**Effects of** ***Bacillus subtilis* and antibiotic growth promoters on the growth performance, intestinal function and gut microbiota of pullets from 0 to 6 weeks**

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Short title: *Bacillus Subtilis* application in pullets

**Supplementary Material S1**

*Intestinal morphology*

According to anatomical characteristics, duodenum appear in the form of “U” and begin with the gizzard; jejunum is between the end of U and Meckel’s diverticulum; ileum is between Meckel’s diverticulum and ileocecal junction. Duodenum, jejunum and ileum were obtained and rinsed using saline to remove their content. About 3 cm samples at the midpoint region of the duodenum, jejunum and ileum were excised. Then these intestinal segments were flushed and fixed in 10% buffered formalin at least 48 h before histology process. After fixing, fixed samples were dehydrated and cleared. Then intestinal samples were cut and inserted into cassettes which were embedded in liquid paraffin. Later, 5 μm paraffin section was gained using the microtome and stained with hematoxylin-eosin. Villus height, crypt depth and wall thickness were determined using phase contrast microscope.

**Supplementary Material S2**

*ELISA assay of Secretory Immunoglobulin A (sIgA) determination*

Supernatant of mucosa homogenate and standards of known concentrations were added into the wells of ELISA plate. Cover with a plate sealer, and incubate for 1 hour at 37℃.Then the solution was discarded, and wells were washed using 250 μL washing buffer for 3 times. Detection reagent was added into the wells for incubating at 37℃ about 30 minutes with plate sealer covering. Later, discard the solution and wash wells for 5 times. Add substrate solution to each well. Cover with a new plate sealer, incubate dark for 20 minutes. Stop solution was added into the wells. Mix the liquid by tapping the side of the plate. Finally, run the microplate reader and conduct measurement at 450 nm immediately. the standard curve was carried out based on standards of known concentrations and their corresponding optical density value (OD). The concentration value for experimental samples was calculated by their OD values referring to the standard curve. The standards, ELISA wells, plate sealer, washing buffer, substrate and stop solution were provided by ELISA Kits.

**Supplementary Tables**

**Table S1** *Composition and nutrient levels of basal diets in pullets (as-fed basis)*

|  |  |
| --- | --- |
| Items | Content |
| 0 to 3 weeks | 4 to 6 weeks |
| Ingredients（%） |  |  |
| Corn | 42.91 | 61.00 |
| Soybean meal | 17.00 | 23.00 |
| Extruded corn | 20.00 |  |
| Fermented soybean meal | 6.00 | 4.00 |
| Fish meal | 3.00 | 2.00 |
| Wheat bran | 3.00 | 5.00 |
| Chicken plasma gluten meal | 2.00 | — |
| Soybean oil | 1.80 | 1.40 |
| Glucose | 1.50 | — |
| CaHPO4 | 1.20 | 1.40 |
| Limestone | 1.00 | 1.20 |
| NaCl | 0.12 | 0.20 |
| Lysine | 0.10 | 0.10 |
| Choline chloride | 0.10 | 0.06 |
| *DL*-methionine | 0.05 | 0.06 |
| Acidifier | 0.04 | 0.40 |
| Complex enzyme | 0.03 | 0.03 |
| Threonine | 0.02 | 0.02 |
| Multi-minerals1 | 0.10 | 0.10 |
| Multi-vitamin2 | 0.03 | 0.03 |
| Total | 100.00 | 100.00 |
| Nutrient levels（%） |  |  |
| CP | 20.00 | 19.70 |
| Ca | 0.82 | 0.91 |
| Total phosphorus | 0.62 | 0.63 |
| Available phosphorus | 0.38 | 0.40 |
| NaCl | 0.37 | 0.34 |
| Lysine | 1.18 | 1.11 |
| Methionine | 0.39 | 0.39 |
| Methionine+Cysteine | 0.75 | 0.72 |

1The trace element premix provided per kg of diets: Cu (as copper sulfate) 10 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 75 mg, I (as potassium iodide) 0.40 mg, Se (as sodium selenite) 0.30 mg.

2The vitamin premix provided per kg of diets: vitamin A, 250 000 IU; vitamin D, 50 000 IU; vitamin K3, 53 mg; vitamin B1, 40 mg; vitamin B2, 120 mg; vitamin B12, 0.50 mg; vitamin E, 600 IU; biotin, 0.65 mg; folic acid, 25 mg; pantothenic acid, 240 mg; niacin, 1 000 mg.

**Table S2**. *Primers for RT-qPCR analysis*

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | Accession number | Primer sequences (5'to3') | Product size (bp) |
| *β-actin* | L08165 | F:ATTGTCCACCGCAAATGCTTCR:AAATAAAGCCATGCCAATCTCGTC | 113 |
| *Sucrase* | XM015291762 | F: CGCAAAAGCACAGGGACAGTR: TCGATACGTGGTGTGTGCTCAGTT | 140 |
| *aminopeptidase* | NM204861 | F: TTGGCAACAAGGAGCGAGTGR: AGTGGGTGGGAGGTGGTCAG | 238 |
| *Maltase**ZO-1**Mucin-2**Occludin* | XM015273018XM0152789XM421035NM205128 | F: ACGCTCACGGGCAGAAATACR: GCACTCCTCTATCCACCAACGF: GAATGATGGTTGGTATGGTGCGR: TCAGAAGTGTGTCTACTGTCCGF: TTCATGATGCCTGCTCTTGTGR: CCTGAGCCTTGGTACATTCTTGTF: TCATCGCCTCCATCGTCTACR: TCTTACTGCGCGTCTTCTGG | 22420993240 |

Note: ZO-1= tight junction protein 1; RT-qPCR= Real-time Quantitative PCR.