01	0.14±0.02	0.15±0.02
	Shear force (N)	11.1±0.42	10.9±0.34	11.2±0.24
	Fat content (%)	4.52±0.53 ^b	6.27±0.70 ^a	3.62±0.33 ^c
	Water content (%)	68.2±2.58	63.9±3.28	69.5±3.20
	pH value	5.68±0.05	5.77±0.08	5.76±0.15

Means in the same line with different letters were significantly different ($p < 0.05$). Data are presented as means ± SD.

Effects of *L. salivarius* and *P. pentosaceus* supplementation on microenvironment of chickens

Compared with the control group, *L. salivarius* and *P. pentosaceus* supplementation significantly increased the number of *Lactobacillus* and significantly decreased plasma ammonia content, pH value and the number of *E. coli* in ceca, and fecal ammonia emission on days 12 and 24 ($p < 0.05$) (Table 3). Fecal ammonia emission of *P. pentosaceus* supplementation was significantly

higher than that of *L. salivarius* supplementation on day 24 ($p < 0.05$).

Effects of *L. salivarius* and *P. pentosaceus* supplementation on ammonia content in medium

Compared with the control group, *L. salivarius* and *P. pentosaceus* supplementation significantly decreased ammonia content in medium at 12 h, 24 h, 36 h and 48 h ($p < 0.05$). *P. pentosaceus* supplementation significantly decreased ammonia content in medium

Table 3 – Effects of *Lactobacillus salivarius* and *Pediococcus pentosaceus* supplementation on microenvironment in chicken

Items	<i>L. salivarius</i>	<i>P. pentosaceus</i>	Control
Plasma ammonia content (mmol/L)	75.8±7.46 ^b	71.7±9.27 ^b	123.5±9.77 ^a
Plasma uric acid (mg/L)	34.6±2.23	35.5±2.11	32.8±3.95
Plasma urea nitrogen (mmol/L)	2.26±0.51	2.71±0.20	2.54±0.30
No. of <i>E. coli</i> in ceca (\log_{10} CFU/g)	7.28±0.18 ^b	7.19±0.31 ^b	8.20±0.23 ^a
No. of <i>Lactobacillus</i> in ceca (\log_{10} CFU/g)	8.86±0.42 ^a	8.32±0.22 ^a	7.80±0.16 ^b
pH value	5.60±0.14 ^b	5.62±0.14 ^b	6.14±0.27 ^a
Ammonia emission (12 d, mg/L)	2.42±0.15 ^b	2.62±0.44 ^b	7.64±1.06 ^a
Ammonia emission (24 d, mg/L)	3.89±0.22 ^b	2.39±0.46 ^c	8.49±0.16 ^a

Means in the same line with different letters were significantly different ($p < 0.05$). Data are presented as means ± SD.



more than *L. salivarius* supplementation did at 24 h and 36 h ($p < 0.05$) (Figure 2).

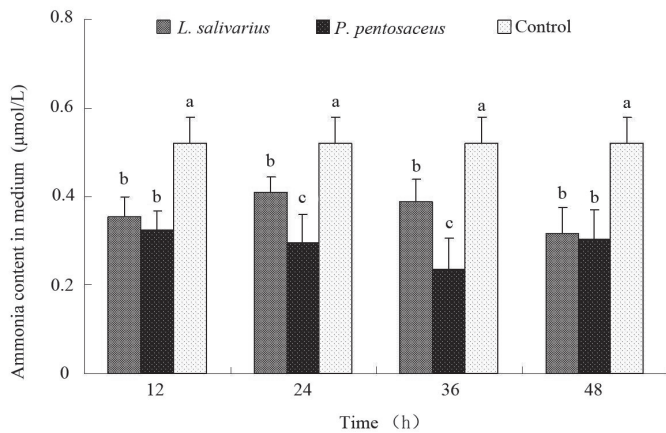


Figure 2 – Effects of *Lactobacillus salivarius* and *Pediococcus pentosaceus* supplementation on the ammonia content in medium. Data are presented as means \pm SD.

DISCUSSION

One of the most common methods to isolate and identify novel probiotic strains is phylogenetic and homology analysis of 16s rDNA sequences after cultivation in isolation medium (Chen *et al.*, 2013; Tulini *et al.*, 2013; Yanagida *et al.*, 2008). In the present study, six strains of lactic acid bacteria were isolated from the cecal content of SPF chickens on MRS Agar under anaerobic conditions. PCR products of the six strains were sequenced, and nucleotide sequences were most similar to the sequences of *P. pentosaceus* or *L. salivarius* with the highest sequence identity by BLAST sequence analysis and placed in the *P. pentosaceus* branch or the *L. salivarius* branch with the highest sequence homology by phylogenetic and homology analysis of 16s rDNA sequence. These results demonstrated that the six strains of lactic acid bacteria isolated from the cecal content of chickens belonged to the species *P. pentosaceus* and *L. salivarius*.

In this study, *L. salivarius* and *P. pentosaceus* supplementation improved growth performance by increasing BW, ADG, DP, AD-CP and HEYP and decreasing FCR of chickens, which is consistent with some previous studies of probiotics. Suo *et al.* (2012) reported that *Lactobacillus plantarum* ZJ316 significantly improved pig growth at a dose of 10^9 CFU/d, and ADG and FCR were better. Foo *et al.* (2003) reported that feeding *Lactobacillus* I-UL4 and the metabolites to weaned rats improved growth. Peng *et al.* (2015) reported that the basal diet supplemented with 2×10^9 CFU/kg *L. plantarum* B1 enhanced ADG and FCR in broilers. However, Lahteinen *et al.* (2014) reported that *Lactobacillus brevis* supplementation induced

a non-significant increase in BW of piglets. Many factors may contribute to the different performance, including the bacterial species and dosage, laboratory animals, and experimental conditions. We considered that the main reason may be that *L. salivarius* and *P. pentosaceus* isolated from chickens were used in the same laboratory animals.

L. salivarius and *P. pentosaceus* supplementation significantly improved *in vivo* and *in vitro* microenvironment by increasing the number of *Lactobacillus* in ceca and decreasing plasma ammonia content, pH value and the number of *E. coli* in ceca, and fecal ammonia emission. Many studies have reported that *Lactobacillus* spp. and *Bacillus* spp. had better effects on controlling ammonia emission and intestinal flora (Biagi *et al.*, 2007; Jeong and Kim, 2008; Zhang *et al.*, 2013; Ahmed *et al.*, 2014; Altaher *et al.*, 2015). Zhang *et al.* (2013) considered that ammonia emission was related to nutrient utilization and the intestinal microflora ecosystem, and some probiotics might reduce ammonia emission by improving enzyme activity and nitrogen utilization. Otherwise, some probiotics supplementation will result in a significant reduction in ammonia emission without any accompanying improvement in nitrogen digestibility. We considered that *L. salivarius* and *P. pentosaceus* improved the microenvironment in the following aspects. (i) *L. salivarius* and *P. pentosaceus* might secrete some antimicrobial compounds, such as organic acids, hydrogen peroxide and bacteriocins, which might have a potential modulating effect on the intestinal microflora and pathogen inhibition and decrease pH value in ceca. These compounds are unsuitable for the activity and proliferation of pathogens such as *E. coli*, but suitable for *L. salivarius* and *P. pentosaceus* populations. (ii) *L. salivarius* and *P. pentosaceus* could produce high levels of ammonia nitrogen and then increase AD-CP, which might be one of the reasons for reducing ammonia level. Moreover, the *in vitro* experiment showed that *L. salivarius* and *P. pentosaceus* significantly decreased the ammonia level in the medium. Therefore, we considered that *L. salivarius* and *P. pentosaceus* had a capacity for ammonia nitrogen fixation and enhanced ammonia nitrogen transformation. Further study is needed to elucidate other possible mechanisms of reducing ammonia emission.

L. salivarius and *P. pentosaceus* supplementation could improve meat quality; especially increasing the fat content in muscles. Many studies have demonstrated the potential effects of other probiotics on meat



quality, including *Clostridium butyricum* in pigs (Meng *et al.*, 2010) and *Bacillus coagulans* (Zhou *et al.*, 2010), *Bacillus subtilis* (Fan *et al.*, 2013; Park and Kim, 2014), *Enterococcus faecium* (Zheng *et al.*, 2014), *L. plantarum* (Loh *et al.*, 2013) and *Rhodopseudomonas palustris* (Xu *et al.*, 2014) in chickens. These results showed a lot of different indicators of the effects of probiotics on meat quality (Suo *et al.*, 2012). Zhang *et al.* (2015) considered that the shear force of raw drumstick decreased in the whole yeast treatment relative to the controls. Zhou *et al.* (2010) demonstrated that *B. coagulans* probiotic affected meat quality by decreasing the drip loss of broiler breast meats of Guangxi Yellow chickens. Meng *et al.* (2010) demonstrated that the probiotic treatment groups had increased meat color scores, marbling scores, and redness values in growing–finishing pigs. Until now, the functioning mechanism of probiotics (especially *Lactobacillus*) on meat quality (fat content in the study) remains unclear, although some investigators have attributed the effects of probiotics on meat quality to the role of bacteriocins or extracts (Zheng *et al.*, 2014). We considered that further study of the effects of *L. salivarius* and *P. pentosaceus* on meat quality was required.

In conclusion, the present study demonstrates that *L. salivarius* and *P. pentosaceus* improve growth performance, meat quality, ammonia emission and intestinal environment in chickens and are promising probiotic properties.

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