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# Construction of Recombinant *Pichia pastoris* Carrying a Constitutive AvBD9 Gene and Analysis of Its Activity

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Avian beta-defensin 9 (AvBD9) is a small cationic peptide consisting of 41 amino acids that plays a crucial rule in innate immunity and acquired immunity in chickens. Owing to its wide antibacterial spectrum, lack of a residue, and failure to induce bacterial drug resistance, AvBD9 is expected to become a substitute for conventional antibiotics in the livestock and poultry industries. Using the preferred codon of *Pichia pastoris*, the mature AvBD9 peptide was designed and synthesized, based on the sequence from GenBank. The *P. pastoris* constitutive expression vector pGHKα was used to construct a pGHKα-AvBD9 recombinant plasmid. Restriction enzyme digestion was performed using *Sac*I and *Bgl*II to remove the ampicillin resistance gene, and the plasmid was electrotransformed into *P. pastoris* GS115. High-expression strains with G418 resistance were screened, and the culture supernatant was analyzed by Tricine-SDS-PAGE and western blot assay to identify target bands of about 6 kDa. A concentrate of the supernatant containing AvBD9 was used for determination of antimicrobial activity. The supernatant concentrate was effective against *Escherichia coli*, *Salmonella paratyphi*, *Salmonella pullorum*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Enterobacter cloacae*. The fermentation product of *P. pastoris* carrying the recombinant AvBD9 plasmid was adjusted to $1.0 \times 10^8$ CFU/ml and added to the drinking water of white feather broilers at different concentrations. The daily average weight gain and immune organ indices in broilers older than 7 days were significantly improved by the AvBD9 treatment.

**Keywords:** AvBD9, *Pichia pastoris*, constitutive expression, antibacterial activity, white feather broiler, productivity

## Introduction

The use of antibiotics in feed additives produces significant economic benefits for the livestock and poultry industries. However, antibiotic residue and severe bacterial drug resistance pose potential threats to human health. Therefore, new feed additives free from the problems of residue, drug resistance, and pollution are needed to replace conventional antibiotics. Antimicrobial peptides have a wide spectrum of antibacterial activity and little potential to produce drug resistance owing to their unique antibacterial mechanisms. Antimicrobial peptides do not leave residues in animals following treatment or produce toxic effects, and thus serve as good alternative antibiotics. Avian beta-defensin (AvBD) is an endogenous antibacterial peptide that is widely expressed in poultry. AvBD has a stable molecular structure and diverse biological activities, and it plays a major role in the poultry immune system. Chicken AvBD9 was discovered by Xiao *et al.* [15] and Lynn *et al.* [5] through bioinformatic analysis. AvBD9 is widely present in various avian tissues, such as the liver, gall bladder, kidney, and genitourinary tract. Van Dijk *et al.* [11] showed that AvBD9 is extensively expressed in the chicken digestive tract, as well as in the esophagus, ingluvies, bursa, kidney, liver, trachea, and muscle, where the expression is usually high. AvBD9 is expressed at a moderate level in the anterior stomach, uropygial gland, skin, brain, ovaries, and testis. Moreover, AvBD9 expression is also detected in the intestinal tract, lung, thymus, and spleen.

AvBD9 possesses a wide variety of biological functions. Sugiarto and Yu [7] found that AvBD9 can effectively inhibit or kill gram-positive and gram-negative bacteria, mycoplasma, spirochetes, fungi, protozoa, and enveloped viruses. In addition, AvBD9 also has a strong killing effect on malignant tumor cells, as well as immunoregulatory and hormonal regulation functions. Van Dijk *et al.* [11] investigated synthetic and recombinant AvBD9 peptides and found strong antimicrobial activities against *E. coli*, *Salmonella*, *Staphylococcus*, *Campylobacter jejuni*, *Clostridium perfringens*, and *Candida albicans*.

AvBD9 is widely expressed in various tissue types and plays an important role in resistance against the invasion of pathogenic microorganisms. Defensins are usually expressed at low levels, but secretion of AvBD9 can be induced by some substances, which thus prevent diseases and the invasion of pathogenic microorganisms. Sunkara *et al*. [9] found that butyric acid produced in the intestinal tract of chickens can induce high levels of AvBD9 expression without producing cytotoxicity. Feeding experiments have been carried out using different doses of butyric acid, and its optimal dose as a feed additive was determined. At an appropriate dose, butyric acid can induce sufficient secretion of AvBD9 to increase disease resistance in chickens.

However, AvBD9 secretion is limited, and high secretion levels reduce the feed conversion ratio of the animals, prolonging the feeding period and increasing the cost of production [11]. In recent years, genetic engineering techniques have been increasingly applied to produce poultry that express AvBD9 at a high level. The *E. coli* expression system is the most thoroughly studied gene expression vector, and many types of avian defensin have been successfully expressed using this system [4, 13]. However, exogenous gene products are likely to form insoluble inclusion bodies in *E. coli* cells. Although Xing *et al*. [16] suggested that normal proteins can be produced by inclusion body renaturation, additional processing is required, which increases the complexity of the process of inducing gene expression. Because of the toxicity of AvBD9 to the host *E. coli*, product separation and extraction are difficult, and the quality of the product may be affected by the introduction of endotoxins during the extraction process.

Yeast is among the most well-studied eukaryotic expression systems. Wang *et al*. [12] used an *AOX1* promoter to induce expression of chicken AvBD2 with significant antibacterial activity. However, methanol must be used to induce the *AOX1* promoter, leading to potential environmental pollution and damage to human health; therefore, this method is not suitable for the production of food and feed additives. Moreover, using a large amount of methanol in high-density fermentation carries a significant risk of fire. For this reason, promoters that do not require methanol for induction have become a focus of research attention. In comparison with the *AOX1* promoter, use of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter can simplify the fermentation process using recombinant *Pichia pastoris* and shorten the fermentation cycle. Moreover, this process precludes the need to store and transport a large amount of methanol, which makes it suitable for industrial production. Therefore, the *GAPDH* promoter allows for continuous fermentation and cost reduction in heterologous protein production [14].

Most studies on the expression of chicken β-defensin in *P. pastoris* have utilized methanol-induced expression systems [12, 18], whereas constitutive expression systems have been rarely discussed. A constitutive expression vector based on pGAP was used in this study to construct a constitutive expression system for chicken AvBD9 in *P. pastoris*. The effects of this expression system on the activity of the gene products and the productivity of the broilers were analyzed, with the aim of laying a foundation for the application of eukaryotic expression of AvBD9.

## Materials and Methods

### Strains, Reagents, and Experimental Animals

*P. pastoris* GS115 was donated by Anhui Anke Biotechnology Group Co., Ltd (Hefei, China). The goat anti-AvBD9 polyclonal antibody was synthesized by Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China). The rabbit anti-goat IgG-HRP was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The pGHKα plasmid [12] was constructed by and preserved at our laboratory. One-day-old white feather broilers (males and females) were purchased from Guangde Rongda Poultry Development Co., Ltd. (Xuancheng, China).

### Primer Synthesis

The primer sequences used in the experiment are given in Tables 1 and 2. The primer synthesis was performed by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

### Sequence Optimization and Synthesis of Mature Chicken AvBD9 Peptide

The AvBD9 gene sequence was downloaded from GenBank (Accession No. AY534894.1). The preferred codon of *P. pastoris* was used to optimize the gene sequences using DNASTAR software without changing the amino acid sequence (Fig. 1). The *Eco*RI cleavage site and the coding sequence AAAAGA at the Kex2 cleavage site were added to 5′-end of the gene, while TAA

**Table 1.** Primers used for this study.

| Primers | Sequences (5′ → 3′) | Restriction sites |
|---|---|---|
| GAP1 | GCGAGCTCAGATCCTTTTTTGTAGAAATGTC | *Sac*I |
| GAP2 | GGCGGATCCTGTTTTGATAGTTGTTCAATTG | *Bam*HI |
| pG1 | GTCCCTATTTCAATCAATTGAA | |
| pG2 | GCAAATGGCATTCTGACATCC | |
| N1 | CGGAATTCTCAACTAAAAAATTACATAAAGAACC | *Eco*RI |
| N2 | ATTGCGGCCGCTCAGATCTAAAAATTATAAAAGT | *Not*I |

**Table 2.** The average daily weight gain of the different groups chicks.

| Groups | 1–7 d | 8–14 d | 15–21 d | 21–42 d | 1–21 d | 1–42 d |
|---|---|---|---|---|---|---|
| C | 31.46 ± 0.33 | 31.47 ± 0.35 B | 32.15 ± 0.54 B | 38.16 ± 0.70 B | 31.28 ± 0.59 B | 34.69 ± 0.51 B |
| D | 31.48 ± 0.31 | 31.46 ± 0.37 B | 32.14 ± 0.55 B | 38.15 ± 0.69 B | 31.30 ± 0.60 B | 34.72 ± 0.50 B |
| T | 30.96 ± 0.44 | 35.00 ± 0.91 A | 39.23 ± 0.34 A | 47.99 ± 0.66 A | 35.24 ± 0.81 A | 40.63 ± 0.73 A |

C\D: control group. T: Test group. Data in the same column followed by different capital letters donates an extremely significant difference ($p < 0.01$).

and *Not*I cleavage sites were added to the 3′-end. The length of the optimized gene was 146 bp. The AvBD9 gene was synthesized and cloned into vector pUC57 by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

**Preparation of Recombinant *P. pastoris* GS115/pGHKα-AvBD9**

The pUC57-AvBD9 plasmid and the pGHKα vector were extracted. Double enzyme digestion was performed sequentially using *Eco*RI and *Not*I restriction endonucleases. The target products were recovered from the gel, connected by T4 DNA ligase, and transformed into *E. coli* DH5α. The positive recombinants were screened using an LB plate containing 100 μg/ml ampicillin (Amp). The positive recombinants were identified by PCR analysis using primers pG1 and pG2 (Table 1), followed by agarose gel electrophoresis. The positive strains were sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The recombinant vector identified as correct by sequencing was named pGHKα-AvBD9.

The recombinant strains of *E. coli* DH5α carrying the recombinant pGHKα-AvBD9 plasmid were cultured in large quantities. The plasmid was extracted and linearized by digestion with *Sac*I and *Bgl*II. After removal of the Amp resistance gene, the target fragments were recovered by gel extraction. The target fragments were electroporated into competent *P. pastoris* GS115 cells. After sieving using minimal dextrose (MD) medium plate, single colonies were picked. The yeast cells were lysed to obtain genomic DNA, which was used as a template for PCR detection using primers pG1 and pG2. The positive transformants were inoculated onto a yeast extract-peptone-dextrose (YPD) medium plate containing 0.5 mg/ml G418, as well as onto an MD plate. The cells were cultured for 2–6 days at 28°C until the colonies appeared.

The positive transformants were picked and inoculated into a triangular flask containing 5 ml of YPD medium. The cells were cultured overnight at 28°C with shaking at 200 rpm, after which each culture was inoculated into a 500 ml triangular flask containing 50 ml of fermentation medium at a ratio of 1%. The cells were cultured for 48 h at 28°C with shaking at 200 rpm. After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatants

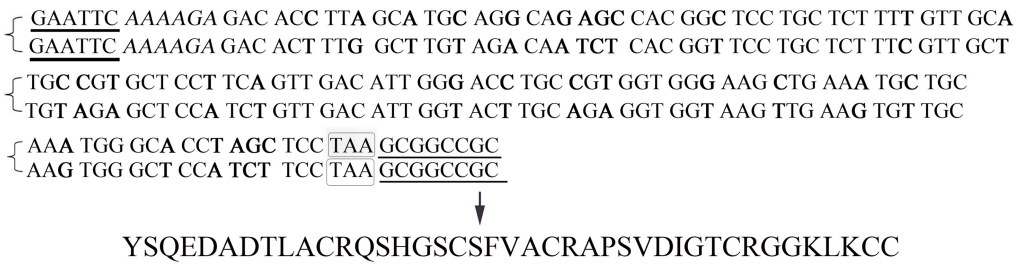


**Fig. 1.** Nucleotide sequences of the AvBD9 gene before and after codon optimization.
The upper sequence is the original sequence, and the lower sequence is the optimized sequence. Bold font indicates modified bases, underlining indicates endonuclease recognition sites, and italics indicate the Kex2 cleavage site coding sequence. The stop codon is enclosed in the box.

were collected and tested for total protein concentration using a BCA protein quantification kit. The total protein concentrations of different strains were compared and the high-expression strains were selected.

**Detection of Recombinant AvBD9**

The supernatant from the culture of the high-expression Y3 strain was collected, freeze-dried, and concentrated. After dissolution in double-distilled water, the proteins were isolated by 16.5% Tricine-SDS-PAGE [17]. The percentage of the target proteins in the total protein was analyzed by optical density scanning. The expression level of target protein AvBD9 in the supernatant was calculated according to the total protein concentration of the sample solution. The isolated proteins were transferred to a membrane and blocked with 5% skimmed milk at room temperature for 2 h, after which the cells were incubated with goat anti-AvBD9 polyclonal antibody (1:500 dilution) overnight at 4°C. After rinsing with TBS, the cells were incubated with the horseradish peroxidase-labeled rabbit anti-goat IgG antibody at room temperature for 2 h (1:5,000 dilution). After the incubation, the cells were rinsed and developed using ECL. The protein concentration was determined using a BCA protein quantification kit.

**Antibacterial Spectrum and Antibacterial Ability Analyses of Recombinant AvBD9**

The agar diffusion method was employed. The experimental bacteria were as follows: *E. coli* CMCC44102, *Salmonella paratyphi* ATCC9150, *Salmonella pullorum* S2, *Enterococcus faecalis* ATCC29212, *Enterobacter cloacae* CMCC45301, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Citrobacter* sp. CMCC48017, and *Candida albicans* ATCC10231. During the middle logarithmic phase of growth, 20 µl of each bacterial solution was added to 20 ml of LB solid medium at 50°C and mixed well. The resulting solution was poured into a 9-cm plate for solidification, and a 7-mm sterilized puncher was used to punch holes, into which 40 µl of supernatant (concentrated 30-fold by dilution of freeze-dried solution) was added. After culture for 6–12 h at 37°C, the diameter of the bacteriostatic ring was observed. Ampicillin (40 µl at 100 µg/ml) or kanamycin (Kan, 40 µl at 250 µg/ml) was added as a positive control treatment. The concentrate of the supernatant containing the proteins expressed by GS115/pGHKα after the same treatment procedures and PBS were used as the negative control.

**Influence of Recombinant AvBD9 on the Productivity of White Feather Broilers**

The *P. pastoris* strains carrying recombinant AvBD9 were inoculated into the fermentation medium at the inoculum of 10%. The cells were cultured for 48 h at 28°C with shaking at 200 rpm, and the density was adjusted to $6 \times 10^8$ CFU/ml. Sixty white feather broilers were randomly divided into an experimental group (group T) and two control groups (group C and group D), with 20 animals in each group. The fermentation liquid of recombinant *P. pastoris* GS115/pGHKα-AvBD9 was added into the drinking water of group T. The ratio of fermentation liquid to water for 1-week-old broilers was 1:50, whereas that of 2-week-old broilers was 1:25, and the broilers in group C were fed the same concentration of supernatant fluid from *Pichia pastoris* cultures without the recombinant plasmid and grown. Group D broilers were controlled with normal drinking water. The experiment lasted for 42 days. The broilers were allowed free access to food and water.

**Influence on the average daily weight gain of broilers.** The broilers were weighed on days 1, 7, 14, 21, and 42. No feed or water was given for 12 h before weighing, which was performed at 08:00. The average daily gain (ADG) was calculated by the following formula: ADG (g) = (average body weight at the end of the experiment − average body weight at the beginning of the experiment) ÷ number of days. The data were subject to multiple comparison tests using the data processing system (DPS).

**Influence on immune organ indices of broilers.** For each group, five broilers were randomly selected on days 7, 21, and 42. The broilers were killed by inerting $N_2$, and the thymus, bursa of Fabricius, and spleen were harvested and weighed. The immune organ indices were calculated by the following formula: immune organ index = fresh weight of immune organ (g) ÷ live weight before slaughter (kg). The data were subject to multiple comparison tests using DPS.

**Influence on the bacterial colonies in the cecum and ileum of broilers.** On days 7, 14, 21, 28, and 42, five broilers were randomly selected from each group and killed by inerting $N_2$. The contents of the cecum and the middle segment of the ileum were collected surgically. The intestinal florae were obtained by pretreatment of the samples.

Bacterial DNA extraction was carried out using a Genomic DNA Extraction Mini Kit, and enterobacterial repetitive intergenic consensus (ERIC)-PCR was performed according to the literature [17]. The PCR products were detected by 2.0% agarose gel electrophoresis and photographed using a gel imaging system. The bands were counted from the ERIC-PCR profiles of two different sections of the intestines on different days using NTSYS 2.1. The coefficient of similarity (Cs) was calculated by the Dice method [17]. Cluster analysis was performed to assess the diversity of intestinal flora using the unweighted pair group mean average [17].

## Results

**Preparation of recombinant *P. pastoris* GS115/pGHKα-AvBD9**

**Construction of recombinant plasmid pGHKα-AvBD9.** The positive recombinants from the LB medium plates (100 µg/ml Amp) were selected and the PCR products

were analyzed by agarose gel electrophoresis using primers pG1 and pG2. The vector without the plasmid had a length of 462 bp, and the recombinant vector had a length of 572 bp, in agreement with the theoretical values. The transfected bacteria containing positive recombinants were sequenced, and the results showed that the plasmids contained the target gene fragments within the correct reading frame, indicating that the recombinant plasmid pGHKα-AvBD9 was constructed successfully.

**Screening and identification of recombinant *P. pastoris* GS115/pGHKα-AvBD9.** Single colonies from the MD plates were selected and their genomic DNA was extracted as the PCR template. The PCR products were analyzed by agarose gel electrophoresis; the positive transformants had the expected length of 572 bp, whereas the control without the vector had a length of 462 bp. The protein concentrations of the supernatant from eight positive yeast strains were determined and designated as Y1–Y8. And Y3 had the highest protein concentration and was thus designated as a high-expression strain. Therefore, Y3 was used for subsequent experiments.

### Detection of Recombinant AvBD9

Y3 fermentation liquid was centrifuged at 10,000 rpm for 10 min, and the supernatant was freeze-dried, concentrated, and analyzed by Tricine-SDS-PAGE. Bands of specific proteins with relative molecular masses of 5,100 and 6,500 were detected (Fig. 2A), indicating that the target proteins were expressed successfully. Analysis using a gel imaging system revealed that the target protein accounted for 11.38% of the total protein expression in the supernatant. Based on the total protein concentration in the Y3 culture solution, the concentration of AvBD9 protein in the supernatant was determined to be 66.18 mg/l. Western blot assays confirmed the presence of AvBD9 protein at the concentration of 2 or 3 mg/l in the supernatant of the fermentation liquor of the recombinant yeast (Fig. 2B).

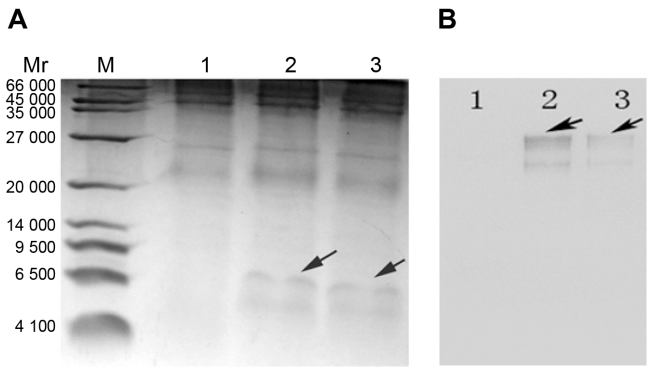


**Fig. 2.** Tricine-SDS-PAGE and western blot analysis of the recombinant AvBD9.

(**A**) Tricine-SDS-PAGE analysis of the recombinant AvBD9. Lane M: protein marker; Lane 1: Supernatant of GS115/pGHKα; Lanes 2, 3: Supernatant of recombinant S115/pGHKα-AvBD9. (**B**) Analysis of recombination proteins by western blotting. Lane 1, empty vector as control. Lane 2, recombinant proteins at the dilution of 3 mg/l. Lane 3, recombinant proteins at the dilution of 2 mg/l.

### Antimicrobial Spectrum and Antimicrobial Ability Analyses of Recombinant AvBD9

The antimicrobial spectrum of recombinant AvBD9 and its antimicrobial effects against indicator bacteria were analyzed using an agar diffusion method [3]. Recombinant AvBD9 at the concentration of 2 mg/l had a broad spectrum with a strong antimicrobial effect on many pathogens, including *E. coli*, *Salmonella*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Enterococcus faecalis*, and *Citrobacter*, forming distinct bacteriostatic rings. However, recombinant AvBD9 had no apparent inhibitory effect on *Candida albicans*, indicating that its antimicrobial effect was restricted to prokaryotes. Based on the diameters of the bacteriostatic rings corresponding to the tested strains, recombinant AvBD9 had a stronger inhibitory effect than the control antibiotic on *Salmonella, Pseudomonas aeruginosa,* and *Enterobacter cloacae*.

### Influence of Recombinant AvBD9 on the Productivity of White Feather Broilers

**Average daily weight gain of white feather broilers.** As shown in Table 2, at the age of 1–7 days, there were no significant differences in the average daily weight gain of the T, C, and D broiler groups ($p > 0.05$), and the average daily weight gain of group T was slightly less than that of groups C and D. From 8–42 days, the average daily weight gain of group T was significantly greater than that of groups C and D ($p < 0.01$). The average daily gain during the first 3 weeks of the study was less than the weight gain during the last 3 weeks, indicating that the broilers entered a rapid growth period during the latter period. These results demonstrated that recombinant AvBD9 can improve the average daily weight gain of white feather broilers.

**Immune organ indices.** The immune organs from the killed broilers showed no abnormalities in appearance. As shown in Table 3, the spleen index of 7-day-old broilers in group T was significantly higher than that of groups C and D ($p < 0.05$), but there was no significant difference ($p > 0.05$) in spleen index between the 21- and 42-day-old broilers. The organ indices of the thymus and bursa of Fabricius in group T were higher than those of groups C

**Table 3.** The effect of different groups on immune organ index of chicks.

| Organ index | Group | Age (Days) | | |
|---|---|---|---|---|
| | | 7 | 21 | 42 |
| Spleen | C | 0.19 ± 0.06 | 0.63 ± 0.39 | 1.24 ± 0.22 |
| | D | 0.19 ± 0.05 | 0.62 ± 0.40 | 1.25 ± 0.21 |
| | T | 0.22 ± 0.02b | 0.62 ± 0.13 | 1.28 ± 0.23 |
| Thymus | C | 1.23 ± 0.06A | 2.93 ± 0.14 | 1.79 ± 0.27A |
| | D | 1.22 ± 0.06A | 2.94 ± 0.13 | 1.78 ± 0.26A |
| | T | 1.40 ± 0.09B | 3.01 ± 0.11 | 2.28 ± 0.19B |
| Bursa of Fabricius | C | 0.55 ± 0.09 | 1.49 ± 0.14A | 0.79 ± 0.16 |
| | D | 0.54 ± 0.08 | 1.47 ± 0.15A | 0.80 ± 0.15 |
| | T | 0.65 ± 0.11 | 1.88 ± 0.23B | 0.92 ± 0.24 |

CD: control group. T: Test group. Data in the same column followed by different capital letters donates an extremely significant difference ($p < 0.01$), and by different lowercase letters donates a significant difference ($p < 0.05$).

and D on days 7, 21, and 41, while the thymus index of the two groups was significantly different on days 7 and 42 ($p < 0.01$), and the index of bursa of Fabricius showed a significant difference on day 21. These results indicate that the recombinant AvBD9 had little effect on the spleen index of the broilers but clearly improved the organ indices of the thymus and bursa of Fabricius.

**Microflorae in the cecum and ileum of white feather broilers.** As shown in Fig. 3, the PCR product bands of samples collected from the ceca of each broiler group were between 100 and 1,000 bp. Abundant product bands were detected in 7-day-old broilers, indicating that bacterial strains had colonized the cecum. The number of bands obtained from the intestinal samples of group T was higher than that of groups C and D, indicating that the intestinal florae of group T were rich in diversity and had high population density. On days 7, 14, and 21, the position of the main band and the number of characteristic bands in the profiles varied by age within each group, demonstrating the instability of the intestinal florae. However, the number of bands showed an increasing trend, indicating that the diversity of the microbial community gradually improved over time. The main bands in the profiles of each group had changed little on day 28, indicating that the dominant florae were relatively stable.

As shown in Fig. 4, the PCR product bands obtained from samples from the ilea of the experimental groups were between 250 and 2,000 bp. At the various tested ages, the number of bands of the intestinal samples from group T was clearly higher than that from groups C and D, indicating that group T had a high diversity of intestinal florae with a high population density. With increasing age, the number and brightness of the characteristic bands at different ages in each group showed upward trends, indicating that the diversity of the microbial communities and their population density improved gradually over time. After 28 days, the intestinal florae of each group were highly similar, showing that the dominant bacteria species of the intestinal microflora were relatively stable.

As shown in Figs. 3 and 4, during the course of the experiment, the number and brightness of the bands, the position of the main band, and the number and position of characteristic bands in the profiles of the ilea from both groups were significantly different from the corresponding bands from the ceca, and fewer bands were identified in the ilea. The cecal and ileal microflorae of the broilers had different species and dominant species, and the abundance and population density of microbial communities in the ileum were reduced in comparison with those of the cecum. The yeast with recombinant AvBD9 had an obvious beneficial effect on the intestinal environment and microfloral abundance.

## Discussion

### Optimization of the Synthesis of the AvBD9 Gene

Chicken AvBD9 is a cationic, antimicrobial peptide that is rich in cysteine residues, with a relative molecular mass of 4,285 Da. The chicken AvBD9 precursor is composed of 67 amino acid residues and the mature peptide contains 41 amino acids. Owing to the degeneracy of genetic codons, the same amino acid often has multiple codons, but codon preference varies with species, leading to significant differences in usage frequency of codons across different

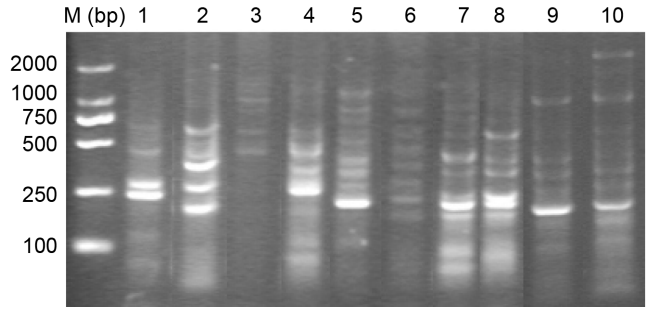


**Fig. 3.** ERIC-PCR results for the cecum contents on different days for chickens in each group.
M: DNA size marker; lanes 1, 3, 5, 7, and 9: the control group at 7, 14, 21, 28, and 42 days, respectively; lanes 2, 4, 6, 8, and 10: the experimental group at 7, 14, 21, 28, and 42 days, respectively.

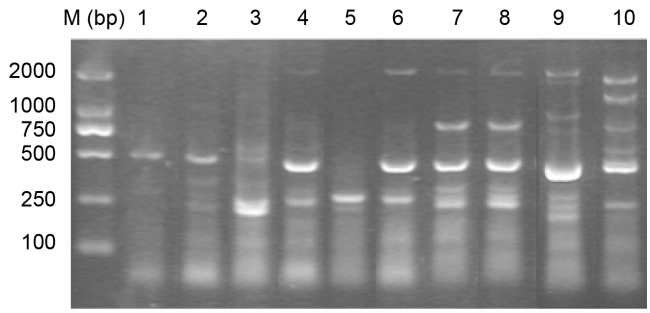


**Fig. 4.** ERIC-PCR results for the ileum contents on different days for chickens in each group.
M: DNA size marker; lanes 1, 3, 5, 7, and 9: the control group at 7, 14, 21, 28, and 42 days, respectively; lanes 2, 4, 6, 8, and 10: the experimental group at 7, 14, 21, 28, and 42 days, respectively.

species. A codon used with high frequency in a particular species may be a rare codon in another species [8], resulting in an inability to achieve effective expression of exogenous genes in the host because of differences in codon preference [1]. The usage frequency of preferred codons in *P. pastoris* is positively correlated with the expression level of exogenous genes, suggesting that codon optimization can enhance such expression [2]. Zhang *et al.* [19] achieved expression of *Penicillium expansum* lipase in *P. pastoris* through codon optimization, and the enzyme activity was 2.3–2.5 times greater than that of the wild lipase. In this study, we optimized the AvBD9 gene without changing the original amino acid sequences by utilizing the codon preference of *P. pastoris* as determined by Zhao *et al*. [20]. A total of 25 codons were optimized. The 5′ end of the gene was added with AAAAGA, the coding sequence of the cleavage site of proteinase Kex2, which allowed the signal peptide sequence to be cut efficiently and correctly. In addition, a stop codon was added to the 3′ end to effectively terminate translation, thus guaranteeing the correctness of the amino acid sequence. The culture supernatant was assessed using Tricine-SDS-PAGE analysis, and the target protein was detected as two bands in the gel, possibly because of partial glycosylation of AvBD9 or incomplete cleavage of the secretory signal peptide. The apparent molecular mass of the two bands in the Tricine-SDS-PAGE gel was approximately 6 kDa, which was slightly larger than the theoretical value of AvBD9 (4.3 kDa). It is probably because the small AvBD9 fragment had fewer amino acids, but contained a large number of polar amino acids, which affected band mobility in the Tricine-SDS-PAGE gel. As a result, the apparent molecular mass was different from the calculated value [6].

**Analysis of Antibacterial Activity of Recombinant AvBD9**

In this study, a constitutive secretion expression system was constructed using *P. pastoris*, and the target protein AvBD9 was secreted into the culture supernatant. Protein secretion by *P. pastoris* occurs at a constant, low rate [10], and as a result, the target protein was dominant in the culture supernatant of the recombinant strains. The total protein concentration was used to determine the concentration of the target protein AvBD9. In this study, the antibacterial activity of recombinant AvBD9 from fermentation was studied, and it was found to have a broad antibacterial spectrum, with a strong killing effect on *E. coli*, *Salmonella*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Staphylococcus*, which conforms to the results of previous research [7]. The major difference between this study and previous research was the lack of a significant inhibitory effect of recombinant AvBD9 against *Candida albicans* in this experiment.

**Effect of *P. pastoris* with Recombinant AvBD9 on Broiler Growth**

The immune organs are the primary structure responsible for immune function in animals and are the main site of immune responses. The immune organs of poultry include the thymus, bursa of Fabricius, and spleen. The bursa of Fabricius and thymus are central immune organs, and the spleen is a peripheral immune organ. The bursa of Fabricius is a unique organ in poultry that is responsible for the humoral immune response and is the birthplace of B lymphocytes. The thymus is a central organ of cellular immunity and the place of differentiation and maturation of T lymphocytes. The spleen is the largest peripheral poultry immune organ and it participates in the systemic immune response, including cellular immunity and humoral immunity. The development status, weight, and performance of poultry immune organs directly determine the effectiveness of the avian immune response.

The normal intestinal flora is formed during the gradual maturation of animals, and it interacts with the internal environment to form a relatively stable and balanced microecosystem, which significantly influences the growth and development of host animals. In this study, the white feather broiler was used as an animal model to investigate the effects of recombinant yeast on average daily weight gain, immune organ indices, and cecal and ileal microflorae of broilers at different ages. The results showed that the

average daily weight gain of broilers and the organ indices of the thymus, spleen, and bursa of Fabricius were considerably improved in the groups treated with *P. pastoris* with recombinant AvBD9 in comparison with the control groups. Moreover, treatment with the recombinant AvBD9 yeast also had an effect on the intestinal dominant flora and flora composition of the broilers, by enriching the diversity of intestinal flora, changing the dominant intestinal flora, improving population density, and reducing the number of harmful microorganisms. These changes caused the intestinal flora to enter a stable state at an earlier time.

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