Effects of dietary supplementation with *Bacillus* spp. and *Debaryomyces* spp. on broiler’s growth performance, serum characteristics, intestinal microflora and antioxidant activity

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**Introduction**

There are about 80% of antibiotics in the world used in animals. As this phenomenon is likely to cause drug resistance (Al-Khalifa 2018; Chang et al. 2020; Huang et al. 2021), more people pay attention to this issue (Lee et al. 2022). In 2006, the European Union (EU) began to prohibit the use of antibiotics in promoting animal growth. In 2017, the United States imposed restrictions on antibiotics, and in 2020, China began to ban the use of antibiotics in animal feed. Accordingly, the use of antibiotics has been reduced and replaced by beneficial feed additives like probiotics (Lutful-Kabir 2009). Probiotics are beneficial for improving feed intake, digestion and intestinal microflora, which could increase the health status and growth performance of chickens (Chang et al. 2019b; Chuang et al. 2021; Lee et al. 2022). Moreover, probiotics are regarded as natural sources to enhance broiler chickens’ antioxidant and immune capacity (Aluwong et al. 2013; Teng et al. 2017; Gong et al. 2018).

It is well-known that *Bacillus* spp. is resistant to gastric and intestinal juices because when *Bacillus*...
suffered from a poor environment, it would produce spores to protect itself, allowing *Bacillus* spp. to pass through gastric and intestinal juices to the host (Barbosa et al. 2005; Tsai et al. 2021; Lee et al. 2022). In addition, *Bacillus* spp. is capable of secreting a variety of enzymes that could improve feed digestion and nutrient absorption (Mingmongkolchai and Panbangred 2018).

Furthermore, *Debaryomyces* spp. have been reported to have potential probiotic properties (Kumura et al. 2004). It is approved by the European Food Safety Authority (EFSA) and included in their Qualified Presumption of Safety status list. *Debaryomyces* spp. DI 02 can be a potential probiotic candidate because it could safely pass through the gastrointestinal tract, adhere to Caco-2 cells, and enhance immune responses (Ochangco et al. 2016). The cell wall of *Debaryomyces hansenii* is composed of β-glucan and mannan. These substances have the ability to enhance the immune system (Angulo et al. 2020). Thus, the strains of yeast *D. hansenii* are considered potential probiotic candidates for the poultry industry.

It is known that male and female parrots secrete the hormone prolactin, increasing the thickness of the inner wall of the crop, which will fall off to form the crop milk before the young bird hatches (Silver 1984). Crop milk is semi-solid, and its main component are proteins (60%). Therefore, crop milk is an important source necessary for the growth of young parrots. A previous study found that using crop milk to feed broilers, the body weight gain is higher than the control group at 7, 14, 21, 28 and 42 days (Pace et al. 1952). Therefore, it may have hypothesised that crop milk contains some probiotics which positively affected the growth of broilers.

However, fewer studies have screened probiotics from parrots’ crop milk. Hence, the purpose of this study is to screen potential *Bacillus* spp. and *Debaryomyces* spp. from parrots’ crop milk as a supplement in the broilers’ diet to investigate the growth performance, serum characteristics, intestinal microflora and antioxidant activity for broilers.” in the Introduction section.

### Materials and methods

**Bacillus** spp. and *Debaryomyces* spp. culture conditions

All animals use protocols were followed in accordance with the Animal Care and Use Committee (IACUC: 107-042). The 22 strains of *Bacillus* spp. and the 4 strains of *Debaryomyces* spp. were screened from *Melopsittacus undulates* crop milk. *Bacillus* strains were cultured in LB broth (Neogen, Lansing, MI, USA) at 37°C for 24h, while *Debaryomyces* spp. was cultured in YM broth (Neogen, Lansing, MI, USA) at 28°C for 48h. *Saccharomyces cerevisiae* spp., a commercial probiotic, was used as a positive control. Comparison of sequencing by NCBI/Basic Local Alignment Search Tool (BLAST) after *Bacillus* strains and *Debaryomyces* spp. were identified by biochemistry, 16s rDNA and 18s rDNA gene sequencing (Table 1).

### In vitro adherence ability

The adherence ability method was modified by Fuller (1978). The fresh crop was taken from a chicken, and the contents of crop epithelium were cleaned with pH 7.2 sterilised phosphate-buffered saline (PBS). Then, the epithelial cells were scraped with sterilised slide glass and dissolved in PBS. The 100 µL of epithelium cells were mixed with 5 x 10⁶ cfu/mL of *Bacillus* spp. and *Debaryomyces* spp. (100 µL), respectively. Then, they were separately cultured at 37°C and 28°C, shaken by a water tank for 30 min. After 30 min, the sample was placed into a microcentrifuge (Labnet, spectrafuge™ 24D Edison, NJ, USA) at 3000 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in 100 µL sterilised PBS. Subsequently, the bacteria were observed with a microscope camera (OPTI, item EL600 HD camera) after a small amount of mixed solution was taken on the

| Numbers | Identity (%) | Strains |
|---------|--------------|---------|
| P3-1    | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P3-2    | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P3-4    | 99.20        | *Bacillus siamensis* KCTC 13613 |
| P3-5    | 99.64        | *Bacillus velezensis* CR-502T |
| P3-6    | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P2-1    | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P2-2    | 99.20        | *Bacillus siamensis* KCTC 13613 |
| P2-3    | 99.64        | *Bacillus velezensis* CR-502T |
| P2-4    | 99.76        | *Bacillus subtilis* subsp. *stercoris* D7XPNT |
| P2-5    | 99.31        | *Bacillus siamensis* KCTC 13613 |
| P1-1    | 99.31        | *Bacillus velezensis* CR-502T |
| P1-3    | 99.31        | *Bacillus siamensis* KCTC 13613 |
| P1-8    | 99.60        | *Bacillus subtilis* subsp. *stercoris* D7XPNT |
| P1-9    | 99.31        | *Bacillus siamensis* KCTC 13613 |
| P1-10   | 99.32        | *Bacillus siamensis* KCTC 13613 |
| P1-16   | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P1-17   | 99.08        | *Bacillus siamensis* KCTC 13613 |
| P1-18   | 99.43        | *Bacillus siamensis* KCTC 13613 |
| P1-20   | 99.32        | *Bacillus siamensis* KCTC 13613 |
| P1-22   | 99.43        | *Bacillus siamensis* KCTC 13613 |
| YD1     | 99.50        | *Debaryomyces hansenii* strain BRDCYB-01 |
| YD2     | 99.49        | *Debaryomyces hansenii* isolate HBLC10 |
| YD6     | 99.50        | *Debaryomyces hansenii* strain YFA122113 |
| YD11    | 99.49        | *Debaryomyces hansenii* strain YFA122113 |
slide glass and stained with gram stain and methyl blue.

**Gastric and intestinal fluid tolerances**

The *Bacillus* and *Debaryomyces* spp. were incubated in the LB broth and YM broth for 1 day and 2 days, respectively. After cultivating, the bacterial liquid with concentration of $5 \times 10^6$ cfu/mL was centrifuged at 7500 x g for 10 min, and the supernatant was removed. The method for gastric and intestinal fluid tolerance was modified by Guo et al. (2006). For gastric tolerance, samples were added with 1 mL PBS (pH 2.0, containing 0.3% pepsin (Merck, CAS NO. 9001-75-6, Darmstadt, Germany). For intestinal fluid tolerance, on the other hand, samples were added with 1 mL PBS (pH 8.0, containing 0.3% bile bovine (Sigma, CAS NO. 8008-63-7, ST. Louis, USA) and 1 mg/mL trypsin.

Then, the samples were incubated at 37°C for 10 min, and the supernatant was removed. The method for gastric and intestinal fluid tolerance was modified by Guo et al. (2006). For gastric tolerance, samples were added with 1 mL PBS (pH 2.0, containing 0.3% pepsin (Merck, CAS NO. 9001-75-6, Darmstadt, Germany). For intestinal fluid tolerance, on the other hand, samples were added with 1 mL PBS (pH 8.0, containing 0.3% bile bovine (Sigma, CAS NO. 8008-63-7, ST. Louis, USA) and 1 mg/mL trypsin. Then, the samples were incubated at 37°C. Surviving microorganisms were plated in LB and YM agars, and the count was expressed in colony-forming units (CFU) per millilitre.

**Enzyme activity assay**

The protease, cellulase and mannanase activities of *Bacillus* spp. (U/mL) methods were modified by previous study (Raut et al. 2012; Yopi et al. 2017; Abu-Gharbia et al. 2018). Protease activity was measured using substrate 0.3 mL of 1% (w/v) casein (dissolved in 20 mM Tris-HCl buffer, pH 7.5) as one unit of protease is defined as the amount of enzyme that hydrolyses casein (Sigma, CAS NO. 9005-46-3, ST. Louis, USA) to produce equivalent absorbance to 1 μmol of tyrosine/min with tyrosine (Alfa Aesar, CAS NO. 60-18-4, Massachusetts, USA) as standard. Cellulase activity was measured using substrate 5 mg/mL of CMC (Sigma, CAS NO. 9004-32-4, ST. Louis, USA) as one unit of cellulase activity is defined as the amount of enzyme that releases 1 μmol of β-glucose per minute under 37°C pH 5.5. Mannanase activity was measured using substrate of 6 mg/mL Locust Bean Gum (Sigma, CAS NO. 9000-40-2, ST. Louis, USA) which was dissolved in 50 mM of sodium phosphate buffer, pH 5.5 as one unit of mannanase activity is defined as the amount of enzyme that liberates 1 μmol of β-mannose per minute under 37°C pH 5.5. Meanwhile, the methods for xylanase, cellulase, mannanase, β-glucanase and α-galactosidase activities of *D. hansenii* were modified by previous study (Vink et al. 2004; Carrasco et al. 2016; Yopi et al. 2017; Liu et al. 2018; Cao et al. 2019). Xylanase activity was measured using 250 μL of the substrate solution of 10 mg/mL xylan (Sigma, CAS NO. 9014-63-5, ST. Louis, USA) as one unit of xylanase activity is defined as the amount of enzyme capability in releasing 1 μmol of xylose per minute under 30°C pH 5.0. One unit of β-glucanase activity is defined as the amount of enzyme required to release one μmole of glucose-reducing-sugar equivalents per minute in the presence of β-glucan (8 mg/mL) (Sigma, CAS NO. 9008-22-4, ST. Louis, USA) in sodium acetate buffer (100 mM), pH 5.0 at 50°C. One activity unit (U) of α-galactosidase was defined as the amount of enzyme that releases one μmol of p-nitrophenol from 10 mM p-nitrophenyl-α-D-galactopyranoside (Sigma, CAS NO. 3767-28-0, ST. Louis, USA) per minute under 40°C pH 4.0.

**Animal experiments**

A total of 480 1-day-old male and female polyculture Ross 308 broiler chickens were randomly assigned for 35 days to 6 groups, namely the control group (fed with corn-soybean meal), BH group (basal diets with $5 \times 10^6$ cfu/kg feed *Bacillus velezensis*), B5 group (basal diet with $5 \times 10^6$ cfu/kg feed *Bacillus subtilis*), DH group (basal diet with $5 \times 10^6$ cfu/kg feed *D. hansenii*), BV + DH group (basal diet with $5 \times 10^6$ cfu/kg feed *B. velezensis* and *D. hansenii*), BV + SC group (basal diet with $5 \times 10^6$ cfu/kg feed *B. velezensis* and *S. cerevisiae*). All the bacteria added into the diet is in powder form. There were 20 chickens per pen and 4 replicates per treatment. During the test period, the feed and drinking were ad libitum. Diets were divided into starter and finisher (Table 2). The average body weight (47.1 ± 0.60 g/bird) was initially similar among the groups. Both were formulated to meet the nutrients of broilers (NRC, 1994). The body weights of the broiler were measured at 21 and 35 days of age to calculate the broiler’s body weight, average weight gain, average feed consumption and feed conversion ratio (FCR).

**Serum characteristics**

At 5th weeks of the experiment, blood samples were randomly collected from broilers in each treatment group. The samples were collected into a blood collection vessel (about 5 mL), placed at 4°C for 3–4 h to precipitate the blood. Then, it was centrifuged at 3000 x g at 4°C for 10 min to extract the serum for analysis. Each treatment 4 replicates (each replicate 2 birds, totally 8 birds for half male and female) was measured by an automatic biochemical analyzer.
Intestinal morphology

At 5 weeks, a total of 30 broiler chickens were randomly taken from 6 treatments (each treatment has 4 broiler chickens) to analyse a total of lactic acid bacteria and coliform counts in the ileum and caecum. The broiler chickens were euthanized, 0.1 g of digesta were taken from the ileum and caecum, and mixed with 0.9 mL sterilised PBS for serial dilution. The number of lactic acid bacteria and coliform were calculated using Lactobacilli MRS agar and CHRO Magar™ ECC as the selected media.

Statistical analysis

Collected data were analysed for significance by ANOVA using the GLM procedure in the SAS software (SAS® 9.4, 2018, SAS Institute Inc., Cary, NC, USA). Differences between the treatment means were analysed using Duncan’s multiple range test, where p < .05 was considered significantly different.

Results

In vitro adhesion of probiotics to epithelial cells

The results of different probiotics adhering to crop epithelial cells are shown in Figure 1. Four different strains of probiotics were used in the adhering assay, namely B. velezensis, B. subtilis, S. cerevisiae and D. hansenii. Based on the pictures, the number of bacteria adhering to the crop epithelial cell was more than on the outside. Thus, all the probiotics had adhered to the crop epithelial ability.

Stimulated gastric fluid tolerance assay

The gastrointestinal fluid tolerance of Bacillus spp. and Saccharomyces spp. are shown in Figure 2. (A) showed that 4 different D. hansenii screened from crop milk decreased within 1 log CFU/mL after 2 h and 4 h of pH 2.0 with 0.3% pepsin-stimulated gastric fluid; hence the ability of all D. hansenii to tolerate gastric juice. On the other hand, (B) showed that the number of 22 Bacillus strains decreased within 1 log cfu/mL after 3 h of pH 2.0 and 0.3% pepsin-stimulated juice, implying

Table 2. Ingredients and compositions of the experimental diets for broiler chickens.1

| Ingredients                        | Starter diet (1–21 days) | Finisher diet (22–35 days) |
|------------------------------------|--------------------------|----------------------------|
| Corn, yellow                       | 524.9                    | 549.5                      |
| Soybean meal (CP-44%)              | 320.0                    | 320.6                      |
| Full fat soybean meal              | 41.4                     | 16.6                       |
| Fish meal (CP-65%)                 | 50.0                     | 30.0                       |
| Soybean oil                        | 30.0                     | 50.0                       |
| Monocalcium phosphate              | 11.2                     | 12.2                       |
| Calcium carbonate                  | 11.6                     | 10.6                       |
| α-Methionine                       | 3.4                      | 3.0                        |
| NaCl                               | 2.9                      | 3.4                        |
| l-Lysine HCl                       | 1.8                      | 1.3                        |
| Choline-Cl                         | 0.8                      | 0.8                        |
| Vitamin premix2                    | 1.0                      | 1.0                        |
| Mineral premix3                    | 1.0                      | 1.0                        |
| Total                               | 1000                     | 1000                       |
| Composition                         |                          |                            |
| ME, kcal/kg                        | 3050                     | 3175                       |
| Crude protein, %                   | 23.0                     | 21.0                       |
| Crude fat, %                       | 6.04                     | 7.56                       |
| Calcium, %                         | 1.05                     | 0.90                       |
| Total phosphorus, %                | 0.73                     | 0.68                       |
| Available phosphorus, %            | 0.50                     | 0.45                       |
| Lysine, %                          | 1.43                     | 1.25                       |
| Methionine, %                      | 0.73                     | 0.65                       |
| Cysteine, %                        | 0.34                     | 0.31                       |
| Analysed nutrient value            |                          |                            |
| Dry matter, %                      | 88.88                    | 88.0                       |
| Crude protein, DM%                 | 23.1                     | 21.4                       |

1Control: corn-soybean meal; BV: basal diet supplemented with 5 x 10^6 cfu/kg feed of Bacillus velezensis; BS: basal diet supplemented with 5 x 10^6 cfu/kg feed of Bacillus subtilis; DH: basal diet supplemented with 5 x 10^6 cfu/kg feed of Debaryomyces hansenii; BV + SC: basal diet supplemented with 5 x 10^6 cfu/kg feed of B. velezensis and Saccharomyces cerevisiae; BV + DH: 5 x 10^6 cfu/kg feed of B. velezensis, and D. hansenii.

2Supplied per kg of diet: Vit. A 15000 U; Vit. D 3000 U; Vit. E 30 mg; Vit. K 4 mg; Riboflavin 8 mg; Pyridoxine 5 mg; Vit. B12 25 μg; Ca 19 mg; Niacin 50 mg; Folic acid 1.5 mg; Biotin 60 μg; Fe (FeSO4.H2O) 90 mg; Zn (ZnO) 68.4 mg; Se (Na2SeO3) 0.18 mg.

3Supplied per kg of diet: Co (CoCO3) 0.255 mg; Cu (CuSO4.H2O) 10.8 mg; Mg (MgCl2.H2O) 34 mg; Mn (MnSO4.H2O) 90 mg; Se (Na2SeO3) 0.18 mg.

Each value represents the mean of 3 replicates.

ME: Metabolizable energy; DM: Dry matter.
that the different *Bacillus* spp. screened from crop milk had the ability to tolerate stimulated juice.

**Stimulated small intestinal fluid tolerance assay**

The small intestinal fluid tolerance of *Bacillus* spp. and *Saccharomyces* spp. are shown in Figure 2(C). Five different *D. hansenii* strains decreased within 1 log cfu/mL after 2 h and 4 h of pH 8.0, 0.3% bile salts (w/v) with 1 mg/mL trypsin-stimulated juice, thereby demonstrating all five strains’ ability to tolerate small intestinal fluid. Figure 2(D) shows *Bacillus* P1-23. P1-24 strains decreased over 1 log cfu/mL after stimulating small intestinal fluid, indicating that *Bacillus* P1-23 and P1-24 could not tolerate such a stimulating environment. However, other strains of *Bacillus* spp. remained at least 7 log cfu/mL after the stimulation of the intestinal fluid environment.

**Enzyme activity assay**

Table 3.1 shows the protease, cellulase and mannanase activities of the 22 *Bacillus* spp. The protease activities of P3-2 and P3-5 had the highest activity—25.9 U/mL and 25.5 U/mL, respectively. Meanwhile, the cellulase activities of P1-8 and P2-4 had the highest activity—respectively, 0.138 U/mL and 0.153 U/mL. In the mannanase analysis, P1-8 and P2-4 had the highest mannanase activities, with 0.388 U/mL and 0.445 U/mL, respectively.

Table 3.2 shows the activities of cellulase, xylanase, β-glucanase, mannanase and α-galactosidase of the strains of *D. hansenii* and *Saccharomyces* spp. In the cellulase test, five strains had similar 0.20 U/mL activity. The highest activity of xylanase, β-glucanase, mannanase and α-galactosidase were YD2, YD1, YD11 and C1 strains, respectively.

**Animal performances**

The results showed five different probiotic formulas (5 × 10⁶ cfu/mL) supplemented with basal diets of broiler chicken in Table 4. At 1–21 days, in terms of the body weight of the chicken, BV, BV + SC, and BV + DH groups were significantly higher than the control group (*p < .05*). The body weight gain significantly increased in the BV + SC and BV + DH groups (*p < .05*). However, all treatments had no significant differences during 22–35 days and 1–35 days.
Serum characteristics

The results of serum characteristics data are shown in Table 5. The analysis of GLU, BUN, CREA, UA, CHOL, HDL-C, LDL-C, SGOT and SGPT showed no significant differences among the groups. Meanwhile, the TG value was significantly decreased in the BV, BV + SC, and DH.
groups (p < .05). The CAT and SOD activities had no significant differences among the groups. However, the GPx activity significantly increased in BV, BS and BV + SC groups than in the control group (p < .05).

**Intestinal morphology**

Table 6 shows the results of the five different probiotic formulas supplemented with basal diets of *Bacillus* spp.

### Table 3.1. The protease, cellulase, mannanase activities of *Bacillus* spp.

| Enzymes | Strains | Protease(U/mL) | Cellulase(U/mL) | Mannanase(U/mL) |
|---------|---------|----------------|-----------------|-----------------|
|         | P1-1    | 13.3 ± 6.08 4  | 0.076 ± 0.005   | 0.670 ± 0.001   |
|         | P1-3    | 21.6 ± 1.55 5  | 0.080 ± 0.007   | 0.143 ± 0.007   |
|         | P1-8    | 4.77 ± 1.25    | 0.138 ± 0.005   | 0.388 ± 0.012   |
|         | P1-9    | 15.9 ± 0.20    | 0.073 ± 0.003   | 0.156 ± 0.005   |
|         | P1-10   | 18.7 ± 5.70    | 0.075 ± 0.004   | 0.148 ± 0.012   |
|         | P1-16   | 17.1 ± 2.10    | 0.076 ± 0.008   | 0.145 ± 0.007   |
|         | P1-17   | 13.4 ± 1.08    | 0.061 ± 0.003   | 0.149 ± 0.007   |
|         | P1-18   | 15.6 ± 0.70    | 0.077 ± 0.003   | 0.143 ± 0.012   |
|         | P1-20   | 7.48 ± 2.48    | 0.076 ± 0.004   | 0.142 ± 0.005   |
|         | P1-22   | 8.34 ± 3.08    | 0.074 ± 0.006   | 0.092 ± 0.007   |
|         | P1-23   | 6.15 ± 0.98    | 0.025 ± 0.003   | 0.064 ± 0.002   |
|         | P1-24   | 0               | 0.027 ± 0.008   | 0.064 ± 0.004   |
|         | P2-1    | 19.0 ± 1.52    | 0.069 ± 0.003   | 0.166 ± 0.003   |
|         | P2-2    | 16.7 ± 2.38    | 0.067 ± 0.008   | 0.189 ± 0.005   |
|         | P2-3    | 9.07 ± 2.52    | 0.041 ± 0.003   | 0.190 ± 0.001   |
|         | P2-4    | 13.1 ± 1.08    | 0.153 ± 0.003   | 0.445 ± 0.001   |
|         | P2-5    | 16.9 ± 5.02    | 0.077 ± 0.008   | 0.141 ± 0.005   |
|         | P3-1    | 14.3 ± 1.08    | 0.069 ± 0.005   | 0.148 ± 0.001   |
|         | P3-2    | 25.9 ± 1.50    | 0.080 ± 0.002   | 0.188 ± 0.001   |
|         | P3-3    | 15.0 ± 1.50    | 0.058 ± 0.007   | 0.172 ± 0.003   |
|         | P3-5    | 25.4 ± 2.05    | 0.073 ± 0.004   | 0.153 ± 0.007   |
|         | P3-6    | 22.01 ± 1.52   | 0.075 ± 0.004   | 0.166 ± 0.005   |

3One unit of protease activity is defined as 1 μmol tyrosine generated from 1% casein in condition of 40 °C and pH 7.5 in a minute.
2One unit of cellulase activity is defined as 1 μmol, where reducing sugar is generated from 5 mg/mL CMC in the condition of 37 °C and pH 5.5 in a minute.
4One unit of mannanose activity is defined as 1 μmol, where reducing sugar is generated from 6 mg/mL locust bean gum in the condition of 37 °C and pH 5.5 in a minute.

Each value represents the mean of 3 replicates.

**Microbial population in ileum and caecum**

Table 7 shows the effect of feed supplement with *Bacillus* spp., *D. hansenii* and *Saccharomyces* spp. on the gut microflora. BV and BS groups increased significantly the lactic acid bacteria compared to the control group in the ileum, whereas BV, BS and DH groups had significantly higher lactic acid bacteria than the control group in the caecum (p < .05). However, coliform had no significant differences among the groups (p > .05).

**Discussion**

The use of antibiotics for growth promotion has been prohibited by the European Union. Therefore, probiotics play an important role in replacing antibiotics. FAO/WHO (2002) defined probiotics as live microorganisms, which could benefit the host. Fuller (1989) listed the basic characteristic of probiotics as (1) it should be a strain that could bring positive effects to the host; (2) it should be non-pathogenic and non-toxic; (3) it should survive in the low pH and bile salts environment. Accordingly, if probiotics pass through the gastrointestinal tract, they would have the ability...
Table 4. The effects of Bacillus spp. and yeast supplemented in diets on growth performance of 1-35d broiler chickens.

| Item                        | Control | BV | BS | DH | BV + SC | BV + DH | SEM  | p Value |
|-----------------------------|---------|----|----|----|---------|---------|------|---------|
| Weight gain (g)             | 1983    | 2115 | 2077 | 2010 | 2123 | 2100 | 56.2 | .408    |
| Feed consumption (g)        | 3015    | 3010 | 3286 | 3108 | 3157 | 3228 | 24.7 | .034    |
| Body weight (g)             | 829b    | 923a | 875ab | 858ab | 926a | 936a | 24.6 | .038    |
| FCR                         | 1.52    | 1.42 | 1.58 | 1.55 | 1.49 | 1.54 | 0.033 | .051    |
| 1–21 d                      | 1119    | 1148 | 1203 | 1147 | 1176 | 1187 | 29.0 | .373    |
| 22–35 d                     | 1986    | 1863 | 2082 | 1961 | 1981 | 2041 | 62.4 | .165    |

1A: corn-soybean meal; BV: basal diet supplemented with 5 × 10⁶ cfu/kg of Bacillus velezensis; BS: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Bacillus subtilis; DH: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Debaryomyces hansenii; BV + SC: basal diet supplemented with 5 × 10⁶ cfu/kg feed of B. velezensis, and 5 × 10⁶ cfu/kg feed of Saccharomyces cerevisiae; BV + DH: 5 × 10⁶ cfu/kg feed of B. velezensis, and 5 × 10⁶ cfu/kg feed of D. hansenii.

2Each value represents the mean of 80 replicates.

3Each value represents the mean of 4 replicates (2 birds in each replicate).

4FCR: feed conversion ratio.

5Means with in the same row with different letters are significantly different (p < .05).

Table 5. The effect of Bacillus spp. and yeast supplemented in diets on serum characteristic of 35d broiler chickens.

| Item                        | Control | BV | BS | DH | BV + SC | BV + DH | SEM  | p Value |
|-----------------------------|---------|----|----|----|---------|---------|------|---------|
| GLU                         | 196     | 207 | 199 | 190 | 226     | 214     | 15.4 | .565    |
| BUN                         | 1.13    | 2.00 | 1.50 | 1.88 | 2.62     | 1.88     | 0.589 | .157    |
| CREA                        | 0.016   | 0.014 | 0.01 | 0.02 | 0.018    | 0.011    | 0.003 | .514    |
| UA                          | 4.81    | 3.46 | 3.03 | 5.6 | 5.11     | 3.40     | 11.5 | .272    |
| CHOL                        | 159     | 147 | 135 | 166 | 156     | 131      | 10.3 | .350    |
| TG                          | 198b    | 129a | 167ab | 155bc | 118ab     | 180ab    | 13.5 | .001    |
| HDL-C                       | 88.9    | 84.4 | 79.8 | 89.4 | 86.3     | 75.8     | 4.79  | .469    |
| LDL-C                       | 66.3    | 60.1 | 53.0b | 73.0 | 66.9     | 49.5     | 5.75  | .179    |
| SGPT                        | 3.75    | 3.13 | 3.62 | 4.12 | 3.88     | 3.63     | 0.368 | .289    |
| SGPT                        | 25.72   | 39.19 | 29.54 | 37.59 | 37.53    | 40.2     | 9.45  | .869    |
| GPx                         | 106.0p | 134.77 | 136.7p | 107.6p | 155.6a   | 110.5p   | 10.82 | .007    |
| CAT                         | 32.7    | 35.9 | 38.0 | 4.24 | 4.63     | 5.08     | 0.526 | .282    |
| TFA                         | 180.0   | 185.8 | 198.0 | 194.0 | 211.8    | 213.2    | 11.61 | .31     |

GLU: glucose; BUN: urea nitrogen; CREA: creatinine; UA: uric acid; CHOL: cholesterol; TG: triglyceride; HDL: high-density lipoprotein; LDL: low-density lipoprotein; SGOT: glutamic-oxaloacetic transaminase; SGPT: serum glutamic-pyruvic transaminase; Alk-P: alkaline phosphatase; SOD: Superoxide dismutase; Gpx: Glutathione peroxidase; CAT: Catalase; IL-6: Interleukin-6; IL-10: Interleukin-10; TNF-α: Tumour necrosis factor alpha.

1Each value represents the mean of 4 replicates (2 birds in each replicate) totally 8 birds.

2Means with in the same row with different letters are significantly different (p < .05).

3A: corn-soybean meal; BV: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Bacillus velezensis; BS: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Bacillus subtilis; DH: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Debaryomyces hansenii; BV + SC: basal diet supplemented with 5 × 10⁶ cfu/kg feed of B. velezensis, and 5 × 10⁶ cfu/kg feed of Saccharomyces cerevisiae; BV + DH: basal diet supplemented with 5 × 10⁶ cfu/kg feed of B. velezensis, and 5 × 10⁶ cfu/kg feed of D. hansenii.

4Significantly different C. L. LIU ET AL. to tolerate gastric juice and small intestinal fluid (Lee et al. 2022). In vitro test showed that 22 strains of Bacillus spp. and 4 different D. hansenii screened from M. undulates (Table 1) could survive in gastric and intestinal fluids because the Bacillus spp. and Debaryomyces spp. counts only decreased within 1.5 log cfu/mL after culturing in the gastric- and intestinal-simulated environment except for P1-23 and P1-24 (Figure 2). Gotcheva et al. (2002) reported that yeast could pass through the gastrointestinal tract. Furthermore, Algbreri et al. (2016) showed B. subtilis KATMIRA1933 exposed to the acid environment (range from pH 2.0 to pH 3.0) and bile salts test for 4 h. In the study, the survival rates of B. subtilis KATMIRA1933 remain changeless. The results showed similar findings in our study.

Furthermore, probiotics should have the ability to adhere to intestinal epithelial cells because they can compete for the binding site with pathogens (Dunne et al. 2011; Teng et al. 2017; Lee et al. 2022). As mentioned previously, probiotics could inhibit the number of pathogens in the host’s intestine and maintain the intestinal microflora ecology balance (Dunne et al. 2011; Chang et al. 2019a; Chang et al. 2020). Both et al. (2010) pointed out that the adhesion ability results depend on the number of adhered bacterial cells on the epithelial cell. According to the in vitro assay with different strains in our study, Bacillus P3-5, P2-4, D. hansenii Y1, and S. cerevisiae (control group for business) were selected to adhere to the epithelial cell assay. The results showed that 4 different strains have adhesion to the epithelial ability because the
Lactic acid bacteria
Each value represents the mean of 3 replicates.

In the enzyme test, Bacillus P3-2 and P3-5 had higher protease activity among other groups – 25.9 U/mL and 25.5 U/mL, respectively. On the other hand, Bacillus spp. had the highest protease activity among other enzymes (Tables 3.1 and 3.2). Chu (2007) pointed out that strain Bacillus APP1 with the highest-yield alkaline proteases improves the high protein contents of feed digestion and enhances broiler’s absorption. Since protease can break protein’s peptide bonds and digest dietary proteins into small molecules, the host can easily absorb dietary nutrients. Additionally, Debaryomyces spp. could secrete enzymes, such as cellulase, xylanase, mannanase, β-glucanase and α-galactosidase; 4 different Debaryomyces spp. had similar results. Park et al. (2020) reported that multi-enzyme, including xylanase, β-glucanase, protease, and amylase, could improve the host’s growth performance and digestibility.

Angulo et al. (2020) reported that yeast D. hansenii has been considered an excellent probiotic in animals because D. hansenii could improve the growth of fish, enhance digestibility and maintain the intestinal population balance. Bai et al. (2017) showed that diets supplemented with Bacillus spp. could increase the average daily gain, improve the feed conversion ratio (FCR) of broiler chickens by reducing the feed intake, and enhance antioxidant activities and immune responses. Above all, D. hansenii and Bacillus spp. are the potential probiotics nowadays, which are beneficial for the growth of broiler chickens. In the current study, BV, BV + SC, BV + DH supplemented in broiler chickens’ diets significantly increased the chickens’ body weight and weight gain at 21 days of age (Table 4). Manafi et al. (2018) showed that adding Bacillus spp. and Debaryomyces spp. in the feed could improve the broilers’ growth performance. Furthermore, Chen et al. (2009) demonstrated that B. subtilis var. natto combined with S. cerevisiae had resulted in a higher broiler chickens body weight, body weight gain, and feed intake than the control group at 1–21 and 21–39 days.

The serum characteristic of 35 d broiler chickens showed that BV, DH and BV + SC groups had a lower value than the control group (129, 155 and 118 vs. 198 mg/dL) (Table 5). Rajput et al. (2013) reported that Saccharomyces boulardii and B. subtilis decreased TG content in broilers. Ahmadi et al. (2017) reported that probiotics could decrease serum triglycerides and VLDL-cholesterol because they can produce short fatty acids, which can suppress the synthesis of fatty acids and decrease the triglycerides secretin rate. The antioxidant assay and glutathione peroxidase (GPx) activity significantly increased in BV, BS, and BV + SC groups than the control group (134.7, 136.7 vs. 155.6 nmol/min/mL) (Table 5). GPx is a cytosolic enzyme that catalyses hydrogen peroxide to water

### Table 6. The effect of Bacillus spp. and yeast supplemented in diets on intestinal morphology of 35 d broiler chickens.1

| Item                  | Control | BV | BS | DH | BV + SC | BV + DH | SEM | p Value |
|-----------------------|---------|----|----|----|---------|---------|-----|---------|
| Jejunum               |         |    |    |    |         |         |     |         |
| Villus height (µm)    | 1350    | 1367 | 1430 | 1322 | 1518 | 1474 | 27.20 | <.0001 |
| Crypt depth (µm)      | 202     | 162 | 181 | 173 | 263 | 186 | 8.23 | .0132  |
| Villus/Crypt ratio    | 6.91    | 8.85 | 8.79 | 8.28 | 7.67 | 8.80 | 0.83 | .1101  |
| Ileum                 |         |    |    |    |         |         |     |         |
| Villus height (µm)    | 100     | 1065 | 1029 | 1054 | 1140 | 1047 | 20.80 | .0002  |
| Crypt depth (µm)      | 178     | 161 | 169abc | 183a | 184 | 153 | 6.61 | .0034  |
| Villus/Crypt ratio    | 6.13    | 7.00 | 6.93 | 7.02 | 6.85 | 6.53 | 0.32 | .3459  |

1The data were means of 50 spots corresponding to 5 birds for each group.

### Table 7. The effect of Bacillus spp. and yeast supplemented in diets on intestinal microflora concentration of 35d broiler chickens.

| Item                  | Control | BV | BS | DH |BV + SC |BV + DH |SEM | p Value |
|-----------------------|---------|----|----|----|--------|--------|-----|---------|
| Jejunum               |         |    |    |    |        |        |     |         |

1: a corn-soybean meal; BV: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Bacillus velezensis; BS: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Bacillus subtilis; DH: basal diet supplemented with 5 × 10⁶ cfu/kg feed of Debaryomyces hansenii; BV + SC: basal diet supplemented with 5 × 10⁶ cfu/kg feed of B. velezensis, and 5 × 10⁶ cfu/kg feed of D. hansenii.

*± Means with in the same row with different letters are significantly different (p < .05).

Each value represents the mean of 3 replicates.
and oxygen. Rajput et al. (2013) pointed out that B. subtilis can increase the serum antioxidant enzyme, which could support host scavenging free radicals and maintaining healthy states. Excessive oxidative stress will cause DNA hydroxylation, protein denaturation, and lipid oxidation on the host. Wang et al. (2017) showed that probiotics might modulate the redox status of the host via metal ion chelating and antioxidant enzymatic system and stimulate the Nrf2-keap1-ARE pathway, which can stimulate phase II detoxifying antioxidant enzymes, such as haem oxygenase-1 (HO-1) and GPx. Thus, the supplementation of BV, BS, and BV + SC probiotics to the feed could help balance the oxidative stress for broilers.

In this study, BV + SC and BV + DH groups had higher villus height, and the BV group had lower crypt depth in the jejunum. The BV + SC group had higher villus height in the ileum, and BV + DH groups had lower crypt depth than the control group (Table 6). The results indicated that BV + SC and BV + DH groups might influence the improvement of nutrient absorption because increasing villus height could expand the villus surface area, which affects the host's nutrient absorptivity. Manafi et al. (2018) pointed out that Bacillus spp. and yeast supplements could increase villus height and decrease crypt depth. Decreasing crypt depth can reduce energy consumption because animals can spend less energy to form crypt depth (Manafi et al. 2018).

BV and BS groups significantly increased the total lactic acid bacteria in the ileum. However, BV, BS, and DH groups increased significantly the total lactic acid bacteria in the caecum (Table 7). Giang et al. (2011) pointed out that Bacillus spp. and yeast can enhance the number of lactic acid bacteria counts in the intestinal. Phuoc and Jamikorn (2016) also demonstrated that probiotics could enhance the organic acid concentration in the intestine, which is expected to reduce pH. Furthermore, a lower pH value could inhibit the pathogens, allowing probiotics to enhance intestinal health.

Conclusions
The Bacillus spp. and Debaryomyces spp. from M. undulataes's crop milk were screened, whose properties were examined in vitro accordingly. The in vitro test shows that the best B. velezensis, B. subtilis, and D. hansenii supplements in the broiler feed additive were selected. In terms of the broilers’ growth performance, BV, BV + SC, and BV + DH groups significantly increased the broilers’ body weight and body weight gain at 21 days of age. BV and BS treatments can increase the lactic acid bacteria in the ileum and caecum. In intestinal morphology, BV + SC group significantly increased the villus height in the jejunum and ileum. The BV group significantly decreased the crypt depth in the jejunum, whereas the BV + DH group significantly decreased in the ileum. BV, BS, and BV + SC groups significantly enhanced the GPx activity than the control group. According to the results, BV and BV + SC treatments possess potential the probiotic feed additives for broilers.
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