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Effects of *Bacillus subtilis* DSM 32315 on Immunity, Nutrient Transporters and Functional Diversity of Cecal Microbiome of Broiler Chickens in *Necrotic Enteritis* Challenge

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ABSTRACT

This study was conducted to determine the effects of *Bacillus subtilis* DSM 32315 probiotic and antibiotic enramycin in broiler chickens with *Clostridium perfringens* induced-Necrotic enteritis on cecal microbial populations, functional diversity, nutrients transporters and cytokines mRNA expression. Day-old broilers (n= 360), Arbor Acre were randomly assigned to three dietary treatments such as control, basal diet fed-group only; antibiotic, basal diet plus enramycin 5 mg/kg; and probiotic group, basal diet plus *Bacillus subtilis* 2 x10^9 CFU/g. Antibiotic and probiotic fed groups was challenged with *Clostridium perfringens* at day1, and from day 14 to day 21. The results of present study showed that broiler chickens supplemented with antibiotic and probiotic significantly exhibited higher abundance of gut beneficial bacteria at the 21 and 35 days of age, while upregulated the expression of anti-inflammatory cytokine enterleukin-10 and secretory immunoglobulin-A. Expression of proinflammatory cytokines interleukin-6 tumor necrosis factor alpha, and interferon gamma were downregulated. Nutrient transporters of Peptide transporter-1, L amino transporter-2 and Cationic amino acid transporter-2 were upregulated in supplemented groups. More so, glucose transporter-2 Sodium glucose transporter-1, Solute carrier family 3, member 1, carbohydrates and vitamin metabolism cofactor enriched in probiotic fed-group, while control group exhibited up-regulation in interleukin-6, tumor necrosis factor alpha, and interferon gamma. Overall, supplementation of *Bacillus subtilis* DMS 32315 reduced the negative impact of necrotic enteritis in broiler chickens, and enhanced the gut-microbial community.

Keywords: Antibiotic growth promoter, *Bacillus subtilis*, *Clostridium perfringens*, Immune response, probiotic

INTRODUCTION

Antibiotic growth promoters (AGPs), are substantially used in Agriculture to improve the economic value of animals through increased growth and feed efficiency, while indirectly they are used to control some enteric diseases (Gadde et al., 2017). Mismanagement in the use of AGPs in agricultural animals leaded to drug-resistant bacteria "superbugs", drug residue and its possible negative consequences in the intestinal microbial homeostasis (Bai et al., 2017). Moreover, regulatory agencies and consumer demands on animals produced free from antibiotics mandated agricultural industry to make changes in the use of AGPs in animal production (WHO, 2017). Thus, it led to withdrawal or restrictions of antimicrobial use in poultry production in many parts of the world, this resulted to many consequences, among others are prevalence of enteritis related diseases commonly Necrotic Enteritis (NE) and widespread of ill-defined intestinal dysbacteriosis which is associated with reduced nutrients digestibility in poultry (Latorre et al., 2014). Necrotic enteritis is a serious disease in poultry caused by *Clostridium perfringens*, a gram positive, ubiquitous and anaerobic bacterium, found at a level less than 10^5 CFU/g of the intestinal contents of healthy birds, but disturbances in normal intestinal

Microflora may cause rapid proliferation of *C. perfringens*, increasing bacterial numbers to 10^7 to 10^9

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CFU/g of digesta resulting in the development of clinical NE (Opengart et al., 2013). Similarly, NE was characterized as the 'clinical and subclinical form', the subclinical form of NE does much more harm to animals than the clinical form due to its persistent in the flock without any clinical manifestation, no peak of mortality, and are associated with reduced feed intake and weight gain, and increased feed conversion ratio (Dahiya et al., 2006; Timbermont and Immerseel, 2011). Besides the health risk imposed by NE to animals, it was assessed to cost $ 6 billion loss to the poultry industry annually (Wade and Keyburn, 2015). Thus, it was imperative to explore other alternatives to AGPs that can maintain both productive potentials and gut health of the animals (Bai et al., 2017). Previous studies proved that, among the basic strategies that can be employed to cope with the loss of AGPs, and to control NE in broiler chickens, is the incorporation of probiotic into the animal's diet, which maintains animal health, growth and feed efficiency (Zhao and Kim, 2015; Musa Bodinga et al., 2019). An increase in performance of animals can be related to the changes in the microbial population dynamics of the gastrointestinal tract (GIT) of the animals creating favorable microbial environment as a result of shift in balance between beneficial and harmful microbes (Cao et al., 2012). Beneficial bacteria such as ''*Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus,* and *Streptococcus*'' were commonly used as probiotics in animal production (Simon et al., 2001). Nevertheless, in recent time, *Bacillus* based probiotics are gaining acceptance as potential alternative to AGPs in the poultry industry. Perhaps this could be due to their distinct advantage in the production of naturally synthesized peptides, production of beneficial metabolites, modification of gut microbial structure, and stimulation of various immune modulators (Lee et al., 2010; Sumi et al., 2015). Bacillus spores were resistant to antimicrobial low pH, bile salts, and harsh conditions of the GIT. (Shivaramaiah et al., 2011). The nutrient transport system played a vital role in the small intestine of the birds, where specific nutrients were conveyed by specific transporters found at the brush border of the small intestines for absorption by enterocytes (Shivaramaiah et al., 2011). Monosaccharides (glucose, galactose, fructose and mannose) were mediated at the brush border membrane by Sodium dependent glucose and galactose transporter1 (SGLT1), and exit of glucose across the basolateral membrane was mediated by facilitated transporter Na^+ - independent glucose, galactose and fructose transporter 2 (GLUT2) the cell via glucose transporter-2 (Shivaramaiah et al., 2011). Where peptide transporter-1 was responsible for absorption of the most di and tri-peptides, and functions as obligatory exchangers of cationic amino acids, CAT1 was responsible for the efflux of cationic amino acids (Shivaramaiah et al., 2011). Similarly, the composition of gut microbiota were hypothesized to affect many host functions including nutrients' utilization of gut epithelium and development of gut immune system (Hill et al., 2010). In our previous study, it was discovered that supplementation of *Lactobacillus fermentum* 1.2029 probiotic in *C. perfringens* challenged broiler, modulated toll-like receptors and some cytokines related genes (Cao et al., 2012). Nonetheless, the immunopathology of NE in broiler chickens was not fully understood (Oh and Lillehoj, 2016), but it was believed that, NE infection caused numerous changes to immunological structure of cytokines and toll-like receptors (Collier et al., 2008). Despite the fact that, a number of probiotics which have been extensively studied here and others in the poultry industry, yet there are many probiotics without clearly defining their mode of action. Therefore, the main objective of this study, was to evaluate the effect of *Bacillus subtilis* DSM 32315 probiotic and antibiotic (enramycin) on *Clostridium perfringens*-induced Necrotic enteritis on immunomodulators, nutrient transporters, distribution of bacterial population and functional diversity in broiler chickens.

MATERIAL AND METHODS

Ethical approval

The present study was approved by the Institutional Animal ethics Committee at the Northwest Agriculture and Forestry University (protocol number NWAFAC1008 Yangling, Shaanxi, China).

Animals and Trial Design

Test strain information

Bacillus subtilis DSM 32315 used in this study was provided by Evonik Nutrition and Care GmbH, (Hanau, Germany) throughout the experimental period, the product contained a spray-dried spore forming bacteria at a concentration of 2 x 10^9 CFU/g, while AGP (Enramycin) was obtained from Wuhan Guangtu Technology Coop., Ltd. (China).

A total of 360 one-day-old Arbor Acre mixed sexed chickens were randomly assigned to three treatment groups (each one had ten cages of twelve birds). One group was supplemented with basal diet only; control (CON); The second group was antibiotic group (AB)

supplemented with basal diet supplemented with 5 mg/kg enramycin, challenged with *C. perfringens*; and the third group was probiotic group (PB) supplemented basal diet with 2× 10^9CFU/g *Bacillus subtilis* DSM 32315, challenged with *C. perfringens*. The broiler chickens were kept in a closed, ventilated and wire-floor caged broiler house (100 cm long × 80 cm wide × 50 cm height/cage). The cages had a linear feeder at the front and a nipple drinker at the back to provide feed and water *ad libitum* throughout the experimental period. Thirty-three ^{O}C room temperature was maintained for the first week, and then reduced by 3 ^{O}C per week until it reached 24 ^{O}C. Light was provided 24 hour/day.

***Clostridium perfringens* infection**

The *C. perfringens* challenge was carried out as previously described (Dahiya et al, 2007). *C. perfringens* type-A (CPA) (CVCC2030) was obtained from China Veterinary Culture Collection Center (CVCC52) of China Institute of Veterinary Drug Control (Beijing, China).The bacteria was cultured under anaerobic condition at 37 ^{O}C in peptone yeast broth overnight (Shoemaker and Pierson, 1976). The cultured medium was centrifuged at 6000 x g at 4^{o}C for 10 minutes, and re-suspended in 0.01 M Phosphate Buffered Saline (PBS). The concentration of *C. perfringens* was adjusted to 10^8 CFU/mL, and each chicken in the challenged groups was orally gavaged with 0.5 mL at day 1 of age, and 1mL from day 14 to day 21 of age, once per day, to induce the NE infection.

Tissue sample collection

At each time of sample collection, one bird/pen and ten birds/treatment were randomly selected and euthanized for the intestinal tissue sample collection, and five samples were used for testing out of the ten collection. The intestinal tissue samples were collected at day 7 and day 21 post challenge (PC) (21 and 35 days of age) for cytokines and nutrient transporters mRNA expression and cecal samples were collected at 14, 21 and 35 days of age for qRT-PCR and at 21 and 35 days of age for cecal microbiota composition. The contents of the intestine from duodenum, jejunum and ileum were separated and squeezed out, and segments were rinsed with PBS, and mucosal scrapping from each segment (duodenum, jejunum and ileum) were collected for RNA extraction for cytokines and nutrients transporter genes expression. Cecal luminal contents were also collected for qRT-PCR and microbiota composition. All the samples were collected into an aseptic 2mL tubes and frozen immediately in liquid nitrogen, later stored at −80°C for further analysis.

DNA Extraction and Cecal Bacterial Determination by Absolute qPCR

The genomic bacterial DNA was extracted using 80 ±10 mg of cecal contents using a modified Cetyltrimethylammonium Ammonium Bromide (CTAB) method as described by Minas et al. (2011). However, the total DNA quantity and quality were determined using Nanodrop® ND-2000 spectrophotometer (Thermo scientific, MA, USA), while the quality of the DNA was determined using agarose gel (1%) electrophoresis. All DNA samples were diluted to 30ng/µL and stored in -20°C thereafter. The cecal abundance of *Bifidobacterium bifidum*, Pediococcus, Enterobacter, *Lactobacillus salivarius Escherichia coli*, and *Clostridium perfringens* were determined using SYBR Green I based absolute qPCR contained the specific primers for each gene (Table 1). The qPCR plasmid standard was prepared as previously described by Liu et al. (2017). The concentration of plasmid standard of the aforementioned bacteria was diluted to 20 ng/µL, and then subjected to a serial of tenfold dilutions (10^{-1}~10^{-6}) to obtain the standard curve. The copy number of the diluted plasmid standard obtained were used and calculated as previously describe by Li et al. (2018). The abundance of the aforementioned bacteria in each sample with 30 ng metagenomic DNA where calculated with the equation: X log [(M_{DNA}/M_C) × (C30/30)×D], M_{DNA} represented the weight of total DNA in the sample (ng); M_C represented the weight of the content used (g); C30 represented the copy number of 30ng metagenomic DNA (plug Ct values into the standard curve); where D represented the dilution ratio. The copy number of each bacterial population was calculated from the standard curves, and finally the population of the bacteria was expressed as $\log_{10}$ CFU/g content.

RNA Isolation and Quantitative RT-PCR for relative expression

The total RNA and complementary DNA from the mucosal scrapping were obtained using TRIzol Reagent following manufacturer's instruction (Invitrogen, Carlsbad, CA). Quantity and purity of RNA were determined using a Nanodrop® ND-2000 spectrophotometer (Thermo scientific, MA, USA), and RNA quality was assessed by Agarose gel (1%) electrophoresis, the cDNA was synthesized from the total RNA using cDNA reverse transcription kit (TaKaRa, Dalian, China) according to manufacturer's instructions, as previously described by Li et al. (2018). A summary of the gene sequences forward and reverse primers used for PCR are shown in table 2. The average threshold cycle

values for relative quantification after normalizing with β-actin were used for each target gene using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Metagenomic sequencing

The total DNA from the cecal samples were extracted using QIAamp DNA Stool Mini Kit (QIAFEN, Germany), according to manufacturer's instructions, and then were eluted with double-distilled water and stored at -20°C for further use. Ultrasonic genomic DNA was randomly disrupted, and purified by agarose gel electrophoresis. The sequencing linker was ligated, and the ligation product was separated by 2% agarose gel electrophoresis. The recovered product of the appropriate size was cut and amplified using qPCR and purified to obtain the final library; the DNA library was constructed and sequenced using illumina Hiseq 2500. To ensure high-quality reads, the raw image data obtained by sequencing were subjected to preliminary mass analysis, and second-generation sequencing data quality filtering were used to remove the low-quality and linker sequences (removal of adapter-containing reads; removal of N-containing reads with a ratio greater than 10%; remove low-quality reads mass value Q ≤ 20 bases account for more than 40 % of the entire read. The final reads (Last HQ Clean Reads) and high-quality sequences were used for subsequent analysis. Default selection parameter: -id 0.90, -id was similar (90% similarity). The software deduplicated all the Scaftigs assembled from each sample. Statistical summarization was carried out and the abundance table of the redundant genes was combined to perform functional abundance analysis. The putative amino acid sequence was aligned from the gene catalogue Kyoto Encyclopedia of Genes and Genomes (KEGG). The database KEGG Pathway divided the biological metabolic pathway into six categories named Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, and Human Diseases; each of which was also classified into B, C, and D levels by the system. Among them, the B class used in this study currently included 43 seeds functions; the C class which was the metabolic pathway map; and the D class was the specific annotation information for each metabolic pathway map. The version of the KEGG database used in the current annotation was Released 76.0, October 1, 2015, and the comparison software used was BLAST+ (version: 2.2.29, parameter: -evalue 1e-5).

Statistical analyses

With the exception of 16S rRNA gene sequencing, all data were analyzed by one-way Analysis of Variance (ANOVA) using the General Linear Model (GLM) Procedure of Statistical Analysis System (SAS) (SAS Institute Inc. Base SAS® 9.4, 2015). Post-hoc Duncan's Multiple Range test was carried out with a P-value < 0.05 as significant. Results were expressed as mean ± Standard Error of the Mean (SEM).

Table 1. Primers for absolute qPCR analysis by 16S rRNA

Target species	Amplicon size (bp)	Sequence (5'–3')[1]	References
Bifidobacterium bifidum	290	F: CCACCGTTACACCGGGAA R: GGGTGGTAATGCCGGATG	Self-designed
Lactobacillus salivarius	108	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Self -designed
Pediococcus	341	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Heiling et al. (2002)
Enterobacter	198	F: ATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	Walter et al. (2001)
Escherichia coli	96	F: CGGGTAACGTCAATGAGCAAA R: CATGCCGCGTGTATGAAGAA	Self-designed
Clostridium perfringens	101	F: GCATAACGTTGAAAGATGG R: CCTTGGTAGGCCGTTACCC	Wise and Siragusa (2005)

[1]F: Forward, R: Reverse.

Table 2. Oligonucleotide primers used for quantitative RT-PCR.

Gene	Gene full name	Accession number	Primer Sequence (5'–3')[1]	References
GLUT-2	Glucose transporter-2(SLC2A1)	XM_205209.1	F: CACACTATGGGCGCATGCT R: ATTGTCCCTGGAGGTGTTGGTG	Hayat et al., 2019
SGLT-1	Sodium glucose transporter-1(SLC5A1)	XM_415247	F: AGCATTTCAGCATGGTGTGTCTTC R: GATGCTCCTATCTCAGGGCAGTTC	Hayat et al., 2019
rBAT	Solute carrier family 3, member1(SLC3A1)	XM_426125	F: CCCGCCGTTCAACAAGAG R: AATTAAATCCATCGACTCCTTTGC	Hayat et al., 2019
CAT-2	Cationic amino acid transporer-2 (SLC7A2)	EU360448	F: CAAGTCTTCTCGGCTCTAT R: GTGCCTGCCTCTTACTCA	Su et al., 2014
PepT-1	Peptide transporter1(SLC15A1)	NM_204365.1	F: GGCCCACAGTTCACCAACAG R: CAAAAGAGCAGCAGCAACGA	Su et al., 2014
Y^+LAT-2	y+ L amino acid transporter-2 (SLC7A6)	NM_001005832	F: CCCCTGAGGAGGATCACTGTT R: TTCAGTTGCATTGTGTTTTGGTT	Su et al., 2014
IL-6	Interleukin-6	AJ309540	F: GCTCGCCGGCTTCGA R: GGTAGGTCTGAAAGGCGAACAG	Rajput et al., 2017
IL-10	Interleukin-10	EF554720.1	F: GACCAGCACCAGTCATCAG R: CCGTTCTCATCCATCTTCTCG	Palamidi et al., 2016
IFN- γ	Interferon gamma	NM_205149	F: AGCTCCCGATGAACGAC R: CAGGAGGTCATAAGATGCCA	Palamidi et al., 2016
SigA	Secretary immunoglobulin A		F: GCGGCACACAATTGCACTGA R: GTTAAGGGGTAAGGTGGCCG	Self designed
TNF-α	Tumor Necrosis Factor-alpha	JN942589.1	F: GACATCCTTCAGCATCTCTTCA R: AGGCGCTGTAATCGTTGTCT	Rajput et al., 2017
B-Actin	Beta-Actin	NM_205518.1	F: ATTGTCCACCGCAAATGCTTC R: AAATAAAGCCATGCCAATCTCGTC	Musa Bodinga et al., 2019

[1]F: Forward, R: Reverse.

RESULTS

Data and sequencing

Cecal chicken samples from Control, Antibiotic and Probiotic groups were freshly collected, and sent for sequencing. The total DNA was extracted using QIAamp DNA Stool Mini Kit (QIAFEN, Germany), and was eluted with ddH_2O and stored at -20°C for further use. Ultrasonic genomic DNA in the sample was randomly disrupted, and the target fragment was recovered and purified by agarose gel electrophoresis. The ligation product was separated using 2% agarose gel electrophoresis, and the recovered product of the appropriate size was cut and amplified by PCR, and purified to obtain a final library. The constructed library was sequenced using Illumina HI sequence TM 2500. Illumina, in cooperation, San Diego, CA USA, A total of 776,294,600 higher quality classifiable reads were generated from all samples with the average of 129,328,433 sequence per sample, and a maximum number of sequences of 143,723,136, and a minimum of 122,474,100 (Table 3).

Variation of cecal bacterial microbiome

The sample richness and alpha diversity of the cecal microbiome of boiler chickens are shown in Table 4. Alpha diversity was compared among the three fed-groups at twenty first and thirty fifth days of age respectively. The results revealed that the species richness of Chao1 and Ace indices were highest in AGP and PB, and lowest in CON group at both 21_{st} and 35_{th} days of the age.

Bacterial taxonomic composition

The bacterial compositions at the phylum level are presented in figure 1a and 1b respectively. At 21_{st} day of age, Firmicutes was the dominant microbiota in all the three fed-groups, 81.3%, 80.9% and 81.2% in CON, AB and PB respectively, followed by proteobacteria, Actinobacteria as well as other unclassified group respectively with no significant variation between the groups (Figure 1a). However, at 35_{th} day of age, the dominant microbiota were likewise Firmicutes. Proteobacteria, and Actinobacteria with phylum Firmicutes were having the highest abundance in AB and PB fed-groups than in CON group, 73.3%, 76.5% and 77.3% (Figure 1b), while in addition to the three major phylum detected, 6.8% of Bacteroidetes were detected in PB fed group, nonetheless, observed decreased of 8%, 4.4% and 3.9% of Firmicutes in CON, AB and PB respectively, and increase in the relative taxa abundance of 3.1%, 2.1% and 0.5% of proteobacteria in CON, AB and PB fed-groups respectively (Figure 1b).

Three relative abundance and microbial functions

The B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) and the orthologues variation for important microbial functions of the cecal microbiome of the three groups at 21_{st} and 35_{th} days of age were identified respectively (Figure 2a and 2b). Six orthologues pathways for the relative abundance of genes encoded in KEGG level B were considered such as translation, replication and repair, amino acid metabolism, carbohydrate metabolism, membrane transport, and vitamin metabolism co-factor (Figure 2a). At 21_{st} day of the age, the gut microbiota in CON group was richest in translation, replication and repair pathways, while AB fed-group showed enrichment in amino acid metabolism. It was also observed that gut microbiota was richest in pathways related to carbohydrate metabolism, and membrane transport in PB fed-group. Similarly, at 35_{th} day of age, PB fed-group maintained the highest enrichment in carbohydrate metabolism of cecal microbiota related pathways. However, membrane transport replication and repairs pathways were relatively richer in AB fed-group, while the CON group was richer in translation pathways. (Figure 2). The distribution of functional components of cecal microbiome B level of KEGG classification pathways, each data represents ten chickens for each five samples from each group (a) at 21_{st} day of age; (b) 35 days of age; 21 and 35 days of age (day 7 and 21 post challenged). The three groups were control group (CON); supplemented with basal diet only, antibiotic group (AB); basal diet supplemented with enramycin (5mg/kg) and challenged with *C. perfringens*, probiotic group (PB); basal diet supplemented with *B. subtilis* DSM 32315 ($2x10^9$CFU/g) and challenged with *C. perfringens*.

Cecal microbial populations detected by absolute qPCR

The mean $\log_{10}$ 16S rRNA gene copies/g of the cecal digesta for *Bifidobacterium bifidum, Escherichia coli, Clostridium perfringens, Enterobacter, Lactobacillus salivarius* and *Pediococcus* of broilers were detected at 14, 21 and 35 days of the age which are shown in figure 3. At 14_{th} day of the age, the bacterial abundance of *B. bifidum*, *Enterobacter*, and *L. salivarius* were significantly higher ($P<0.05$) in AB and PB, while relative abundance of *E. coli* was significantly ($P<0.05$) higher in CON group (Figure 3a). However, at 21_{st} day of the age, no significant difference ($P>0.05$) in the populations of *C. perfringens*, *B. bifidum* and Pediococcus, was detected, but the relative abundance of *Enterobacter* and *L. salivarius* were significantly higher in AB and PB group, while relative abundance of *E. coli* was significantly higher in CON and PB fed-group than in their counterpart groups (Figure 3b). Similarly, at 35_{th} day of the age, no significant difference was observed in the cecal abundance in *Pediococcus* and *C. perfringens*, whereby the abundance of *B. bifidum*, *Enterobacter* and *L. salivarius* were significantly higher in AB and PB than in CON fed-group.

Sugars, amino acids and peptides transporters mRNA genes expression

The mRNA expression levels of nutrient transporters (Monosaccharides) such as Glucose transporter- 2 (GLUT-2), Sodium glucose transporter-1 (SGLT-1) and (Solute carrier family 3, member-1 (rBAT) of the intestinal mucosa from duodenum, jejunum and ileum at 21_{st} and 35_{th} days of the age are presented in figure 4. In the duodenum, at 21_{st} day of age, mRNA expressions of GLUT-2 were significantly ($P < 0.05$) higher in PB and AB, while SGLT-1 and rBAT significantly showed high expression in PB than in CON and AB fed-groups (Figure 4a). Similarly, at 35_{th} day of age, PB fed-group showed significantly ($P < 0.05$) higher expression of GLUT-2 and SGLT-1, whereas, rBAT was significantly higher in AB and PB than in CON fed-group (Figure 4b). Likewise, in jejunum, AB and PB showed high expression of GLUT-2 and rBAT, while, at 35_{th} day of age, SGLT-1 and GLUT-2 significantly showed down regulation with the highest expression in PB than in CON and AB fed-groups (Figure 4d). However, in ileum, at 21_{st} and 35_{th} days of age, GLUT-2 and rBAT were significantly ($P<0.05$) expressed in PB than in AB and CON group (Figure 4e).

The amino acids and peptides mRNA genes expression

The amino acid and peptide transporters; Oligopeptide tansporter-1(PepT-1), L amino acid transporter-2 (LAT-2) and Cationic amino acid transporter-2 (CAT-2*)* from duodenum, jejunum and ileum at 21_{st} and 35_{th} days of age are presented in figure 5. Results showed the expression of LAT-2 at 21_{st} and 35_{th} days of age in duodenum, and CAT-2 at 35_{th} days was significantly ($P < 0.05$) higher in AB and PB fed-groups. Also, PB showed significantly ($P<0.05$) higher expression of PepT-1 than in AB, while CON group shows the lowest expression (Figure 5a and 5b). In jejunum, at 21_{st} day of age, groups fed AB and PB significantly ($P<0.05$) showed a higher expression of PepT-1 and LAT-2, while CAT-2 at 21_{st} days of age and PepT-1 at 35_{th} day of age, were significantly ($P < 0.05$) higher in AB than in PB fed-group, with the lowest expression in CON group (Figure

5c). Similarly, expression of CAT-2 was significantly ($P < 0.05$) higher in AB and PB than in CON group (Figure 5d). In Ileum at 21_{st} day of age, only LAT-2 showed substantial difference with AB fed-group having the highest expression (figure 5e), On the other hand, at 35_{th} day of age, LAT-2 and CAT-2 showed significantly ($P < 0.05$) higher expression in AB and PB than in the CON group, while expression of PepT-1 was meaningfully ($P< 0.05$) higher in PB than AB with the lowest expression in CON group (Figure 5f).

The Cytokines mRNA genes Expression

There were also some changes in mRNA expression of proinflammatory cytokines of interleukin-6, tumor necrosis factor alpha and interferon gamma from duodenum, jejunum and ileum of the three groups at 21_{st} and 35_{th} days of the age (Figure 6). In duodenum at 21_{st} day of age (figure 6a), expression of IL-6, TNF-α and IFN-γ, and at 35_{th} day of age (figure 6b), IL-6 and TNF-α were significantly higher compared to AB and PB challenged groups. Also, in jejunum, at 21_{st} day of age (figure 6c), IL-6, TNF-α, IFN-γ and at 35_{th} day of age (figure 6d), TNF-α and IFN-γ were significantly ($P < 0.05$) higher in CON than in AB and PB. Similar trends were also observed in ileum at 21_{st} day of age (figure 6e), and 35_{th} day of age (Figure 6f). The mRNA expression level of anti-inflammatory cytokine of IL-10, and mucosal secretory immunoglobulin A at 21_{st} and 35_{th} days of the age were measured. At 21_{st} day of age, AB and PB fed-group considerably ($P < 0.05$) indicated higher expression of SigA than in CON group (figure 7a), while at 35_{th} day of age, PB fed-group showed significantly ($P < 0.05$) higher expression of IL-10, and SigA than in AB fed-group, and the lowest expression was in CON group (Figure 7b). In jejunum, at 21_{st} day of age, SigA was upregulated in AB than in PB and CON fed-group, whereas expression of IL-10 was meaningfully ($P < 0.05$) upregulated in AB and PB than in CON fed-group (Figure 7c). However, at 35_{th} day of age, IL-10 was upregulated in PB than in CON, while SigA was upregulated in AB than in CON group (Figure 7d). Similarly, in ileum at 21_{st} day of age, AB and PB showed notably ($P < 0.05$) high expression of IL-10 and SigA than in CON (Figure 7e). Similarly, at 35_{th} day of age, expression of SigA in PB fed-group was substantially ($P <0.05$) up-regulated than in AB and CON fed-groups. Nonetheless, the changes in mRNA expression of proinflammatory cytokines of IL-10 in AB and PB were up-regulated than in CON group (Figure 7f).

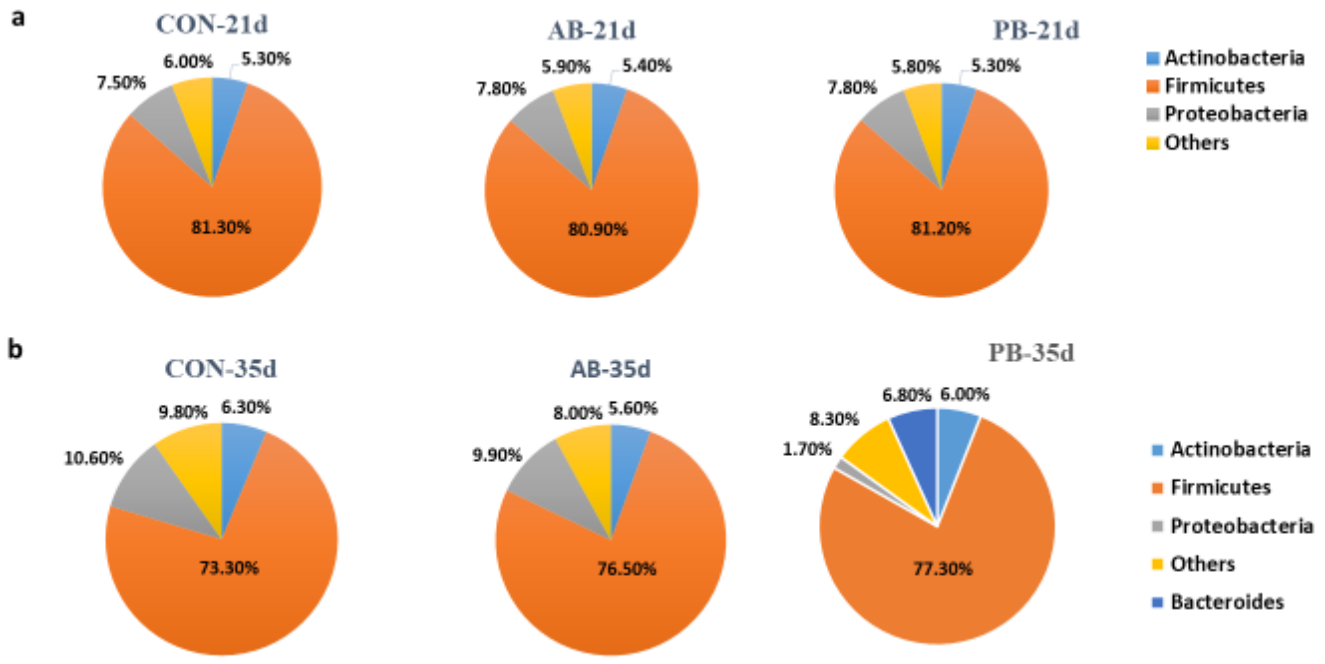

Figure 1. Cecal bacterial composition of broiler chickens. Relative taxa abundance (%), **a:** at 21 days of age and; **b:** at 35 days of age; 21 and 35 days of age means (day 7 and 21 post challenged respectively). The three groups contained: CON: control group supplemented with basal diet only; AB: antibiotic group (enramycin 5mg/kg) challenged with *Clostridium perfringens;* PB: probiotic group contained *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

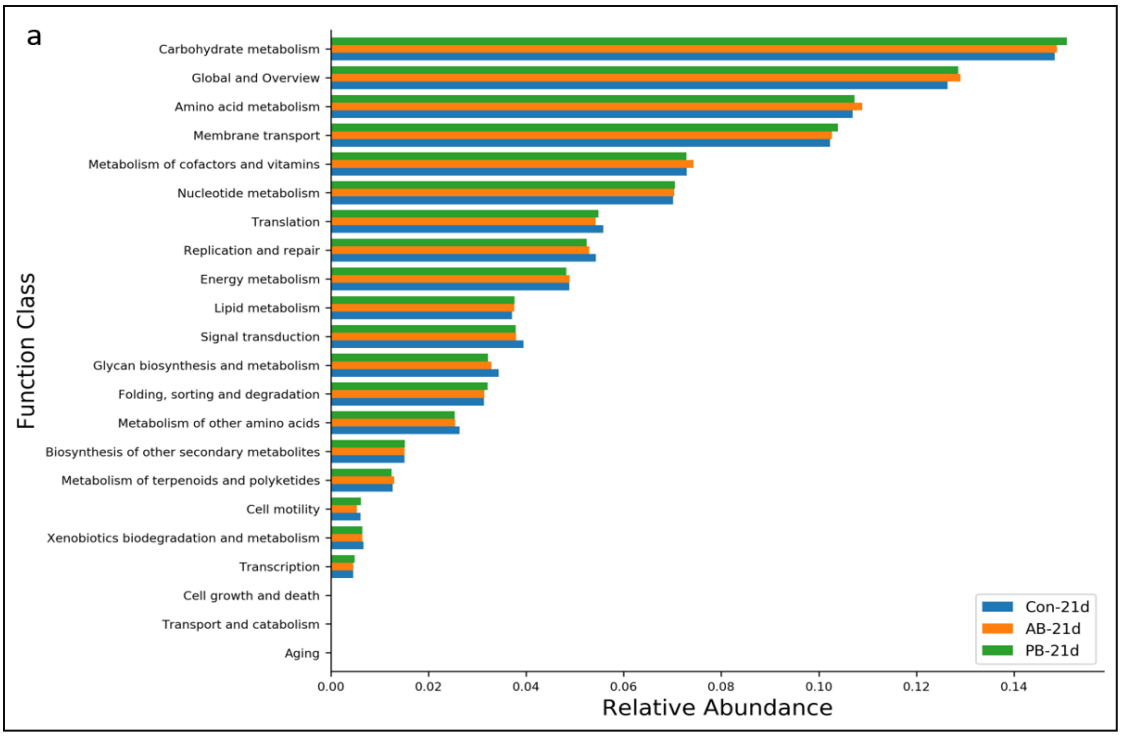

Figure 2a. Distribution of functional components of cecal microbiome B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) classification pathways. Each data represents ten chickens for each five samples from each group; at 21 days of age means (day 7 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5mg/kg) challenged with *Clostridium perfringens*; probiotic group (PB): basal diet supplemented with *Bacillus subtilis* DSM 32315 ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

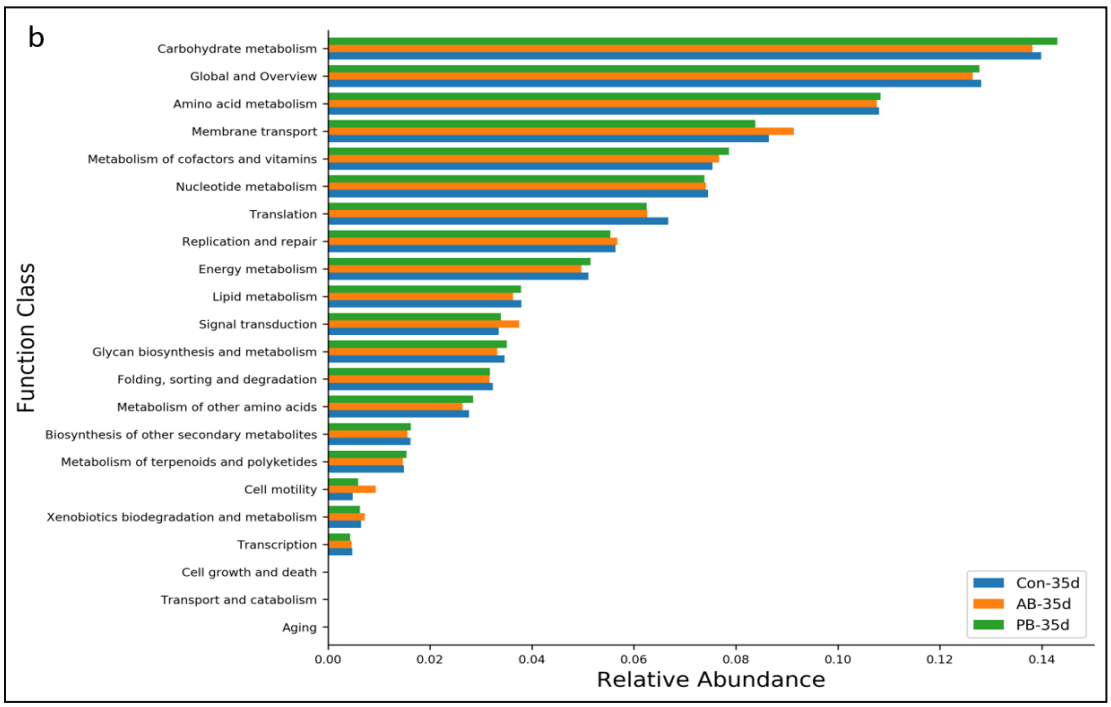

Figure 2b. Distribution of functional components of cecal microbiome B level of Kyoto Encyclopedia of Genes and Genomes (KEGG) classification pathways. Each data represents ten chickens for each five samples from each group; at 35 days of age means (day 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5mg/kg) challenged with *Clostridium perfringens*; probiotic group (PB): basal diet supplemented with *Bacillus subtilis* DSM 32315 ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*

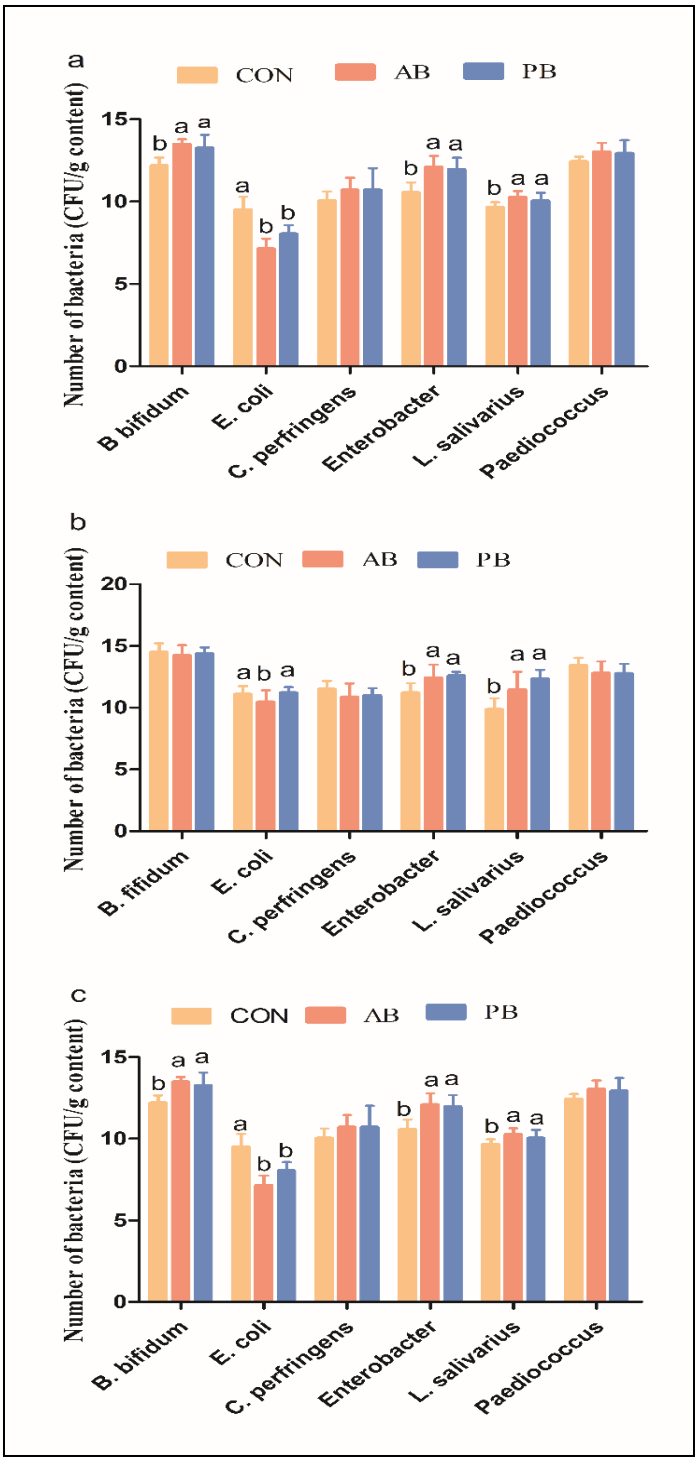

Figure 3. Composition of cecal microbiome number of 16S rRNA gene copies broilers from three groups (a); at 14 days of age; (b); at 21 days of age (c); at 35 days of age**;** 14 days of age (before challenge); 21 days of age (7 days after challenged); and 35 days of age (21 days after challenged). The three groups are CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotics group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, each data represents five replicates intestinal cecal samples for each group. The error bars indicate the standard error of the mean. Data was presented as log10CFU/g cecal content. a-c Means with different letters are significantly different ($P < 0.05$).

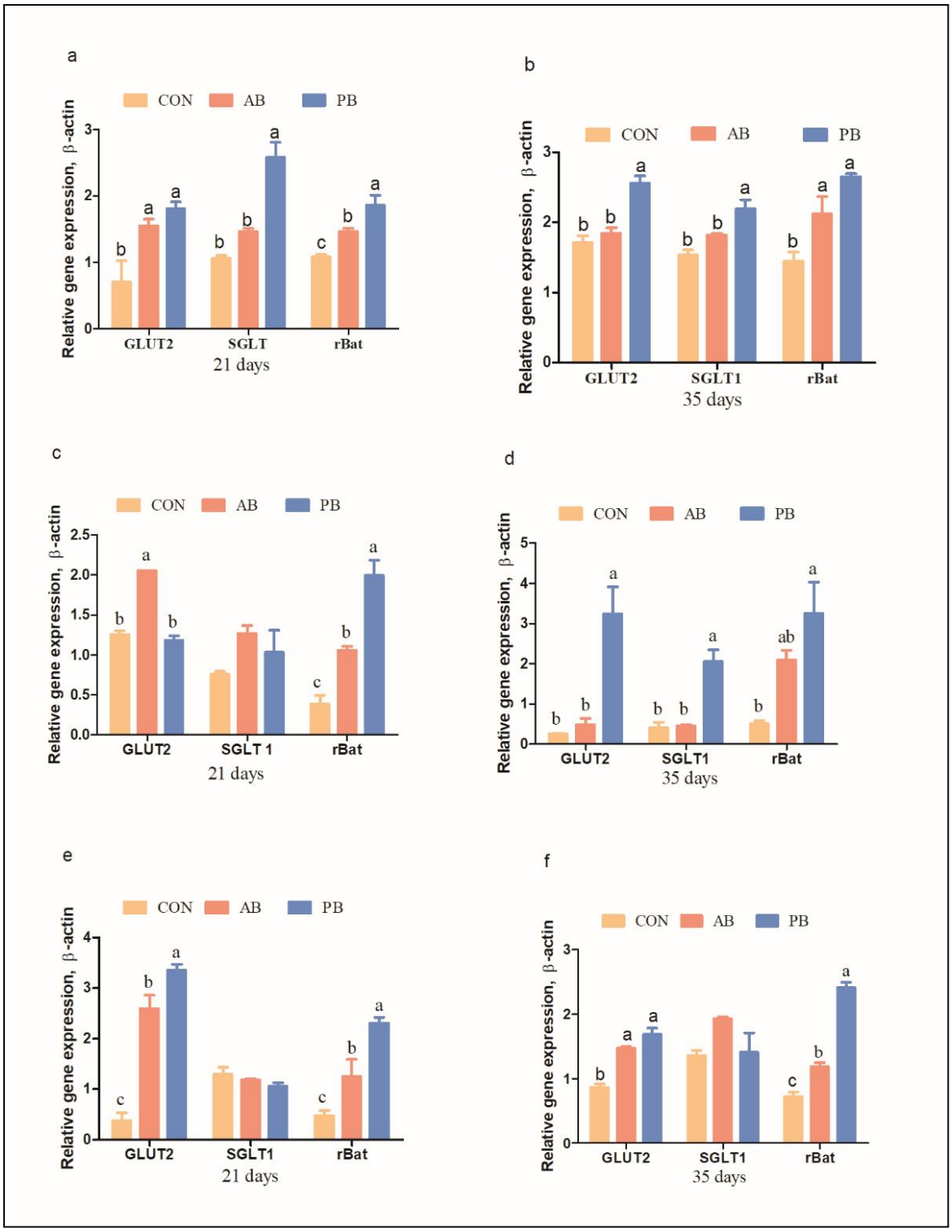

Figure 4. Relative mRNA expression levels of sugars transporters-related genes of the intestinal mucosa of broiler from duodenum, (Figure 4a and 4b) jejunum (Figure 4b and 4c) and ileum (Figure 4E and 4f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotic group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*, GLUT-2= glucose transporter-2, SGLT1= sodium glucose transporter-1, rBAT = Solute carrier family 3, member1 Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05)

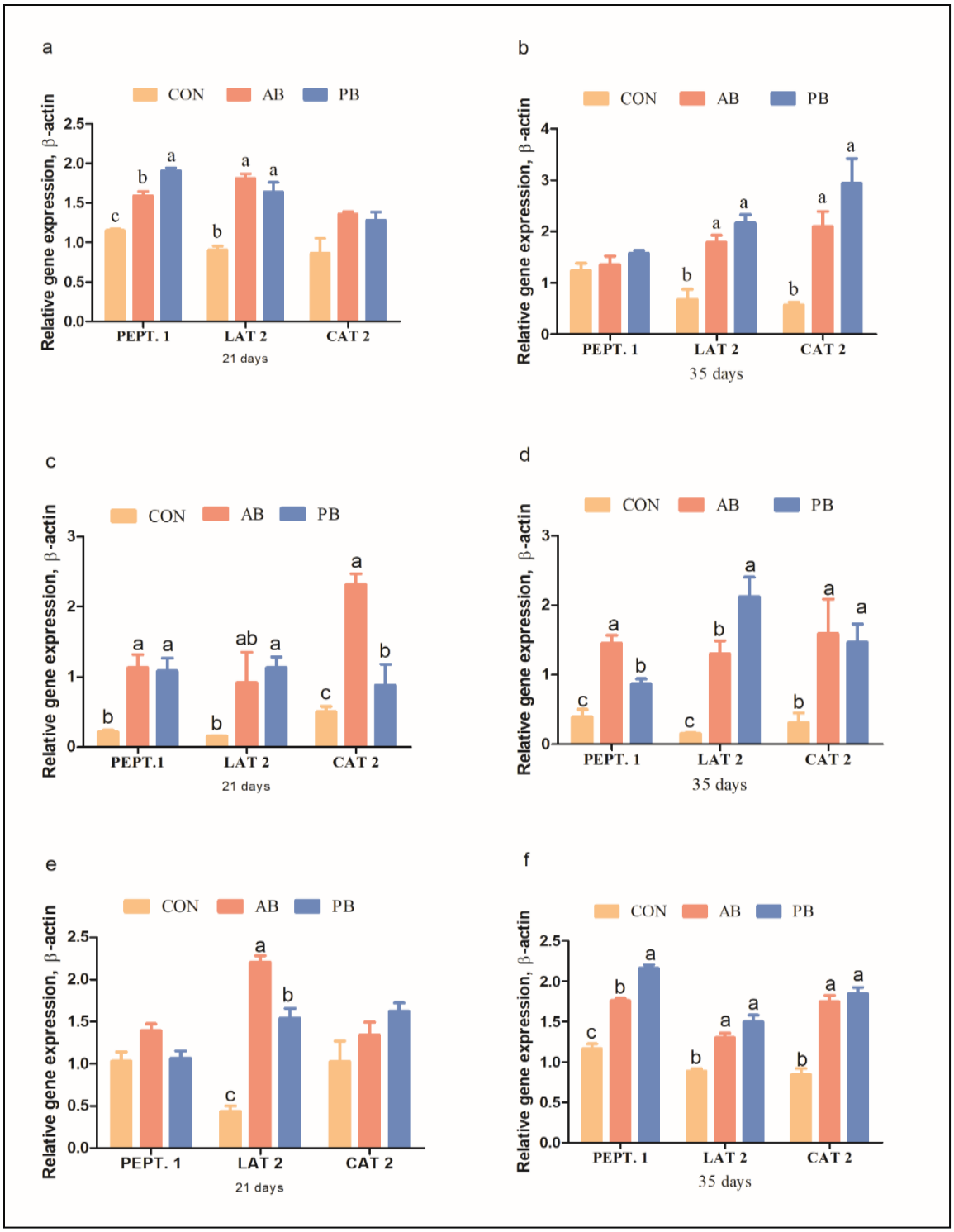

Figure 5. Relative mRNA expression levels of amino acid and peptide transporter related-genes of the intestinal mucosa of broiler from duodenum (Figure 5a and 5b), jejunum (Figure 5c and 5d) and ileum (Figure 5e and 5f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotics group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, CAT-2= cationic amino acids transporter-2; PepT-1= peptide transporter-1, Y^+LAT-2 =amino acid transporter-2. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different ($P < 0.05$).

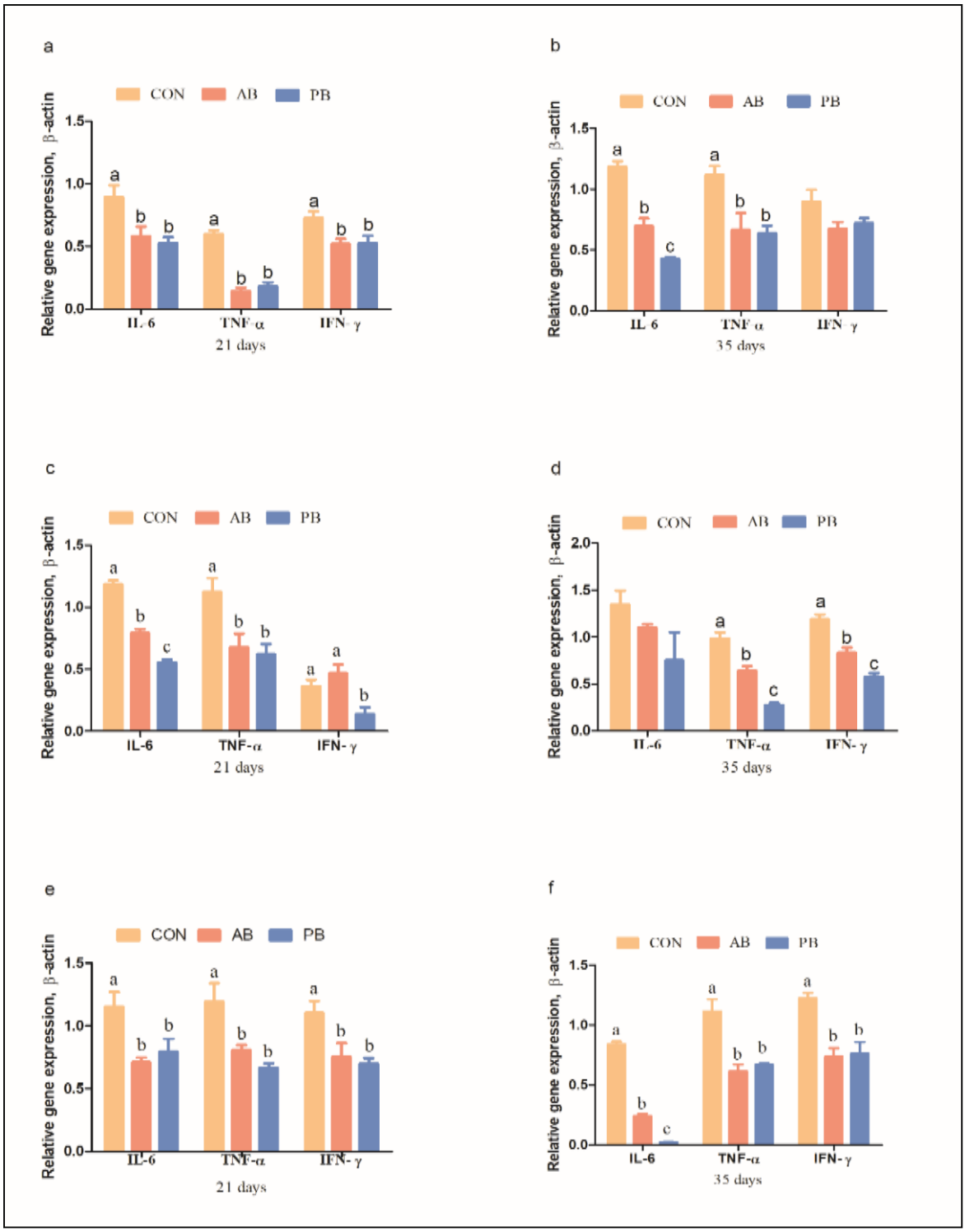

Figure 6. Relative mRNA expression levels of cytokine-related-genes of the intestinal mucosa of broiler from duodenum (Figure 6a and 6b), jejunum (Figure 6c and 6d) and ileum (figure 6e and 8f) at 21 and 35 days of age, 21 and 35 days of age means (d 7 and d 21 post challenged). The three groups are contained CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with; (enramycin 5mg/kg) challenged with *Clostridium perfringens;* probiotic group (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$ CFU/g) challenged with *Clostridium perfringens*, IL-6= interleukin 6; TNF-α=Tumor necrotic factor alpha; IFN-γ = Interferon gamma. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05).

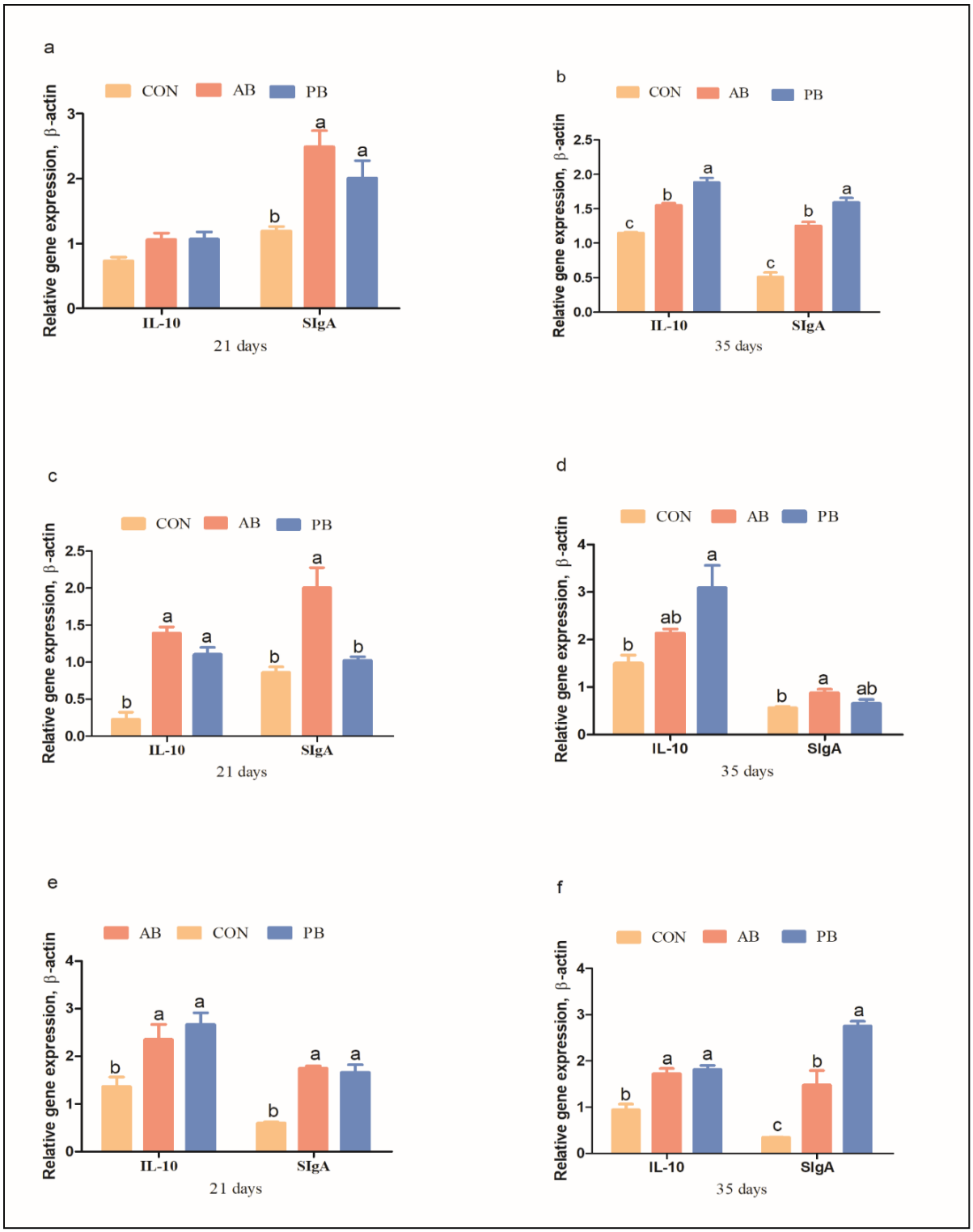

Figure 7. Relative mRNA expression levels of cytokine-related-genes in the intestinal mucosa of broiler from duodenum, (Figure 7a and 7b) jejunum (Figure 7c and 7d) and ileum (Figure 7e and 7f) at 21 and 35 days of age,21 and 35 days of age means (d 7 and d 21 post challenged). The three groups contained; CON: control group supplemented with basal diet only; antibiotic group (AB): basal diet supplemented with (enramycin 5 mg/kg) challenged with *Clostridium perfringens*; probiotic group: (PB): basal diet supplemented with *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*, IL-10= Interleukin 10; SigA= Secretory immunoglobulin A. Data are the means ± SEM of five chicks in each group. a-c Means with different letters are significantly different (P <0.05).

Table 3. Metagenome Illumina HI Sequencing raw and validated data statistics

Groups	Total length (read)	Total base (bp)	Q20Base (%)	Q30 Base (%)
Control 21d (Raw data)	143723136	21558470400	95.82	90.84
(Validated data)	137267732	20095347393	97.90	93.80
Antibiotic 21d (Raw data)	124568192	18685228800	96.24	91.38
(Validated data)	120150374	17579482737	97.94	93.89
Probiotic 21d (Raw data)	126434430	18965164500	96.12	91.14
(Validated data)	121778776	17822523973	97.87	93.71
Control 35d (Raw data)	133089518	19963427700	96.21	91.18
(Validated data)	128706256	18818324785	97.82	93.60
Antibiotic 35d (Raw data)	126005224	18900783600	96.34	91.85
(Validated data)	120660598	17707723742	98.19	94.49
Probiotic 35d (Raw data)	122474100	18371115000	96.90	92.65
(Validated data)	118742952	17442437811	98.27	94.68

Table 4. Alpha diversity indices of the cecal microbiome of broiler chickens

Groups	Number of reads	Chao1	Ace	Observed species	Shannon	Simpson
CON (21d)	54981879	3837.72	3798.27	3715	4.252	0.86
AB (21d)	63691912	3954.03	3933.65	3880	4.189	0.85
PB (21d)	58508325	3922.09	3898.63	3844	4.205	0.84
CON (35d)	52962927	3994.97	3979.22	3917	4.304	0.83
AB (35d)	63280909	4065.55	4059.32	4002	4.455	0.85
PB (35d)	56346246	4060.63	4026.71	3947	4.309	0.84

CON: control group supplemented with basal diet only; AB: antibiotic group supplemented with basal diet and enramycin (5mg/kg) challenged with *Clostridium perfringens*; PB: probiotics group supplemented with basal diet and *Bacillus subtilis* ($2x10^9$CFU/g) challenged with *Clostridium perfringens*

DISCUSSION

Previous studies showed that pathogenic strains of *C. perfringens* can cause NE that disrupts, and lead to significant shift in the normal structure of gut microbiome in broiler chickens (Stanley et al., 2012; Mountzouris et al., 2015). Similarly, it was revealed that gut microbiome can provide the host with nutrients exchange, modulation of immune system and exclusion of pathogens, which had great impact on metabolic reactions (Binek et al., 2012). It was also revealed that, inclusion of *B. subtilis* strains probiotics in broiler diet could improve gut health, in the production of antimicrobial peptides and alteration of gut microbiome (Knap et al., 2011; Lee et al., 2011). Likewise, the use of in feed antibiotics were believed effective in mediating and decreasing the number of gut pathogenic bacteria in animals and humans (Dibner and Richards, 2005). Infeed antibiotics also proved an effective way to prevent animals from diseases, enhance feed efficiency, and to improve animal performance (Hooge et al., 2003; Kamran et al., 2013). Various species of Bacillus genus were known in producing bacteriocins and antimicrobial peptides (Lee et al., 2011; Cochrane and Vederas, 2014; Park and Kim, 2014). *B. subtilis* SP6 showed an anti *C. perfringens* effect *in vitro* (Teo and Tan, 2005). The results of present study demonstrated that supplementation of *B. subtilis* DSM 32315 at $2x10^9$ and antibiotic, enramycin at the dose of 5 mg/kg relatively altered the gut microbial diversity and functions of broiler chickens at 21_{st} and 35_{th} days of age as compared to the control group. This could be as a result of the early and continuous supplementation of *B. subtilis* probiotic from 1 to 42 days of age for the feeding period, which could probably mediate the beneficial effect of the gut microbiota through competitive exclusion (CE) in chickens by colonization and modulation of pathogens, while restoring the normalcy and integrity of gut ecosystem as well as the effect of the antibiotic administered to the chicken. Previous studies confirmed that several strains of *B. subtilis* have antagonistic activities against *C. perfringens in vitro* (Martaet al., 2014; Cochrane and Vederas, 2016). Similarly, the results of this

study disclosed that AB and PB challenged groups had the highest percentage of population of dominant taxa such as *Firmicutes*, *Proteobacteria* and other bacterial groups at 35 days of age. Likewise, we observed a unique group of "*Bacteroidetes*" (6.8%) in PB fed group, the dominant cecal microbiome composition obtained in this study was contrary to study of Xiao et al. (2017), that reported *Bacteroidetes* as the dominant bacterial group which could be to the fact that intestinal microbiome composition could be affected by many factors like surrounding environment, dietary supplementation, and pathological condition (Xiao et al., 2017). *Firmicutes* and *Bacteroidetes* were important gut microbiota in broiler that function in energy production and metabolism, specifically in microbial fermentation and starch digestion (Shaufi et al., 2015). Bacteroidetes primarily functioned in the formation of potential toxins via putrefaction that leads to increase pH of the intestinal contents which is of beneficial to the gut health against acid-sensitive pathogens (Apajalahti, 2005). Other species such as, *B. salanitronis*an which are inhabitant of chickens' cecum was well known for its ability in aiding further digestion of food by making the nutrients available to the host (Gronow et al., 2011). Also, with regards to 16S rRNA sequences, *B. bifidum*, *E. coli*, *C. perfringens*, *L. salivarius* Enterobacter, and Pediococcus, the cecal contents, revealed significantly ($P < 0.05$) higher relative abundance in AB and PB fed groups, with suggestively higher population of *E. coli* in CON group without any supplementation. The higher relative abundance of *L. salivarius* and *B. bifidum* in PB fed-group could be attributed to their tolerance against *C. perfringens* infection. Alike results were found by Pyoung et al. (2007) who isolated several *Lactobacillus* and *Bifidobacterium* from pig gut that had antagonistic action against *C. perfringens*. Teo and Tan (2007) reported higher amount of *Lactobacillus* and *Bifidobacterium* from ileum of chickens supplemented with *B. subtilis* PB6. Similarly, Gilbert et al. (2008) observed a substantial increase in *Lactobacillu*s abundance in the ilea and cecum of chickens following by *B. subtilis* C-3104 supplementation. Stanley et al. (2014) and Jeong and Kim (2015) reported that genus *Lactobacillus* in chickens' gut was predominant, and did not disturb the following *C. perfringens* infection. However, *Lactobacillus* was associated with reduction of intestinal inflammation and enhanced immunological barrier functions in broilers (Chen et al., 2012). Nevertheless, in this study, statistically no significant difference in the cecal population of *C. perfringens* either in supplemented groups or in the CON group fed basal diet only. This could signify that supplementation of either AB or PB directly or indirectly minimizes the proliferation of gut pathogens. More so, corresponding results were also obtained by Barbosa et al. (2005) and Teo and Tan (2005) who recognized *B. subtilis* and *B. licheniformis* as antagonistic species against *C. perfringens* infections. Likewise, in the present study, six enriched KEGG orthologues markers related to replication and repair, translation, amino acids metabolism, vitamin metabolism cofactor, membrane transport, and carbohydrate metabolism were considered. The CON group indicated enrichment in translation related pathways compared to AB and PB challenged groups. This could be attributed to the fact that AB and PB fed groups were challenged with *C. perfringens* which meant to induce NE, whereas, the subclinical form of the disease is characterized by necrosis, inflammation, pseudo-membrane and mucosal hemorrhages (Wielen, 2000). Increase in carbohydrates metabolism related pathways in PB fed-group could be attributed to the high density of the commensals bacteria in the gut, in the sense that, many gut bacteria can additionally hydrolyze indigestible carbohydrate of polysaccharides, oligosaccharides and disaccharides to their compositional sugars, which later be fermented by gut bacteria, producing short chain fatty acids which can be utilized as energy source by the host (Hooper et al., 2002; Tellez et al., 2006). The high relative abundance of *Lactobacillus spp*. and *Bifidobacterium* from both AB and PB fed-groups could influence carbohydrate metabolism, and release energy into the host.

CONCLUSION

In conclusion, *C. perfringens* challenged broilers, with supplementation of AGP enramycin at the dose of 5 mg/kg or probiotic *B. subtilis* DSM 32315 at $2x10^9$ CFU/kg, during broiler production period of 1 to 42 days of age increased the cecal relative abundance of beneficial microbiome, upregulated the expression of some anti-inflammatory cytokines, and downregulated the expression of some proinflammatory cytokines. Thus, it can cause the reduction of gut pathogens. Similarly, the amino acids and peptides transporter genes expressions were also enhanced in both antibiotic and probiotic fed-groups when compared with the control group. In addition, sugar transporter related genes were upregulated by *B. subtilis* probiotic fed-group compared to antibiotic and control group. The overall results clearly confirmed the potential of *B. subtilis* DSM 32315 probiotic against *C. perfringens* bacteria causing necrotic enteritis infection,

and thus it could be used as an alternative to AGPs from day one to day 42 of production period in broiler chickens.

DECLARATIONS

Consent to publish

All authors have approved to publish the work

Competing interests

The authors declare that they have no conflict of interests.

Author`s contributions

The conceptualization was done by Bello M. Bodinga and XiaoJun Yang. The methodology was designed by Rab N. Soomro, Khawar Hayat, JinHui Zhou and Xinshuai Liu. Validation and formal analysis were performed by Abdullahi Ismaila and Zhouzheng Ren.

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