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# Recombinant plectasin elicits similar improvements in the performance and intestinal mucosa growth and activity in weaned pigs as an antibiotic

Jin Wan[a,1], Yan Li[a,b,1], Daiwen Chen[a], Bing Yu[a], Guang Chen[b], Ping Zheng[a], Xiangbing Mao[a], Jie Yu[a], Jun He[a,*]

[a] *Institute of Animal Nutrition, Sichuan Agricultural University, No. 211, Huimin Road, Wenjiang District, Chendu 611130, Sichuan, PR China*
[b] *Cheng Du Hua Luo Bio-Tech Col., Ltd, Chengdu 610062, Sichuan, PR China*

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ABSTRACT

This study was conducted to determine the effects of the recombinant plectasin (Ple) on growth performance and intestinal health in weaned pigs. Twenty-four weaned pigs were randomly assigned to one of four treatments, including a corn-soybean basal diet (Con), the basal diet supplemented with antibiotics (60 mg/kg colistin sulfate, Ant), probiotics (*Bacillus subtilis* $\geq 10^9$ CFU/g, Pro) and recombinant plectasin (60 mg/kg, Ple). The study lasted 21 d. Results showed that dietary supplementation with antibiotics and Ple had positive effects on the average daily feed intake (ADFI) and body-weight gain (ADG) ($P < 0.05$). Compared with the Con, the ratio of feed to gain (F/G) and diarrhea rate in both groups were significantly reduced. Pigs fed the diets containing antibiotics and Ple had a higher villus height ($P < 0.05$) and a higher disaccharidase activity ($P < 0.05$) in small intestinal mucosa. Ple supplementation also increased the abundance of *Bifidobacterium* in the ileum ($P < 0.05$). Interestingly, Ple elevated the absoption of xylose ($P < 0.05$) and the expression levels of tight junction protein *CLDN1* and *ZO-1* ($P < 0.05$) in the small intestinal mucosa. The results suggest Ple may be an effective alternative to antibiotics for the pork industry.



## 1. Introduction

Over the past decades, antibiotics have been widely used in the animal production as growth promoters and therapeutic medicines to decrease the susceptibility to infectious diseases (Barton, 2000). However, their continuous use not only leads to drug resistance (Monroe and Polk, 2000) but also increases the risk of residues in livestock products (Schwarz et al., 2001; Smith et al., 2002). Thus, the development of alternatives for antibiotics has attracted considerable research interest (Turner et al., 2001; Wang et al., 2007, 2011).

Antimicrobial peptides, isolated from a wide range of animals, plants, and bacterial species, are small cationic molecules (Hancock and Diamond, 2000; Brown and Hancock, 2006). Compared with conventional antibiotics, antimicrobial peptides

* Corresponding author. Tel.: +86 13419354223; fax: +86 28 86290922.
*E-mail addresses:* wanjin91@163.com (J. Wan), m18782044216@163.com (Y. Li), dwchen@sicau.edu.cn (D.W. Chen), ybingtian@yahoo.com.cn (B. Yu), cheng@cahic.com (G. Chen), Zpind05@163.com (P. Zheng), acatmxb2003@163.com (X.B. Mao ), jerryyujie@163.com (J. Yu), hejun8067@163.com (J. He).
[1] These authors contributed equally to this work.

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have a broader spectrum, a more rapid killing action and highly selective toxicity (Hancock and Patrzykat, 2002; Zasloff, 2002). Supplementation with lactoferrin, potato protein or antimicrobial peptide-A3 has been reported to have positive effects on growth performance, nutrient digestibility, intestinal morphology and intestinal microflora (Shan et al., 2007; Jin et al., 2008; Yoon et al., 2012). Most importantly, bacteria have difficulty in developing resistance against these peptides due to their ability to disrupt bacterial membranes via non-specific electrostatic interactions with the membrane lipid components (Chou et al., 2010). Therefore, antimicrobial peptides may have potential as an alternative to antibiotics for use in livestock industry.

Plectasin, the first antimicrobial defensin peptide to be isolated from a fungus (Mygind et al., 2005), has attracted considerable research interest due to its actions on multiple pathogens and the increasing resistance of microorganisms to conventional antibiotics (Hancock and Sahl, 2006). However, the production of plectasin from natural microorganisms is still not commercially feasible because of its low expression levels, weak stability and high cost. Currently, heterologous expression represents a potential approach for production of plectasin. In our laboratory, the *plectasin* gene of *Pseudoplectania nigrella*, coding plectasin, was previously expressed in *Pichia pastoris*. To evaluate its potential benefits for industrial applications, especially in the pork industry, high-density fermentation and antimicrobial characterization of Ple has been performed. To our best knowledge, this is the first paper to report the results of a study in which Ple was compared against antibiotics and a probiotic on the performance, gastrointestinal tract development and digestive activity in weaned pigs. Our hypothesis was that Ple would have similar effects on animal performance and health as antibiotics.

## 2. Materials and methods

### 2.1. Micro-organisms and media

The *plectasin* gene of a black saprophytic ascomycete, *P. nigrella*, coding plectasin was previously expressed in *P. pastoris* (PPle) and conserved in our laboratory. PPle was cultivated on either buffered glycerol-complex medium (1% yeast extract, 2% tryptone, 1.34% yeast nitrogen base, 1% glycerol) or buffered methanol-complex medium (1% yeast extract, 2% tryptone, 1.34% yeast nitrogen base, 1% methanol), respectively. Zeocin was added to a final concentration of 100 μg/mL.

### 2.2. Preparation of Ple in 30-L fermenter

The recombinant plectasin (Ple) used in this study was prepared in a 30-L fermenter (Nanjing Rune Bioengineering Equipment Co. Ltd, Nanjing, China) through high-density fermentation. PPle was cultured in 5 L Erlenmeyer flask containing 1 L of buffered glycerol-complex medium at 30 °C on a rotary shaker at 240 rpm. After 18–24 h incubation, cell pellets were harvested by centrifugation at 3000 × *g* for 10 min For Ple induction, the cell pellets were re-suspended in 10 L of buffered methanol-complex medium. The induction was maintained for 3 d by adding methanol (100%) to a final concentration of 1% every day. Relative percentage of dissolved oxygen was maintained above 30% via adjusting the agitation rate. Supernatant fraction samples were collected every day and kept at −80 °C before analysis. At the end of fermentation, all culture supernatant fraction was purified by $Ni^{2+}$-NTA affinity chromatography and subsequently used as the Ple source for the animal trial.

### 2.3. SDS–PAGE and in vitro antimicrobial activity assay

SDS–PAGE on 15% polyacrylamide was performed by the Laemmli method (Laemmli, 1970). The crude protein fractions (induction supernatant fraction) were boiled for 5 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue R 250 staining. The pH stability of Ple was determined after a 10-h incubation at 37 °C in different pH buffers. The buffers used were 100 mM glycine–HCl (pH 2.0) and 100 mM glycine–NaOH (pH 10.0). The proteinase solutions was performed at 37 °C, the Ple was pre-incubated with a bufferd solution containing 2500 U/mg of pepsin in glycine–HCl buffer (pH 2.0). Untreated Ple (pH 6.0) served as the independent control. Subsequent to each of these groups, the antimicrobial activity of Ple against *Staphylococcus aureus* ATCC 25923 was tested by the agar radial diffusion assay as described by Tian et al. (2009).

### 2.4. Animals, experimental design and diets

The experimental protocol used in this study was approved by the Animal Care and Use Committee of Sichuan Agricultural University (Chengdu, China). A total of 24 individually housed pigs (Duroc × Landrace × Yorkshire) weand at 24 d of age with initial body-weight (BW) of 7.67 (±0.32) kg were randomly assigned to one of four treatments (6 pigs/treatment). Treatments consisted of: (1) control without supplementation (Con); (2) antibiotics added at a concentration of 60 mg/kg diet (colistin sulfate, Ant); (3) probiotics added at a $10^9$ CFU/g diet (*Bacillus subtilis*, Pro); (4) recombinant plectasin added at a concentration of 60 mg/kg diet (Ple). The pigs were housed individually in metabolism cages (0.7 × 1.5 m) with woven wire flooring in an environmentally controlled room (25–28 °C). All pigs were given ad libitum access to water through a water nipple. After an adaptation period of 5 d, pigs were fed their respective diets four times per day at 08.00, 12.00, 16.00 and 20.00 h for a 21-d period. The diet adaptation period was followed by a collection period, which included a 4 d collection of feces to determine apparent digestibility of nutrients.

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