Probiotic *Lactobacillus casei* Expressing Porcine Antimicrobial Peptide PR39 Elevates Antibacterial Activity in the Gastrointestinal Tract

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4 Running title: *L. casei* Expressing the Antimicrobial Peptide PR39

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Abstract

PR39, a 4.7-kDa proline-rich antimicrobial peptide, acts as a cationic host defense peptide. In addition to killing bacteria, PR39 mediates inflammatory reactions, including cell proliferation, migration, wound healing, and angiogenesis. Here, we examined the antibacterial effects of this peptide. The synthetic gene fragment PR39 was inserted into the secretory expression vector plasmid pPG:612 of *Lactobacillus casei*, yielding the recombinant strain pPG:612-PR39/*L. casei* 393. In vitro antibacterial tests showed that expression of the PR39 peptide in recombinant *L. casei* resulted in antibacterial activity against *Escherichia coli* and *Salmonella*, but had only minor antibacterial effects in *Staphylococcus aureus*. In addition, BALB/c mice fed the recombinant pPG:612-PR39/*L. casei* 393 grew better and had increased peripheral blood lymphocyte percentages, white blood cell numbers, and spleen indices compared with those of the control group. Scanning electron microscopy showed that jejunum and duodenum villus height, crypt depth, and the ratio of V/C in the intestinal villi also increased. Moreover, mice fed the recombinant strain showed significantly lower mortality rates than the control group when challenged with the enterotoxigenic *E. coli* K88⁺ (ETEC K88⁺). Thus, this recombinant expression system had beneficial characteristics of both *Lactobacillus casei* and PR39, supporting its potential as an animal feed additive.

Keywords: Antimicrobial peptide PR39, *Lactobacillus casei*, recombinant expression, antimicrobial activity

37 Introduction

38 Antimicrobial peptides (AMPs) play fundamental roles in innate immunity owing to their
39 direct antimicrobial activity and other functions (Bals 2000; Zasloff 2002). 2684 AMPs (266
40 bacteriocins from bacteria, 4 from archaea, 8 from protists, 13 from fungi, 329 from plants,
41 and 2018 from animals) have been reported in the Antimicrobial Peptide Database
42 (<http://aps.unmc.edu/AP/main.php>). These AMPs have antibacterial, antiviral (anti-human
43 immunodeficiency virus [HIV]), antifungal, antiparasitic (antimalarial), anticancer, antiprotist,
44 insecticidal, spermicidal, chemotactic, and antioxidant activities and can function as protease
45 inhibitors. Among these endogenous AMPs, the 4.7 kDa PR39, a member of the cathelicidin
46 family, was first identified in pig intestinal tissue and later in neutrophilic granulocytes
47 (Boman 1995; Sang and Blecha 2009; Zanetti 2004). Like other proline-arginine-rich
48 peptides, PR39 has a broad antimicrobial spectrum against *Escherichia coli* ATCC 25922, *E.*
49 *coli* K881, *Salmonella* Typhimurium, *Salmonella* Choleraesuis, *Streptococcus suis*, and
50 *Staphylococcus aureus* (Shi et al. 1996). PR39 is thought to kill bacteria by interfering with
51 DNA and/or protein synthesis (Boman et al. 1993), rather than changing membrane
52 permeability (Litteri and Romeo 1993; Maloy and Kari 1995). However, the mechanism of
53 action has not been fully elucidated (Deslouches et al. 2005; Dürr et al. 2006). Moreover, in
54 addition to its known effects on the neutrophil NADPH oxidase in the context of host-derived
55 inflammation, PR39 has also been shown to be effective for induction of angiogenesis and
56 promotion of syndecan expression in mesenchymal cells (Gallo et al. 1994; Li et al. 2000;
57 Korthuis et al. 1999). PR39 has also been reported to have immunomodulatory functions
58 (Zhu et al. 2012), antitumor activity, and therapeutic effects in the treatment of ischemic heart
59 disease (Gler et al. 2012). Considering its high proline content, PR39 shows high stability in
60 solution and is resistant to serine proteases, elastase, and amino peptidases, resulting in a long
61 half-life (Sang and Blecha 2009); these properties make PR39 a promising therapeutic agent.

The synthesis and purification of AMPs are costly and time-consuming; therefore, an economical expression system that could produce large quantities of AMPs is needed. *E. coli* is one of the most widely used hosts for the production of heterologous proteins (Terpe 2006). However, the expressed proteins are usually found within inclusion bodies and are therefore often inactive, insoluble, and incorrectly folded (Demain and Vaishnav 2009). Alternatively, lactic acid-producing bacteria (LAB) are generally regarded as safe organisms and are therefore widely used in the production and preservation of fermented products by the food industry. *Lactobacillus casei* has been considered a good candidate to serve as a cost-effective live vaccine delivery vehicle for mucosal immunization. *L. casei* participate in the body's metabolic pathways and play important roles in the body's immune system. With excellent colonization ability, *L. casei* exist in the mucosa and continue to multiply, allowing sustained production and release of the target protein. As vaccine vectors, *L. casei* could be taken orally and as they exhibit tolerance to acid and bile salt (Yuki and Kiyono 2009).

In this study, we aimed to express PR39 in *Lactobacillus casei* 393 using a shuttle expression vector to provide both the probiotic effects (Douillard et al. 2013) of *L. casei* 393 and the biological function of PR39.

Materials and methods

Plasmids and strains

To obtain *Staphylococcus aureus* CVCC26003, *E. coli* CVCC10141, *Salmonella enterica* subsp. *enterica* Choleraesuis CVCC79102, and enterotoxigenic *E. coli* K88⁺ (ETEC K88⁺) CVCC230 at mid-logarithmic-phase, these organisms were cultured for 8 h at 37°C in Luria broth (LB). *L. casei* 393 was grown in MRS (De Man et al. 1960) (Sigma, St. Louis, MO, USA) supplemented with 0.1% (v/v) Tween-80 at 37°C without shaking.

A DNA fragment, (*Sac*I) GAGCTCGAACAAAAAGTTGATTTCAGAAGAAGATTTA

87 (Myc-tag)AGGAGACGTCCCCGACCCCATATTTGCCAAGGCCAAGGCCACCTCCGT
88 TTTTCCCACCAAGGCTCCCACCAAGGATCCCACCAGGGTTCCCACCAAGGTTCCC
89 ACCACGGTTCCCCGGATAATGACTCGAG(*Xho*I), encoding PR39 and an extra myc-tag,
90 was inserted into the plasmid pPG:612 (obtained from Prof. Seegers, NIZO Institute,
91 Netherlands; this plasmid contains the secretion signal peptide for secretory expression)
92 between *Sac*I and *Xho*I to construct the expression plasmid pPG:612-PR39. The fragment
93 encoding PR39 was obtained by gene synthesis (DNA2.0 Co. Ltd) based on the bias of *L.*
94 *casei* 393. The ligated product was then transformed into *E. coli* *TG1* cells to screen the
95 positive plasmid before transforming this plasmid into *L. casei* 393.

96 Competent *L. casei* 393 were prepared as previously described (Landete et al. 2014), and
97 2 µL recombinant plasmid was then mixed with 200 µL ice-cold competent cells. The mixture
98 was incubated in an ice bath for 5 min and then transferred to a precooled Gene Pulser
99 disposable cuvette (interelectrode distance: 0.2 cm) before delivery of a single electrical pulse
100 (2,000 V/cm, 2.5 µF). Immediately, 800 µL of recovery medium (MRS broth with 15%
101 sucrose) was added to the suspension, and the bacteria were then incubated for 2 h at 37°C
102 for recovery. Recombinant strains were selected on MRS agar medium containing 5 µg/mL
103 chloramphenicol (Cm). Identification was performed via extraction of recombinant plasmid
104 DNA, followed by restriction analysis and sequencing. The strains and plasmids used in this
105 study are shown in Table 1.

106 *Western blot analysis*

107 To analyze the expression of PR39 by pPG:612-PR39 in *L. casei* 393, recombinant strains
108 were cultured for 20 h in MRS broth containing Cm (10 µg/mL) and xylose (10 g/L) as a
109 supplement at 37°C. Both bacterial and trichloroacetic acid (TCA)-treated supernatants (a
110 10-fold concentration) were analyzed by sodium dodecyl sulfate-polyacrylamide gel
111 electrophoresis on 12% gels. Proteins were then transferred to nitrocellulose membranes,

soaked in blocking solution (5% skim milk in phosphate-buffered saline [PBS]) for 2 h, and immersed in primary antibodies (monoclonal mouse anti-Myc antibodies; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary goat anti-mouse IgG (1:3000 dilution; Sigma) for 2 h at 37°C. Finally, the film was visualised with freshly prepared 4-chloro-1-naphthol-H₂O₂ solution.

Growth performance of the recombinant strain

To investigate the influence of exogenous genes on the growth performance of the recombinant strain, mid-logarithmic-phase pPG:612-PR39/*L. casei* 393 and pPG:612/*L. casei* 393 were inoculated in MRS broth with Cm (1/20 inoculum). When the OD₆₀₀ values of both cultures reached 2, we added 1% xylose to each culture and induced the cultures for 24 h. The OD₆₀₀ was measured every 2 h to create a growth curve. A replicate experiment was performed without induction; in this experiment, mid-logarithmic-phase pPG:612-PR39/*L. casei* 393 and pPG:612/*L. casei* 393 were inoculated in MRS broth with Cm (1/50 inoculum) and cultured for 24 h.

Antibacterial activity in vitro

Staphylococcus aureus CVCC26003, *E. coli* CVCC10141, and *Salmonella enterica* subsp. *enterica* Choleraesuis CVCC79102 were grown for 8 h at 37°C in LB and collected at mid-logarithmic-phase. Next, 1 mL of the concentrated supernatant of each recombinant strain was mixed with 9 mL LB, and the above bacteria were inoculated into the mixture. Cultures were incubated for 8 h on a rotary shaker at 37°C, and the OD₆₀₀ values were measured using a spectrophotometer before plotted the growth curves.

Animal experiments and growth performance

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University, China. Three random groups (n = 9 per

group) of female BALB/c mice (6 weeks old) were housed in individual ventilation cages (IVCs; temperature, $32.70 \pm 2.99^{\circ}\text{C}$; relative humidity, $77.54\% \pm 2.34\%$; THI, temperature-humidity index, 87.27) and fed *ad libitum*. The grouped animals were orally dosed with 200 μL (10^9 CFU/mL) inoculum of pPG:612-PR39/*L. casei* 393, pPG:612/*L. casei* 393, and MRS broth. The recombinant bacteria was induced as previously described. Mice were treated via the oral route for 28 days using pPG:612/*L. casei* 393 and MRS broth as control groups. The mice were maintained under strict conditions according to international recommendations for animal welfare. The initial weights of mice in each group were measured, and food intake and weight gain were determined each day. These growth performance parameters, including average weekly feed intake (AWFI), average daily gain (ADG), and feed/gain (F/G) ratio, were calculated throughout the 28-day experimental period.

Sample collection

On day 28, blood samples and the spleen and small intestine were collected from three randomly chosen mice from each group, blood samples were analyzed the lymphocyte percentages and leukocyte counts. The spleen index was calculated using the following formula: spleen index = (spleen weight / body weight) $\times$ 10. In addition, the morphology of small intestine villi was observed by scanning electron microscopy (SEM). Fecal samples were collected on days 0 and 28 (6 g per group), and fecal DNA was extracted using a gel midi purification kit (TIANGEN Biotech (Beijing) Co., Ltd., Beijing, China) according to the manufacturer's protocol. The V3 region of the 16S-rRNA was amplified for analysis of bacterial diversity using synthetic universal bacterial primers (Sangon Biotech Co., Ltd.), as follows:

forward	primer	GC-338F
(5'-CGCCCGGGGCGCGCCCCGGGGCGGGGCGGGGGCGCGGGGGGCCTACGGGAG		
GCAGCAG-3')		
and reverse primer 518R (5'-ATTACCGCGGCTGCTGG-3') (Yu, Z. and M.		
Morrison. 2004). The polymerase chain reaction (PCR) fragments were separated by		
denaturing gradient gel electrophoresis (DGGE) with the following protocol: 8%		

polyacrylamide (v/v; ratio of acrylamide:bisacrylamide [37.5:1]) in 1× TAE buffer, gradient ranging from 20% to 35% denaturant (100% denaturant acrylamide corresponding to 7 M urea and 40% [v/v] formamide). Electrophoresis was carried out at 65°C for 14 h in 1× TAE buffer, with a constant voltage of 100 V. After electrophoresis, gels were stained with silver nitrate for 30 min, photographed using a UVR-800 instrument (Bio-Rad, Hercules, CA, USA), and analyzed by Quantity One 4.6.2. Using these data, the Shannon-Wiener diversity index, richness, and evenness were calculated.

Challenge with the ETEC K88⁺ strain

To confirm the minimal lethal dose (MLD) of ETEC K88⁺ in mice three random groups (n = 6 per group) of female BALB/c mice (6 weeks old) were housed under the same conditions and challenged with 200 µL different amounts of ETEC K88⁺ before the protection experiments.

Next, we investigated the protective effects of the recombinant strain against ETEC K88⁺. On day 29, the remaining 18 mice (6 per group) from above animal experiments after sample collection were challenged with an MLD oral dose (2×10^8 CFU/mL) of ETEC K88⁺, and death occurring within 24 h was observed.

Statistical analysis

SPSS Statistics v17.0 software (International Business Machines Corporation, NY, USA) was used for single-factor analysis of variance. Bacterial numbers were log transformed using the population estimate. Differences between groups with probability values of less than 0.05 were considered statistically significant using Duncan's multiple comparisons test.

Results

Protein localization

Western blot analysis showed that the target protein was present in both the sediment and

supernatants of the recombinant strain. The protein identified in sediment of pPG:612-PR39/*L. casei* 393 was 36 kDa; this was approximately 5 times higher than the predicted size derived from the amino acid sequence of PR39 and myc tag (total 6 kDa approximately). In contrast, that in the supernatant was 2 times larger at 18 kDa (Fig. 1).

Influence of PR39 on the growth of recombinant L. casei

Growth curves showed that the two strains had similar growth trends, indicating that the exogenous gene encoding PR39 did not alter the growth of the recombinant strain. Because PR39 has antimicrobial activity in some gram-positive bacteria (Freer et al. 1999), the growth curves of induced recombinant strains were also analysed. Similarly, the data suggested that the exogenous protein did not influence the growth of the recombinant strains (Fig. 2a, 2b).

Antimicrobial test in vitro

As shown in Figure 3a and 3b, the recombinant strain had a certain degree of antimicrobial activity against *E. coli* CVCC10141 and *Salmonella enterica* subsp. *enterica* Choleraesuis CVCC79102. However, the recombinant strain did not have antimicrobial activity against *S. aureus* CVCC26003 (Fig. 3c).

Growth performance of mice

Compared with mice in the control groups, mice in the experimental group fed the recombinant strain exhibited higher weight gain. After 28 days, the F/G ratio of mice in the experimental group (pPG:612-PR39/*L. casei* 393) was significantly lower ($P < 0.01$) than that of mice in the control groups (MRS broth and pPG:612/*L. casei* 393 ; Table 2). Thus, these results demonstrated that expression of PR39 by the recombinant strain promoted the growth performance of mice.

Immunomodulatory function

Analysis of lymphocyte percentages and leukocyte numbers in blood samples showed that mice in the experimental group had significantly higher lymphocyte percentages and

leukocyte counts than mice in the control groups ($P < 0.05$; Table 2). Additionally, mice in the control groups exhibited significantly higher spleen indices than mice in the experimental group ($P < 0.05$; Table 2).

Effects on intestinal villus morphology

Observation Measurement of villus height and intestinal crypt depth in the duodenum and jejunum by SEM (Fig. 4) showed that villus height was significantly higher in the experimental group than in the control group ($P < 0.01$). However, there was no obvious difference in intestinal crypt depth. Notably, the V/C value in the experimental group was significantly higher than that in the control group ($P < 0.01$). In addition, observation of the small intestine villus morphology showed that the recombinant antibacterial peptide improved the structure of the intestinal mucosa in mice (Table 3).

Diversity of the intestinal microbiota

The V3 region of 16S-rRNA in fecal samples was amplified with synthetic universal bacterial primers to obtain an amplification fragment of approximately 240 bp (Fig. 5). The PCR fragments were separated by DGGE to investigate the diversity of the intestinal microbiota. Analysis of the DGGE map (Fig. 6) showed that there were no significant changes in richness (Richness, S) during the experiment. However, the diversity (Shannon-Wiener, H) and evenness (Evenness, E) indices of the experimental group were decreased (Table 4).

Protection against ETEC K88⁺

The minimal lethal dose (MLD) of ETEC K88⁺ in mice was 2×10^8 CFU/mL (Table 5). After oral challenge using the MLD, the mortality rates in the MRS, pPG:612/*L. casei* 393, and experimental groups were 100%, 83%, and 33%, respectively (Table 6). These results suggested that the recombinant strains were effective and that peptide PR39 could protect mice from ETEC K88⁺ to some extent.

239

240 **Discussion**

241 In this study, we expressed PR39 in *L. casei* 393 and examined the effects of PR39 on
242 bacterial growth. Our results demonstrated that PR39 could protect mice from bacterial
243 infection when expressed in *L. casei* 393, providing support for the evaluation of this system
244 as a functional food additive.

245 The gene inserted into the plasmid pPG:612 was codon optimized according to the
246 codon bias of *L. casei* and contained a c-myc tag to facilitate protein detection. Notably, we
247 found that the expressed protein was 36 kDa in the pellet of bacteria and 18 kDa in the
248 supernatant of the culture (Figure 1), i.e., approximately 5 and 2 times larger than the
249 predicted molecular weights. Previous data have shown that when PR39 is inserted into the
250 pGEX-4T-1 plasmid, a vector used to produce a fusion protein with GST, and expressed in *E.*
251 *coli*, the targeted protein was 31 kDa, as predicted (Fan et al. 2010). This discrepancy may be
252 explained by the different sizes of the protein tags; for example, the fusion protein is
253 predicted to be a 6-kDa protein, which is much smaller than the predicted 31-kDa protein and
254 therefore may be more prone to formation of polymers. Accordingly, the 36-kDa secreted
255 protein in the pellet from the recombinant *L. casei* may be a hexamer of the fusion protein
256 (Zhu et al. 2012), and that in the supernatant (18 kDa) may be a trimer, produced during
257 ultrasonication.

258 AMPs are well known for their diverse functions, the most important of which is their
259 antimicrobial activity. PR39 exhibits a broad antimicrobial spectrum, affecting *E. coli*,
260 *Salmonella* Typhimurium, *Acinetobacter calcoaceticus*, *Bacillus anthracis*, and *Streptococcus*
261 *pyogenes* (Agerberth et al. 1991). Additionally, in this study, we found that PR39 inhibited
262 the growth of *Salmonella enterica* subsp. *enterica* Choleraesuis and *E. coli*, whereas *S.*
263 *aureus* was insensitive to PR39 (Figure 3). Notably, PR39 did not affect the growth of

recombinant *L. casei* (Figure 3). Studies have indicated that Pro-Arg-rich AMPs are more effective against Gram negative bacteria than against Gram positive bacteria, which are consistent with the expected results of this experiment (Scocchi et al. 2011). Several studies have suggested that PR39 may act through a nonlytic mechanism (Casteels et al. 1989; Boman et al. 1993) by interfering with DNA and/or protein synthesis (Shi et al. 1996). Thus, these data confirmed the antibacterial effects of PR39 *in vivo*.

In addition to killing bacteria directly, AMPs have been shown to have several immunoregulatory functions and may be involved in the clearance of infection, including alteration of host gene expression, function as chemokines, induction of chemokine production, inhibition of lipopolysaccharide induced pro-inflammatory cytokine production (Davidson et al. 2004), promotion of wound healing, modulation of dendritic cell responses, and promotion of the adaptive immune response (Li et al. 2000; Korthuis et al. 1999). Previous studies have indicated that these AMPs also play important roles in protecting the organism from pathogen infection. Moreover, AMPs act as signal transduction molecules between innate immunity and specific immunity to promote the host-specific immune response and expand the biological activity of the immune system. PR39 can reduce inflammation, promote wound healing, and activate neutrophils by inducing syndecans (Huang et al. 1997). Full-length PR39 is capable of inducing the expression of interleukin (IL)-8, whereas N-terminal PR39-derived peptides are sufficient for antimicrobial activity and can stimulate tumor necrosis factor (TNF)- α production by macrophages (Veldhuizen et al. 2014). Moreover, another study showed that PR39 can act as a Ca^{2+} -dependent chemoattractant of neutrophils, demonstrating the important role of PR39 in inflammation (Huang et al. 1997). Consistent with these findings, we found that mice in the experimental group in our study exhibited better growth performance (Table 2) and increased immune capacity following oral dosing with recombinant *L. casei*. Notably, the growth performance

289 and immune status in the *L. casei* control group were better than those in the MRS group,
290 suggesting that improvement of performance in mice in the experimental group could be
291 attributed to both the expressed PR39 and the probiotic *L. casei*.

292 Several reports have described the efficiency of recombinant LAB for both oral and intranasal
293 immunization. Some studies have shown higher immune responses after oral immunization
294 with recombinant LAB (Cheun et al. 2004; Ramasamy et al. 2006). Consistent with this, our
295 study showed that the recombinant strain had antimicrobial activity and immunomodulatory
296 functions and improved intestinal health, as shown by our results of DGGE-PCR (Table 4 and
297 Figure 6) and SEM images (Table 3 and Figure 4). The beneficial effects of recombinant
298 *Lactobacillus* may be explained by several mechanisms. First, changes in metabolites of
299 bacterial fermentation and enhancement of intestinal motility may be explained by changes in
300 the compositions of the intestinal microbiota, which could be attributed to reduction of the
301 pH value or shortening of the transit time. Bacterial production of organic acids (lactic acid,
302 butyric acid, and propionic acid) results in a decrease in pH and affects the reabsorption of
303 electrolytes and water, thereby changing the osmotic pressure and promoting intestinal
304 motility (Salminen and Salminen 1996). After oral challenge using the *E.coli*, the mortality
305 rates was reduced clearly in recombinant strains than MRS group (Table 6), the result
306 suggested that *L.casei*, as nonpathogenic microorganisms in the gastrointestinal microbiota,
307 could be a good choice for enhancing intestinal health. In fact, several AMPs have been
308 successfully expressed by probiotics (Mandal et al. 2014). In our case, *L. casei* 393 was
309 chosen to express PR39, and there was no need for protein purification. This system has
310 advantages of both AMPs and probiotics. More importantly, as a carrier, LAB can be taken
311 orally; this is considered a major advantage and convenience. In recent years, recombinant
312 *Lactobacillus* expressing antibacterial peptides have been shown to have a unique advantage,
313 and the development of food-grade *Lactobacillus* vectors has great potential as an important

food additive.

In conclusion, in our experimental setup, the codon-optimized gene of PR39 was inserted into a shuttle expression vector and expressed in *L. casei* 393 successfully. Our findings confirmed that the expressed fusion protein was capable of inhibiting the growth of two pathogenic bacteria *in vitro* and that the recombinant strain was beneficial to animals owing to both the expression of PR39 and the probiotic properties of *L. casei* 393.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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418 **Table 1.** Strains and plasmids used in this study.

419		
Strain or plasmid	Relevant characteristics or description	Reference or source
Strains		
<i>Lactobacillus casei</i> ATCC 393	Plasmid-free wild-type strain	de Vos 1999
<i>Staphylococcus aureus</i> CVCC26003	Standard strain	China Veterinary Culture Collection Center
<i>Escherichia coli</i> CVCC10141	Standard strain	China Veterinary Culture Collection Center
<i>Salmonella enterica</i> subsp. <i>enterica</i> <i>choleraesuis</i> CVCC79102	Standard strain	China Veterinary Culture Collection Center
Enterotoxigenic <i>E. coli</i> K88 ⁺ CVCC230	Standard strain	China Veterinary Culture Collection Center
Plasmids		
pPG:612	Cmr, repA ⁺ , and repC ⁺	de Vos 1999
pPG:612-PR39	pPG:612 with a copy of PR39 cloned <i>SacI</i> and <i>XhoI</i> sites	This study

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Table 2. Effects of antimicrobial peptides on growth performance, spleen index, lymphocyte percentages in peripheral blood, and white blood cell numbers in mice.

Treat	Growth Index			Spleen index	White blood cell numbers ($10^9/L$)	Lymphocyte percentage in peripheral blood (%)
	Total feed intake (g)	Total weight gain (g)	Feed/gain			
MRS	115.730 $\pm$ 2.6	2.870 $\pm$ 0.14	40.200 $\pm$ 0.78	3.666 $\pm$ 0.20	3.20 $\pm$ 0.10	83.00 $\pm$ 1.30
pPG:612/ <i>L. casei</i> 393	119.000 $\pm$ 4.0	2.795 $\pm$ 0.20	42.576 $\pm$ 0.91	3.747 $\pm$ 0.14	3.45 $\pm$ 0.05	84.40 $\pm$ 1.00
pPG:612-PR39/ <i>L. casei</i> 393	114.474 $\pm$ 2.8	3.695 $\pm$ 0.17	30.935 $\pm$ 0.87 ^{bd}	4.245 $\pm$ 0.27 ^{ac}	4.00 $\pm$ 0.10 ^{ac}	91.25 $\pm$ 1.30 ^{ac}

Values represent the mean $\pm$ SD. ^a $P < 0.05$ and ^b $P < 0.01$ for comparisons of pPG:612-PR39/*L. casei* 393 with MRS; ^c $P < 0.05$ and ^d $P < 0.01$ for comparisons of pPG:612-PR39/*L. casei* 393 with pPG:612/*L. casei* 393.

425 **Table 3.** Effects on the villous height, crypt depth, and V/C of the jejunum and
426 duodenum of mice.

Treatment	Jejunum			Duodenum		
	Villous height	Crypt depth	V/C	Villous height x	Crypt depth	V/C
MRS	275.50 ± 15.00	88.30 ± 5.20	2.88 ± 0.18	308.33 ± 3.80	78.40 ± 3.20	3.54 ± 0.24
pPG:612/ <i>L. casei</i> 393	260.00 ± 14.00	76.97 ± 3.30	3.38 ± 0.28	322.00 ± 13.70	76.60 ± 4.80	4.20 ± 0.11
pPG:612-PR39/ <i>L. casei</i> 393	304.47 ± 11.00 ^{bd}	78.54 ± 4.20	4.70 ± 0.32 ^{bd}	367.73 ± 6.10 ^{bd}	76.70 ± 8.20	4.92 ± 0.48 ^{bd}

427 Values represent the mean ± SD. ^a $P < 0.05$ and ^b $P < 0.01$ for comparisons of
428 Ppg:612-PR39/*L. casei* 393 with MRS; ^c $P < 0.05$ and ^d $P < 0.01$ for comparisons of
429 pPG:612-PR39/*L. casei* 393 with pPG:612/*L. casei* 393.

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430 **Table 4.** Diversity indices calculated from the DGGE banding profiles.

	A ₀	A ₂₈	B ₀	B ₂₈	C ₀	C ₂₈
H	3.98	3.91	3.68	3.76	3.61	3.48
S	45	45	40	40	41	40
E	1.04	1.04	1.08	1.07	1.06	0.85

431 A. MRS group; B. pPG: 612/*L. casei* 393 group; C. pPG:612-PR39/*L. casei* 393
 432 group. H, S and E represents diversity, richness and evenness, respectively. Analyses
 433 of DGGE maps were performed with Quantity One 4.6.2 software; the
 434 pPG:612-PR39/*L. casei* 393 group was compared with the control group, richness was
 435 unchanged, and Shannon-Wiener and evenness were reduced.

436 **Table 5.** Mortality of mice after challenge with different amounts of K88⁺ ETEC.

Group	Number of mice	Challenge dose (CFU/mL)	Number of deaths	Mortality (%)
A	6	1×10^8	2	0
B	6	2×10^8	6	100
C	6	5×10^8	6	100

437 A, B, and C represent groups given different challenge doses of K88⁺ ETEC.

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438 **Table 6.** Effects of antimicrobial peptides on ETEC K88⁺ infection in mice.

439

Group	Number mice	of Challenge (CFU/mL)	dose	Number of deaths
MRS	6	2×10^8		6/6
pPG:612/ <i>L. casei</i> 393	6	2×10^8		5/6
pPG:612-PR39/ <i>L. casei</i> 393	6	2×10^8		2/6

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440 **Figure captions**

441 **Figure 1.** Analysis of the expressed product PR39 by western blotting. Lane 1,
442 molecular mass marker; Lane 2, pellet of pPG:612/*L. casei* 393 induced by xylose;
443 Lane 3, pellet of pPG:612-PR39/*L. casei* 393 induced by xylose, expressing a 36-kDa
444 protein; Lane 4, supernatant of pPG:612/*L. casei* 393 induced by xylose; Lane 5,
445 supernatant of pPG:612-PR39/*L. casei* 393 induced by xylose, expressing an 18-kDa
446 protein.

447 **Figure 2.** Growth curves of recombinant bacteria without (a) and with (b) induction.

448 **Figure 3.** Antimicrobial activity of recombinant antibacterial peptide supernatants
449 against *Escherichia coli* (a), *Salmonella enterica ssp. enterica choleraesuis* (b), and
450 *Staphylococcus aureus* (c).

451 **Figure 4.** Jejunum (A–C) and duodenum (E–F) sections from laboratory mice (100×).
452 A. and E. Medium group; B. and D. Empty vector pPG:612/*L. casei* 393 group; C.
453 and F. Recombinant strain pPG:612-PR39/*L. casei* 393 group. The villus height and
454 intestinal crypt depth were measured in the duodenum and jejunum as representatives
455 of intestinal health.

456 **Figure 5.** Amplification of 16S rDNA V3. Lane M, DNA marker 5000 bp; Lane 1,
457 mouse feces from the medium group at day 0; Lane 2, mouse feces from the pPG:612/
458 *L. casei* 393 group at day 0; Lane 3, mouse feces from the pPG:612-PR39/*L. casei*
459 393 group at day 0; Lane 4, mouse feces from the medium group at day 28; Lane 5,
460 mouse feces from the pPG:612/*L. casei* 393 group at day 28; Lane 6, mouse feces
461 from the pPG:612-PR39/*L. casei* 393 group at day 28; Lane 7, water control.

462 **Figure 6.** DGGE map of intestinal flora 16S rDNA fragments from mouse feces. A0,
463 mouse feces from the medium group at day 0; B0, mouse feces from the pPG:612/*L.*
464 *casei* 393 group at day 0; C0, mouse feces from the pPG:612-PR39/*L. casei* 393

465 group at day 0; A28, mouse feces from the medium group at day 28; B28, mouse
466 feces from the pPG:612/*L. casei* 393 group at day 28; C28, mouse feces from the
467 pPG:612-PR39/*L. casei* 393 group at day 28.

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Figure 1. Analysis of the expressed product PR39 by Western blot. Lane 1, molecular mass marker; Lane2. The sediment of pPG:612/*L.casei* 393 inducing by xylose; Lane3. The sediment of pPG:612-PR39/*L.casei* 393 inducing by xylose, expressed a 36kDa protein; Lane4. The supernatant of pPG:612/*L.casei* 393 inducing by xylose; Lane5. The supernatant of pPG:612-PR39/*L.casei* 393 inducing by xylose, expressed a 18kDa protein.

a

b

Figure 2. Growth curves of recombinant bacteria without (a) and with (b) induction.

Figure 3. Antimicrobial activity of recombinant antibacterial peptide supernatants against *Escherichia coli* (a), *Salmonella enterica ssp. enterica* –choleraesuis (b), and *Staphylococcus aureus* (c).

Figure 4. The jejunum(A,B,C) and duodenum(E,D,F) sections of laboratory mice (1×100) .
A.medium group; B. empty vector pPG:612/*L.casei* 393 group; C. recombinant strain pPG:612-PR39/*L.casei* 393 group; E. medium group; D. empty vector pPG:612/*L.casei* 393 group; F. recombinant strain pPG:612-PR39/*L.casei* 393 group. The villi height and Intestinal crypt depth of duodenum and jejunum were significantly higher in pPG:612-PR39/*L.casei* 393 group than those of control, which demonstrates an improvement of intestinal health.

Figure 5. The amplified results of 16S rDNA V3. Lane M. DNA Marker 5000bp; Lane 1. the mice feces of medium group at 0d; Lane 2. the mice feces of pPG:612/ *L.casei* 393 group at 0d; Lane 3. the mice feces of pPG:612-PR39/*L.casei* 393 group at 0d; Lane 4. the mice feces of medium group at 28d; Lane 5. the mice feces of pPG:612/*L.casei* 393 group at 28d; Lane 6. the mice feces of pPG:612-PR39/*L.casei* 393 group at 28d; Lane 7. water control.

Figure 6. DGGE map of fungal 16S rDNA fragments from the mice feces. A₀. the mice feces of medium group at 0d; B₀. the mice feces of pPG:612/*L.casei* 393 group at 0d; C₀. the mice feces of pPG:612-PR39/*L.casei* 393 group at 0d; A₂₈. the mice feces of medium group at 28d; B₂₈. the mice feces of pPG:612/*L.casei* 393 group at 28d; C₂₈. the mice feces of pPG:612-PR39/*L.casei* 393 group at 28d.