Clays and Clay Minerals, Vol. 69, No. 2:205–216, 2021

EFFECTS OF DIETARY PALYGORSKITE SUPPLEMENTATION ON CECAL MICROBIAL COMMUNITY STRUCTURE AND THE ABUNDANCE OF ANTIBIOTIC-RESISTANT GENES IN BROILER CHICKENS FED WITH CHLORTETRACYCLINE

Rui Jin[1], Yueping Chen[1], Yuru Kang[2,3], Yunfeng Gu[1], Chao Wen[1], Aiqin Wang[2,3], and Yanmin Zhou[1,*]

[1]College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, P.R., China
[2]Center of Eco-material and Green Chemistry, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, P.R. China
[3]R&D Center of Xuyi Palygorskite Applied Technology, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Xuyi 211700, P.R. China

Abstract—Antibiotic-resistant genes (ARGs) have been regarded as emerging contaminants that threaten public health worldwide. Poultry excreta, often used as a fertilizer in agriculture, are a major route for the proliferation and dissemination of ARGs in the environment. The aim of the present study was to assess the potential of dietary palygorskite (Plg) supplementation as nutritional manipulation for the modulation of microbial community structure and the attenuation of ARGs in the cecal contents of broilers fed with chlortetracycline (CTC). In total, 256 one-day-old, mixed-sex, broiler chicks were allocated randomly into a 2×2 factorial design of four treatments, which consisted of two levels of CTC (0 or 50 mg/kg) and Plg (0 or 10 g/kg). By employing in vivo feeding and slaughter experiments, after collecting the cecal contents and extracting the total genomic DNA, 16S rRNA V3-V4 hypervariable amplicon pyrosequencing and quantitative PCR-based approaches were used to address the impact of Plg on microbiota and the abundance of ARGs in broilers. The results showed that broilers given a diet supplemented with Plg had greater α-diversity indices including Chao1, phylogenetic diversity tree, and observed-species index calculations, when compared with those without Plg supplementation. Birds given a diet supplemented with Plg had fewer *Firmicutes* at the phylum level, but a greater abundance of *Alistipes* at the bacterial genus level. Dietary Plg counteracted the CTC-induced increased abundance of ARGs, among which tet(K) had a pronounced decrease, along with a similar decreased tendency for other measured ARGs and intI1. Overall, the results indicated that Plg supplementation caused pronounced changes in cecal microbial diversity and microbiota community composition of broilers, and effectively reduced ARGs, indicating that Plg supplementation is a potential alternative measure for the attenuation of ARGs in the cecal contents of broilers.

Keywords—Antibiotic-resistant genes · Broilers · Chlortetracycline · Microbial community structure · Palygorskite

INTRODUCTION

Antibiotics are used extensively at sub-therapeutic levels in livestock production to promote growth and improve feed conversion efficiency (Zhao et al. 2010). In spite of the non-negligible benefits of antibiotics, their heavy use in animal feed has provoked public health concern and safety problems, as demonstrated by antibiotics residue in animal food and the occurrence of antibiotic-resistant bacteria (Huyghebaert et al. 2011; Moghadam et al. 2016). As a consequence, the European Union has, since January 2006, forbidden the use of antibiotics as growth promoters in the animal-feed industry (Marshall and Levy 2011), and the Ministry of Agriculture and Rural Affairs of the People's Republic of China has also limited the heavy use of antibiotics in animal production since 2020. Antibiotics therapy to resist diseases in animals, especially those under intensive conditions, is sometimes necessary. Intestinal microbes assist the host in resisting pathogens (Stanley et al. 2014) and improving immunity, etc., and thus play an essential role in maintaining the health of the host. The microbiota of ceca have received considerable attention because they are very diverse, and 1 g (wet weight) of cecal content may contain 10^{11} bacteria (Mead, 1997). Antibiotics are perceived generally to affect numbers and compositions of gut microbes, indicating that antibiotics can affect the cecal microbial community structure of animals (Mamber and Katz 1985; Castillo et al. 2006). The occurrence of antibiotic-resistant genes (ARGs) is the primary reason for bacterial resistance to antibiotics, and ARGs have been recognized as emerging contaminants which attract worldwide attention (Pruden et al. 2006; Baquero et al. 2008). Growing evidence has shown that livestock farms could be a potentially significant reservoir for the release of ARGs into the environment (Chee-Sanford et al. 2009; Gao et al. 2012; Berendonk et al. 2015).

Horizontal gene transfer (HGT) is a critical pathway for the proliferation of ARGs (Hall and Collis 1995; Ochman

* E-mail address of corresponding author: zhouym6308@163.com
DOI: 10.1007/s42860-021-00118-9

et al. 2000), and HGT is associated commonly with mobile genetic elements, such as plasmids, transposons, and integrons, which may enable the ARGs to move from species to species and into a wide range of genera by conjugation (Chopra 2001; Roberts 2003). IntI1 has been considered to be the most ubiquitous among resistant bacteria and plays a leading role in the emergence and wide dissemination of ARGs (Gillings et al. 2008; Cambray et al. 2010). Undoubtedly, antibiotics can induce the occurrence of ARGs of intestinal microbes in animals (Gao et al. 2012). What is worse, > 95% of *Escherichia coli* from diseased broilers has been found to be resistant to one or more types of antibiotics in recent decades in China (Chen et al. 2014). These findings suggest that liberal use of antibiotics has led to some pathogens becoming resistant to antibiotics in livestock production and has inspired discussion of effective methods of reducing the occurrence and spread of ARGs.

Palygorskite (Plg), a hydrated magnesium aluminum silicate present in nature as a fibrous silicate clay mineral (Huang et al. 2007), possesses a unique chain-layered crystal structure, which in turn endows it with properties of adsorption, cation exchange, and adhesive ability (Bergaya and Lagaly 2013). Plg has been used as both a selective and an active adsorbent and also as a catalyst support (Zhou et al. 2016). The characteristics of Plg enable its use in the livestock industry as a feedstuff raw material or additive. Previous studies have demonstrated that Plg supplementation could improve growth performance (Zhang et al. 2013), intestinal integrity, and barrier function in animals (Chen et al. 2016). Furthermore, dietary Plg supplementation can adsorb pathogenic bacteria in vitro (Zaid et al. 1995) and modulate cecal microbiota composition in vivo (Slamova et al. 2011; Chalvatzi et al. 2016). These findings have provided evidence that the supplementation of animal feed with Plg may improve the cecal microbiota of broiler chickens. In addition, zeolite, with a porous structure and adsorption characteristics which are similar to those of Plg, could reduce the abundance of ARGs in sludge compost (Zhang et al. 2016a) and decrease the abundance of some ARGs in the cecal content of broilers (Qu et al. 2019). These findings may suggest that dietary Plg supplementation could help to reduce the abundance of cecal ARGs in vivo.

The objective of the present study was to determine whether dietary Plg supplementation would influence the cecal microbiota of Partridge Shank chickens fed with antibiotics, and to assess the potential of Plg for the attenuation of ARGs induced by chlortetracycline (CTC).

MATERIALS AND METHODS

Palygorskite

The Plg used in the present study was provided by Jiangsu Huida Mining Sci-Technology Co., Ltd. (Xuyi County, Jiangsu Province, P.R. China). The main chemical components of the Plg were listed as follows: SiO_2, 54.74%; Al_2O_3, 9.37%; Fe_2O_3, 6.88%; MgO, 7.03%; CaO, 2.82%; K_2O, 1.41%; Na_2O, 0.14%. The X-ray diffraction (XRD) patterns of Plg were collected using an X'pert PRO X-ray power diffractometer equipped with a CuKα radiation source (λ = 0.1541 nm; 40 kV, 40 mA) (PANalytical Co., Ltd., Almelo, The Netherlands). All XRD patterns were obtained over the range 3 to 80°2θ at a scanning speed of 8.34°2θ/min. Scanning electron microscopy (SEM) of Plg was performed using a field emission scanning electron microscope (JSM-6701F, JEOL, Tokyo, Japan), and transmission electron microscopy (TEM) images were captured using a JEM-1200 EX/S TEM instrument (JEOL, Tokyo, Japan).

Experimental Design, Diets, and Management

All experimental procedures involving animals were approved by the Nanjing Agricultural University Institutional Animal Care and Use Committee. A total of 256 one-day-old, mixed-sex, Partridge Shank chicks with similar hatching weights were divided randomly into four groups. This trial consisted of a 2 × 2 factorial design with two levels of CTC (Jinhe Biotechnology Co. Ltd. Hohhot, P.R. China) (0 or 50 mg/kg) and Plg (0 or 10 g/kg) for 50 days. Each of these groups contained eight replicates with eight chicks per replicate. The four treatments were designated as follows: (1) CON group – birds received a basal diet; (2) CTC group – birds received a basal diet supplemented with 50 mg/kg CTC; (3) Plg group – birds were fed a basal diet supplemented with 10 g/kg Plg; and (4) CP group – birds were fed a basal diet supplemented with a combination of 50 mg/kg CTC and 10 g/kg Plg. The basal diet was formulated according to the recommendation by the National Research Council (1994) to meet the nutritional requirements of the chicks. Birds were housed in 3-level cages (120 cm × 60 cm × 50 cm) in an environmentally controlled room. Continuous light was provided in the chicken house, and the temperature of the experimental room was set at 32–34°C for the first 3 days and then reduced by 2–3°C per week to a final temperature of 20°C. Feed and fresh water were available ad libitum throughout the trial.

Sample Collection

One bird from each replicate was selected randomly and weighed after feed deprivation for 12 h after 50 days of the experiment. The birds were euthanized by cervical dislocation. Cecal pouches were opened immediately using sterile scissors, and the contents were recovered into sterile cryovials. All samples were frozen immediately in liquid nitrogen and stored at –80°C for further analysis.

DNA Extraction

Total genomic DNA was extracted from the cecal contents of each chicken using a QIAamp® Fast DNA Stool Mini Kit (QIAGEN, Duesseldorf, Germany), following the manufacturer's instructions. The concentration of DNA was measured thereafter using a NanoDrop ND-1000UV spectrophotometer (NanoDrop Technologies, Wilmington,

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

Delaware, USA) to ensure that an adequate concentration of high-quality genomic DNA had been extracted. The DNA extractions were stored at –20°C for future experiments.

PCR Enrichment of the V3–V4 Region, and Pyrosequencing

The V3–V4 region of the bacteria's 16S ribosomal RNA (rRNA) gene was amplified by PCR with barcode-indexed primers, using TransStart® FastPfu Polymerase. Amplicons were then purified by gel extraction (AxyPrep DNA Gel Extraction Kit, Axygen Biosciences, Inc., Union City, California, USA). The purified amplicons were pooled in equimolar concentrations, and paired-end sequencing was performed using an Illumina MiSeq instrument (Illumina Inc., San Diego, California, USA).

Following sequencing, the reads were de-multiplexed into samples according to the barcodes using the *QIIME* software pipeline (Caporaso et al. 2010) with the default parameters. Primer and barcode sequences were removed. Operational taxonomic units (OTUs) clustering was performed at a 97% similarity threshold using the *QIIME* pipeline (Caporaso et al. 2010). Rarefaction was performed on the OTUs table to prevent methodological artifacts arising from varying sequencing depths. α-diversity was measured by species richness from the rarefied OTU table. β-diversity was estimated by computing weighted UniFrac and was visualized with principal coordinate analysis (PCoA). The relative abundances of the taxa at the phylum and genus levels were then calculated.

Quantification of tet, intI1, and 16S rRNA Genes

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify the presence of tet(A), tet(E), tet(K), tet(M), tet(W), tet(X), and intI1 genes as well as 16S rRNA genes (as a measure of bacterial biomass). As the genes found most often in genomes of culturable bacteria conferring resistance to tetracycline, the aforementioned six genes encoded three known resistance mechanisms as follows; efflux pump mechanism: tet(A), tet(E), and tet(K); ribosomal protection mechanism: tet(M) and tet(W); and enzymatic modification mechanism: tet(X) (Chopra and Roberts 2001). The intI1 gene was also measured because of its essential role in the spread of antibiotic resistance (Mazel 2006).

All target genes including the 16S rRNA gene were quantified by qPCR, performed by utilizing SYBR® Premix Ex Taq TM (TaKaRa Biotechnology, Dalian, P.R. China) according to the manufacturer's protocol, and qPCR was conducted on a QuantStudio 5 Real-time PCR System (Applied Biosystem, Life Technologies, California, USA). The qPCR conditions were: preheat denature at 95°C for 30 s, perform 40 cycles of denaturation at 95°C for 5 s, then anneal at 60°C for 30 s. At the end of the PCR procedure, melting-curve analysis of the amplification products was performed following this process: one cycle of denaturation at 95°C for 10 s, then a temperature increase from 65 to 95°C with a temperature change rate of 0.5°C/s. The primer sequences targeting the tetracycline resistance genes are listed in Table 1. Agarose electrophoresis was used to examine the specificity of PCR products, followed by a purification step using the Geneclean spin kit (TSINGKE Biological Technology, Beijing, P.R. China), according to the manufacturer's instructions.

The purified PCR products of target genes were ligated into pClone007 Simple vector (TSINGKE Biological Technology, Beijing, P.R. China) and then transformed into competent *Escherichia coli* DH5α (TSINGKE Biological

Table 1 Sequences used for real-time PCR

Genes	Primer sequence, (5′→3′)	Product size (bp)	Reference
16S rRNA	CGGTGAATACGTTCYCGG	172	(He et al. 2007)
	AAGGAGGTGATCCRGCCGCA		
tet(A)	GCTACATCCTGCTTGCCTTC	210	(Ng et al. 2001)
	CATAGATCGCCGTGAAGAGG		
tet(E)	AAACCACATCCTCCATACGC	278	(Levy et al. 1999)
	AAATAGGCCACAACCGTCAG		
tet(K)	TCGATAGGAACAGCAGTA	169	(Ng et al. 2001)
	CAGCAGATCCTACTCCTT		
tet(M)	ACAGAAAGCTTATTATATAAC	171	(Aminov et al. 2001)
	TGGCGTGTCTATGATGTTCAC		
tet(W)	GAGAGCCTGCTATATGCCAGC	168	(Aminov et al. 2001)
	GGGCGTATCCACAATGTTAAC		
tet(X)	CAATAATTGGTGGTGGACCC	468	(Ng et al. 2001)
	TTCTTACCTTGGACATCCCG		
IntI1	GGCTTCGTGATGCCTGCTT	146	(Luo et al. 2010)
	CATTCCTGGCCGTGGTTCT		

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

Technology, Beijing, P.R. China). The recombinant plasmids were sequenced and BLAST (Basic Local Alignment Search Tool) was used in the Genbank database to confirm that the target gene had been transformed successfully into the recombinant plasmid. The concentration of recombinant plasmids was determined using a NanoDrop ND-1000UV spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and then tenfold serially diluted to establish the standard curves. The copy number of target genes in unknown samples was calculated by Ct values according to a previous study (Pfaffl 2001). Furthermore, to minimize variance caused by differences in background bacterial abundance and DNA manipulation efficiencies, the relative abundances of tet genes and intI1 were calculated on the basis of their absolute copy numbers normalized to that of 16S rRNA genes.

Statistical Analysis

Two-way analysis of variance (ANOVA) was employed to determine the main effects (CTC level and Plg level) and their interactions by using the general linear model (GLM) procedure of *SPSS* statistical software (SPSS Inc., Chicago, Illinois, USA). Differences among the treatments were examined by one-way ANOVA using the Tukey's multiple comparison tests. Differences were considered significant when P values were <0.05, and P values between 0.05 and 0.10 were regarded as a trend. Pearson bivariate correlation analysis was performed using *SPSS* 25.0 to reveal relevance between tet genes and intI1.

RESULTS

Characterizations of Plg

The XRD patterns of the Plg sample used in the present study contained peaks at 8.42°2θ ($d=1.0496$ nm), 13.84°2θ ($d=0.6395$ nm), 16.46°2θ ($d=0.5383$ nm), 19.82°2θ ($d=0.4477$ nm), and 34.97°2θ ($d=0.2565$ nm) (Fig. 1a), which were attributed to the (110), (200), (130), (040), and (400) planes of Plg. Peaks at 26.72 and 31.03°2θ were attributed to the presence of quartz and dolomite impurities. The rod-like crystals of Plg were observed in the SEM and TEM images (Fig. 1b, c).

Microbial Diversity Indices

Rarefaction curves based on the number of OTUs indicated that the sequences generated per sample were adequate to define and compare the bacterial diversity among the groups (Fig. 2a). The mean of Good's coverage for all the samples was high (>98%). α-diversity was determined using Chao1, Shannon index, phylogenetic diversity tree (PD-whole tree), and observed-species index calculations (Fig. 2b). Broilers receiving Plg exhibited an increase in the α-diversity indices including Chao1, PD-whole tree, and observed-species as compared with those given a diet without Plg supplementation ($P<0.05$). Furthermore, compared with the non-CTC treated group, a trend for greater bacterial richness assessed by the aforementioned α-diversity indices was also observed for birds given a diet supplemented with 50 mg/kg CTC ($P<0.05$). Although values from the Shannon indices were less distinct with addition of dietary Plg, it still showed a definite increasing trend ($P=0.090$).

β-diversity was determined using the phylogeny-based weighted UniFrac distance matrices (Fig. 3b). PCoA was performed to visualize and scale the dissimilarity matrices in a two dimensional space, in which coordinate 1 and coordinate 2 representing 19.64% and 11.65% of the observed variation, respectively, and overlap among the four groups were observed.

Taxonomic Composition

At the phylum level, 28 prokaryotic phyla were found in all groups (Fig. 4a), but only three were core microbiota: *Firmicutes* (50.09%), *Bacteroidetes* (43.03%), and *Proteobacteria* (3.66%). The three phyla accounted for 97% of the total phyla. At the phylum level, *Firmicutes* was found more abundant in the cecal microbiota of chickens provided with dietary Plg supplementation than those without ($P<0.05$), and a similar trend was observed for the abundance of *Proteobacteria* ($P=0.074$).

At the bacterial genus level, unidentified bacteria in the four experimental groups accounted for 15.2%. Broilers with a diet containing Plg supplementation exhibited a greater richness of *Alistipes* compared to their counterparts which received Plg-free diets ($P<0.01$). Neither *Alistipes* nor *Bacteroidetes* was influenced by dietary CTC inclusion, however ($P>0.05$).

Relative Abundance of ARGs

The relative abundance of tet genes and intI1 in the cecal contents of Partridge Shank chickens are shown in Fig. 5. Dietary supplementation with Plg induced a decrease in the relative abundance of tet(K) ($P<0.05$), regardless of CTC administration. Administration of Plg tended to decrease abundance of tet(E) ($P=0.086$), tet(M) ($P=0.054$), and intI1 ($P=0.058$) genes also. Similar trends in reduction were observed in other genes (tet(W), tet(X), and intI1) (Fig. 5). Curiously, a statistically significant increase was found in the relative abundance of tet(A) ($P<0.05$).

Correlation of intI1 with ARGs

In the present study, correlation analysis was performed to relate intI1 and ARGs occurrence. Only tet(K) and tet(X) were found to have a weak positive correlation with the presence of intI1 ($r=0.529$ and 0.590, respectively; $P<0.01$) in the cecal contents of Partridge Shank chickens. No significant correlation was found between intI1 and other ARGs (tet(A), tet(E), tet(M), and tet(W)) ($P>0.05$).

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

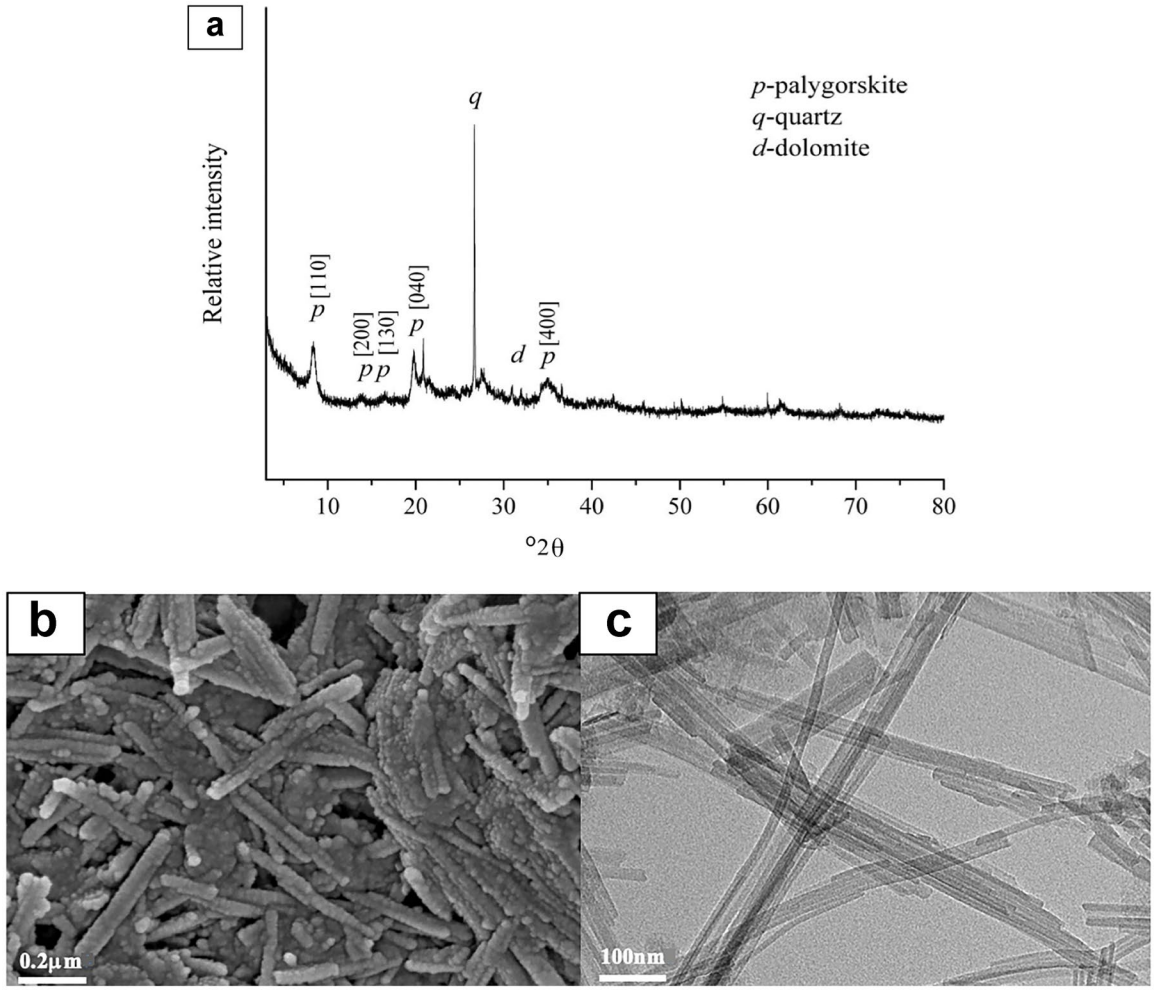

Fig. 1 **a** XRD pattern of Plg; **b** SEM image of Plg; and **c** TEM image of Plg

DISCUSSION

Characterization of the Bacterial Community

The bacterial community was characterized by 16S rRNA V3-V4 hypervariable amplicon sequencing to gain a more in-depth insight into the microbial ecology of the cecal microbiota, which plays an essential role in nutrient preprocessing, assimilation, and energy harvest from food (Ghosh et al. 2014). Species richness and diversity statistics, including Chao1, PD-whole tree, and observed-species, were increased significantly with dietary CTC supplementation in the present study. As is generally accepted, administration of antibiotics may reduce the number of bacteria. Due to the vast diversity of microbiota and different test conditions, the results can vary greatly, however. In an in vitro study, Plg treatment enriched microbial abundance and community diversity in sewage treatment (Duan et al. 2017), and in an in vivo study, dietary montmorillonite increased the cecal microflora diversity of laying hens (Chen et al. 2017). Consistently in the present study, dietary Plg supplementation exerted beneficial effects on cecal microbiota diversity, which may be attributed to the adhesion of microbes onto minerals (Barr 1957; Henao and Mazeau 2008).

Microbial taxon composition and relative abundance varied dramatically among the four groups. At the phylum level, Partridge Shank chickens' microbiota consisted predominantly of *Bacteroidetes* and *Firmicutes*, which had also been found to be the case previously in chickens' cecal populations (Oakley and Kogut 2016). Previous studies had indicated, moreover, that the mechanism of action of antibiotics as growth promoters was related to interactions with the intestinal microbial population (Feighner and Dashkevicz 1987). The use of antibiotics inhibited the growth of certain species, thus enabling selected species to survive (La-Ongkhum et al. 2011), thereby affecting the microbial structure. The results obtained in the present study were not entirely consistent

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

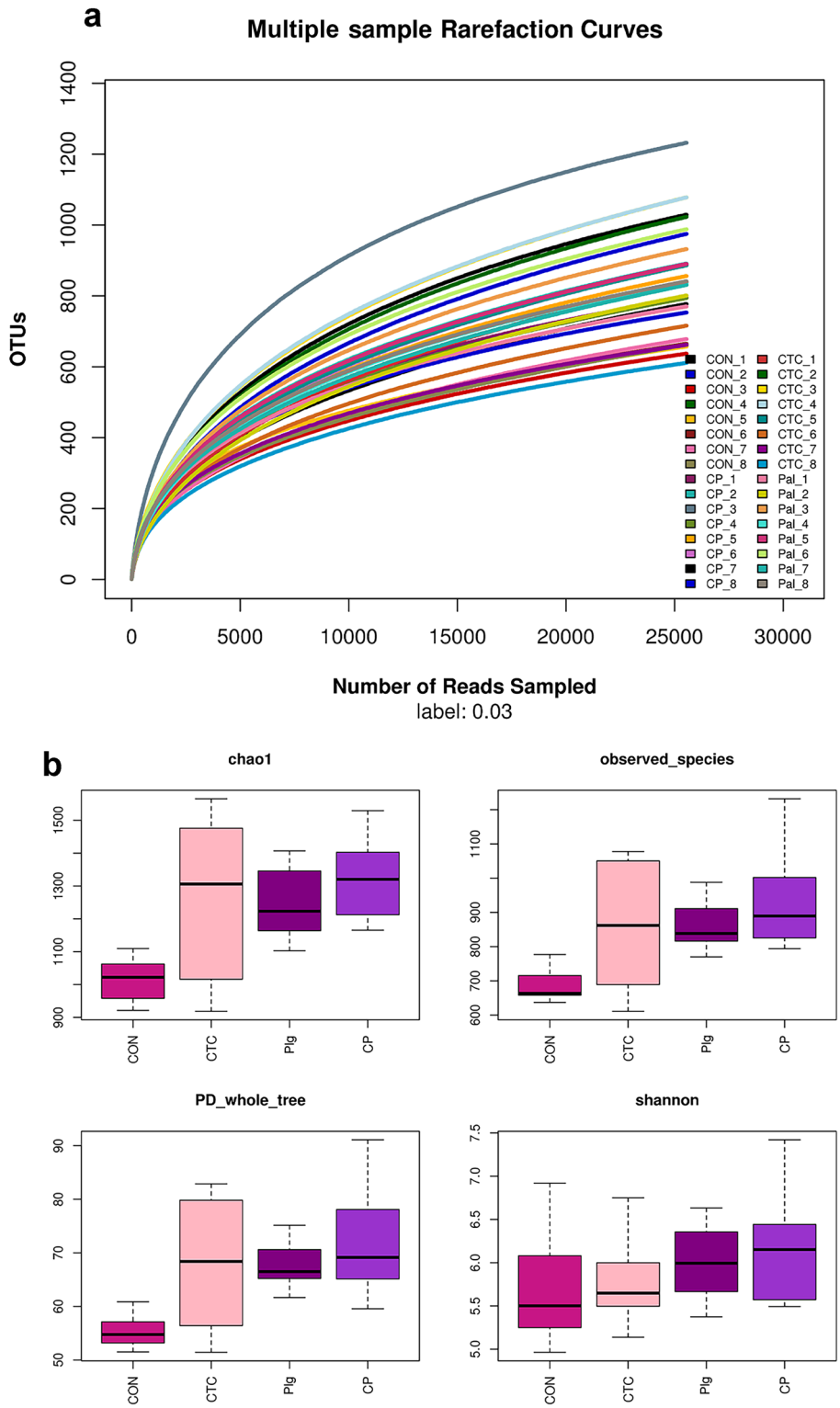

Fig. 2 **a** Richness rarefaction curves for all samples; **b** comparison of α-diversity index in the four groups

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

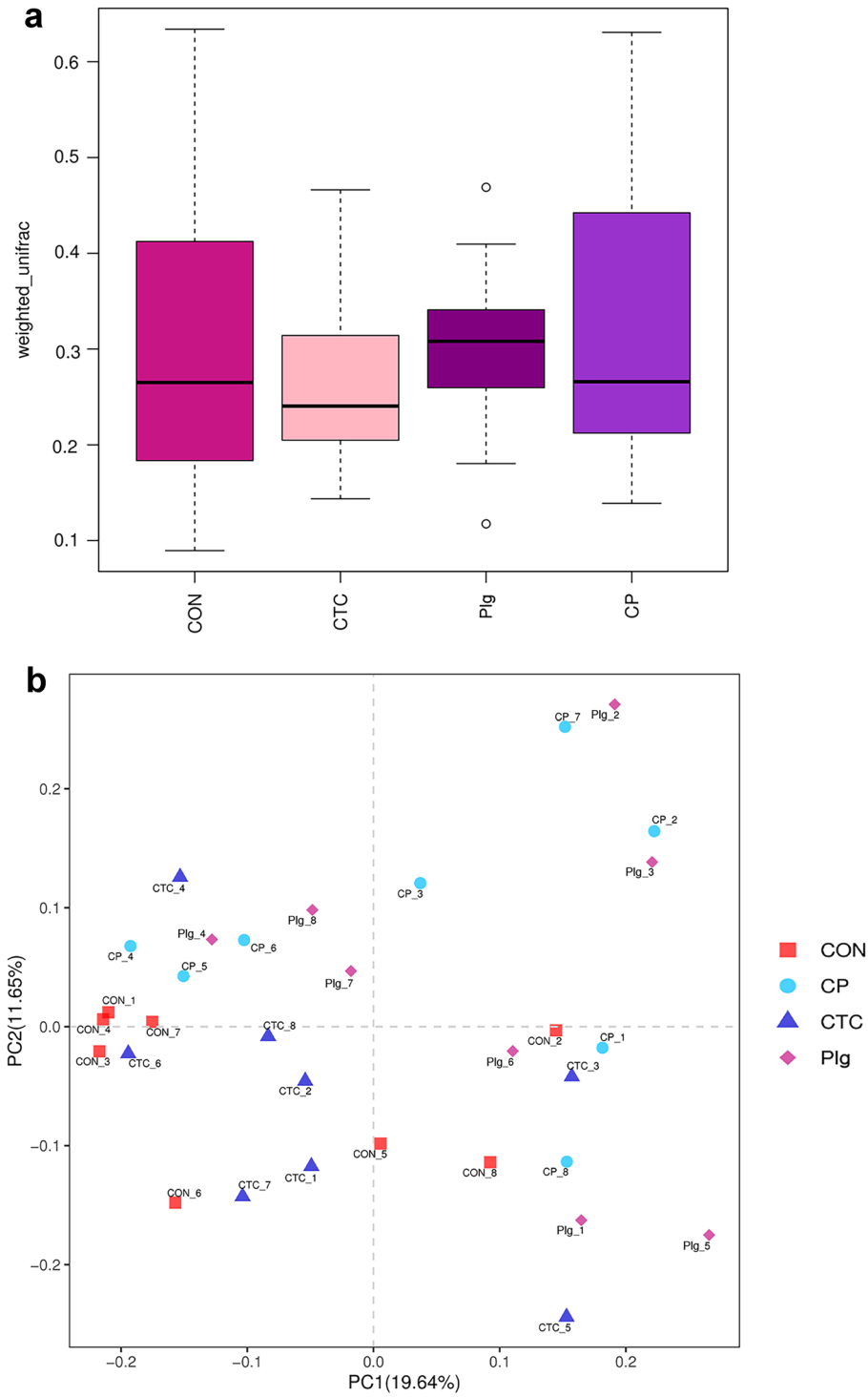

Fig. 3 **a** Comparison of weighted UniFrac in the four groups; **b** based on PCoA analysis of weighted UniFrac

with those findings mentioned above; however, a definite increase was observed in the abundance of *Bacteroidetes* at genus level. The discrepancy may be related to the type or dosage of antibiotics administered. Furthermore, compared with the Plg-free treatment, a dramatic decrease in the number of *Firmicutes* was observed at the phylum

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

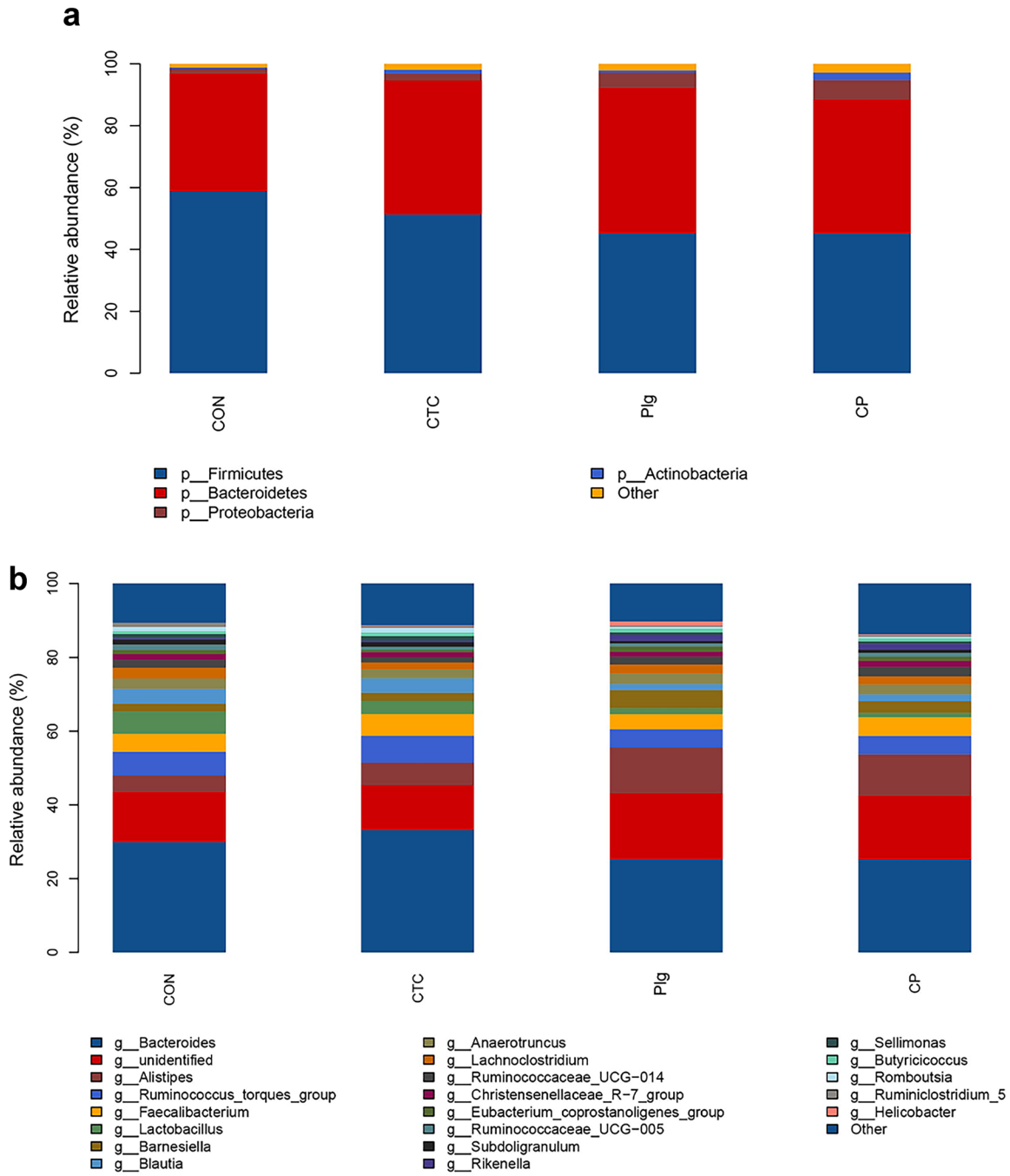

Fig. 4 **a** Relative abundance of cecal microflora at different phyla levels in different groups; **b** relative abundance of cecal microflora at different genus levels in different groups

level classification, even though *Alistipes* was found to be more abundant in the bacterial genus level with dietary Plg supplementation. The previous study (Chalvatzi et al. 2016) provided evidence that dietary inclusion of Plg could alter the growth of particular bacterial groups, thus modulating the cecal microbiota composition of laying pullets (Chalvatzi et al. 2016). The latter authors suggested that the inclusion of Plg in the diets could induce an improvement in feed utilization and nutrient absorption in the proximal intestine that alters the substrate availability, which influences the composition of the cecal bacterial community thereafter.

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

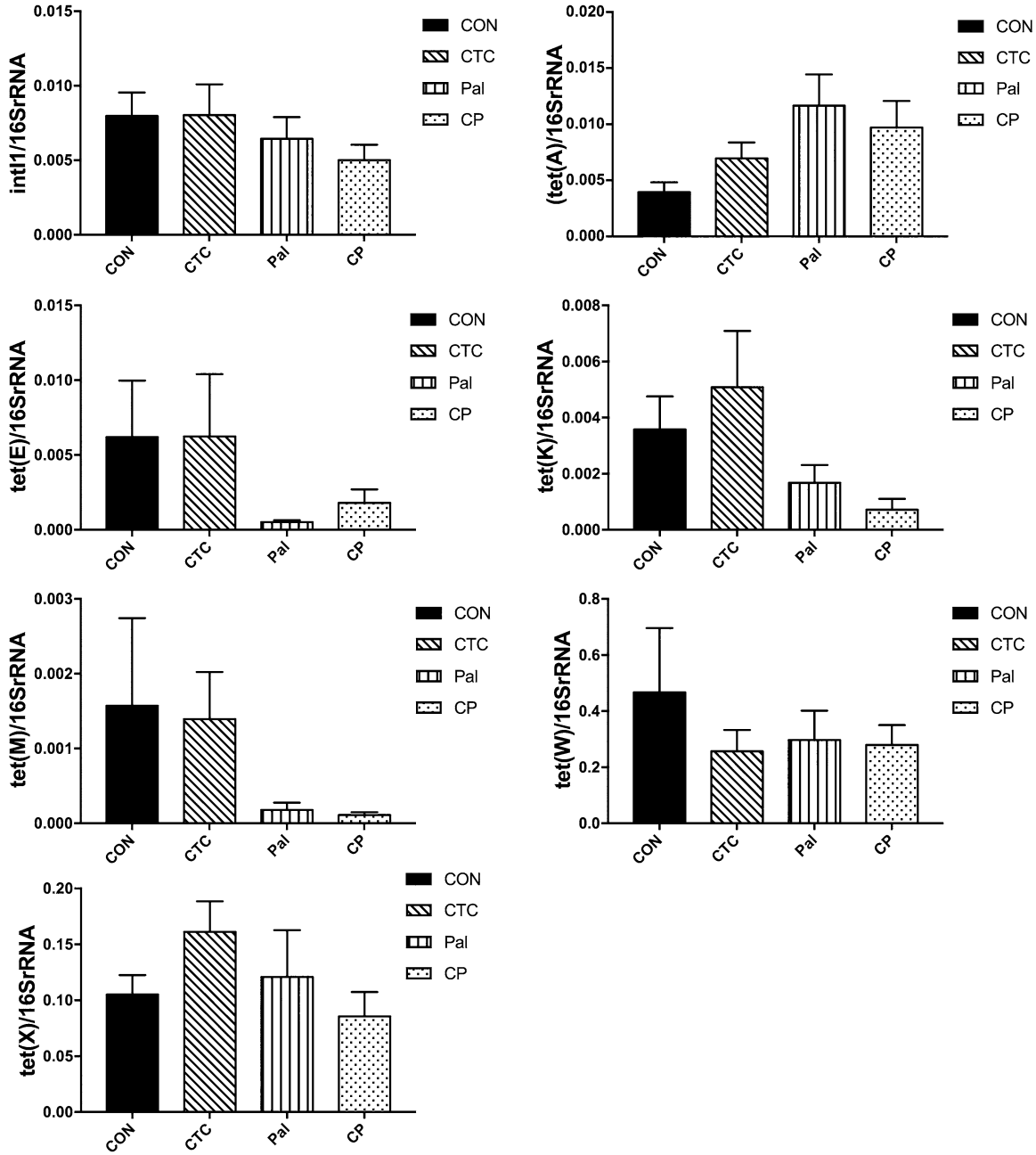

Fig. 5 Abundance (normalized to 16S rRNA gene) of tet and intI1 gene targets in the cecal contents of Partridge Shank chickens

Relative Abundance of ARGs

In the current research, tet(X) and tet(W) were the dominant tet genes. Tet(X) encodes an enzyme that modifies and inactivates the tetracycline molecule (Speer et al. 1991), and its natural host *Bacteroides* (Chopra and Roberts 2001) was confirmed to be the most abundant genus in the present study. In addition, tet(W) was found to be the most abundant genus in feces of cattle (Harvey et al. 2009), supporting the predominance of tet(X) and tet(W) in the present study. The tet(E) gene is associated with large plasmids which are neither mobile nor conjugative (Depaola and Roberts 1995); this may explain its limited predominance in the cecal contents of broilers. The numerical difference in the relative abundance of ARGs could be attributed to host compositions and their potential transferability, therefore.

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

In the present study, the effect of Plg dietary supplementation yielded a reduction in tet(K), and also tended to reduce the abundance of tet(E), tet(M), and IntI1. This finding was in agreement with the results of Qu et al. (2019), who suggested that dietary supplementation of zeolite, possibly due to its porous structure, reduced significantly the relative abundance of some ARGs which might reduce the rate of microbe contact and, therefore, of HGT through conjugation (Zhang et al. 2016a). Beyond that, speculation that kaolinite could regulate the gene expression pattern and metabolism of bacteria against low-dose antibiotic stress was verified by Lai et al. (2019), thereby reducing the possibility of ARGs development and transmission. Furthermore, previous research confirmed that the change in ARGs is closely related to the shift in microbial communities (Qian et al. 2016), which has also been altered by Plg administered in the present study. Plg has a similar porous structure to zeolite and kaolinite, and has the ability to adsorb both *Escherichia coli* (Cai et al. 2013) and antibiotics (Chang et al. 2009), both relevant to the development of ARGs, thus indicating that Plg administration could reduce the selective pressure, eliminate potential host bacteria for ARGs, and, consequently, decrease the occurrence of ARGs. The relative abundance of tet(A) increased significantly with dietary Plg supplementation, which may be associated with the antibiotic selection and the relative growth of hosts. In other words, the host bacteria containing tet(A) is more resistant to CTC administered throughout the trial period. The related underlying mechanism requires further study.

Integrons are bacterial mobile elements that are responsible for genetic transfer between the environmental resistome and both commensal and pathogenic bacteria (Gillings et al. 2015). IntI1, encoding the integrase of class 1 integrons, can facilitate the occurrence of HGT, which is frequently reported to carry one or more gene cassettes that encode antibiotic resistance (Henriques et al. 2006; Mendes et al. 2007). A former study showed that removal of ARGs may also be associated with a reduction in HGT (Zhang et al. 2016c) and intI1 can be used as an essential indicator of the removal and reduction of ARGs in the environment (Wang et al. 2014). In the present experiment, only a weak correlation between intI1 and some of the ARGs was observed, indicating that the potential of lateral transfer for ARGs is relatively small, consistent to some extent with previous studies in which no correlation was observed (Zhang et al. 2016b). In the present study, Plg addition tended to reduce the abundance of intI1, suggesting that the trend for HGT could also be attenuated. Dietary Plg supplementation decreased the relative abundance of ARGs, and the removal rates of tet(E), tet(K), tet(M), tet(W), tet(X), and intI1 were 81.35%, 72.29%, 90.20%, 19.4%, 23.09%, and 35.29%, respectively, which may be because Plg could adhere to or kill harmful bacteria, thereby reducing the possibility of gut bacteria producing ARGs and preventing the spread of ARGs by HGT.

CONCLUSIONS

The currently available results revealed that dietary Plg supplementation caused an increase in the microbial diversity of broilers, along with the changes in the microbial community structure of cecal microbes. Furthermore, administration of Plg reduced the relative abundance of the six representative tet genes encoding different resistance mechanisms, and especially in the case of tet(K). Moreover, Plg addition had a downward trend in the relative abundance of intI1 involved in proliferation of ARGs. The overall results showed that Plg may represent a potentially practical approach to attenuate ARGs and limit dissemination to the environment, thus reducing potential risks to animal and human health.

ACKNOWLEDGMENTS

The study was funded by the National Natural Science Foundation of China (No. 31872405), P.R. China.

Funding

Funding sources are as stated in the Acknowledgments.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

Aminov, R. I., Garrigues-Jeanjean, N., & Mackie, R. I. (2001). Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Applied and Environmental Microbiology, 67*, 22–32.

Baquero, F., Martinez, J. L., & Canton, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology, 19*, 260–265.

Barr, M. (1957) Adsorption studies on clays II. The adsorption of bacteria by activated attapulgite, halloysite, and kaolin. *Journal of the American Pharmaceutical Association, 46*(8), 490–492.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M. N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., & Martinez, J. L. (2015). Tackling antibiotic resistance: The environmental framework. *Nature Reviews Microbiology, 13*, 310–317.

Bergaya, F., & Lagaly, G. (2013). *Handbook of Clay Science*. 2nd edition. Developments in Clay Science, *5*, 243–345.

Cai, X., Zhang, J. L., Ouyang, Y., Ma, D., Tan, S. Z., & Peng, Y. L. (2013). Bacteria-adsorbed palygorskite stabilizes the quaternary phosphonium salt with specific-targeting capability, long-term antibacterial activity, and lower cytotoxicity. *Langmuir, 29*, 5279–5285.

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

Cambray, G., Guerout, A. M., & Mazel, D. (2010). Integrons. *Annual Review of Genetics, 44*, 141–166.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Pena, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7*, 335–336.

Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., & Gasa, J. (2006). The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *Journal of Animal Science, 84*, 2725–2734.

Chalvatzi, S., Kalamaki, M. S., Arsenos, G., & Fortomaris, P. (2016). Dietary supplementation with the clay mineral palygorskite affects performance and beneficially modulates caecal microbiota in laying pullets. *Journal of Applied Microbiology, 120*, 1033–1040.

Chang, P. H., Li, Z. H., Yu, T. L., Munkhbayer, S., Kuo, T. H., Hung, Y. C., Jean, J. S., & Lin, K. H. (2009). Sorptive removal of tetracycline from water by palygorskite. *Journal of Hazardous Materials, 165*, 148–155.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., Maxwell, S., & Aminov, R. I. (2009). Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality, 38*, 1086–1108.

Chen, J. F., Peng, C. Y., Qu, X. Y., & Ji, F. (2017). Effects of montmorillonite on performance and cecal microflora of laying hens. *Chinese Journal of Animal Nutrition, 29,* 4026–4035.

Chen, X., Zhang, W. Q., Yin, J. J., Zhang, N., Geng, S. Z., Zhou, X. H., Wang, Y. H., Gao, S., & Jiao, X. N. (2014). *Escherichia coli* isolates from sick chickens in China: Changes in antimicrobial resistance between 1993 and 2013. *Veterinary Journal, 202*, 112–115.

Chen, Y. P., Cheng, Y. F., Li, X. H., Zhang, H., Yang, W. L., Wen, C., & Zhou, Y. M. (2016). Dietary palygorskite supplementation improves immunity, oxidative status, intestinal integrity, and barrier function of broilers at early age. *Animal Feed Science and Technology, 219*, 200–209.

Chopra, I. (2001). Glycylcyclines: Third-generation tetracycline antibiotics. *Current Opinion in Pharmacology, 1*, 464–469.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews, 65*, 232–260.

Depaola, A., & Roberts, M. C. (1995). Class-D and class-E tetracycline resistance determinants in gram-negative bacteria from catfish ponds. *Molecular and Cellular Probes, 9*, 311–313.

Duan, W. S., Niu, Q. G., Xu, X. G., Li, W., & Fu, D. F. (2017). Influence of attapulgite addition on the biological performance and microbial communities of submerged dynamic membrane bioreactor. *Journal of Water Reuse and Desalination, 7*, 488–501.

Feighner, S. D., & Dashkevicz, M. P. (1987). Subtherapeutic levels of antibiotics in poultry feeds and their effects on weight gain, feed efficiency, and bacterial cholyltaurine hydrolase activity. *Applied and Environmental Microbiology, 53*, 331–336.

Gao, P., Munir, M., & Xagoraraki, I. (2012). Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of the Total Environment, 421*, 173–183.

Ghosh, T. S., Sen Gupta, S., Bhattacharya, T., Yadav, D., Barik, A., Chowdhury, A., Das, B., Mande, S. S., & Nair, G. B. (2014). Gut microbiomes of Indian children of varying nutritional status. *PLoS ONE, 9*, e95547.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., & Stokes, H. W. (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *Journal of Bacteriology, 190*, 5095–5100.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., & Zhu, Y. G. (2015). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME Journal, 9*, 1269–1279.

Hall, R. M., & Collis, C. M. (1995). Mobile gene cassettes and integrons - capture and spread of genes by site-specific recombination. *Molecular Microbiology, 15*, 593–600.

Harvey, R., Funk, J., Wittum, T. E., & Hoet, A. E. (2009). A metagenomic approach for determining prevalence of tetracycline resistance genes in the fecal flora of conventionally raised feedlot steers and feedlot steers raised without antimicrobials. *American Journal of Veterinary Research, 70*, 198–202.

He, J. Z., Shen, J. P., Zhang, L. M., Zhu, Y. G., Zheng, Y. M., Xu, M. G., & Di, H. J. (2007). Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. *Environmental Microbiology, 9*, 2364–2374.

Henao, L., & Mazeau, K. (2008). The molecular basis of the adsorption of bacterial exopolysaccharides on montmorillonite mineral surface. *Molecular Simulation, 34*, 1185–1195.

Huang, J. H., Liu, Y. F., Jin, Q. Z., Wang, X. G., & Yang, J. (2007). Adsorption studies of a water soluble dye, reactive red MF-3B, using sonication-surfactant-modified attapulgite clay. *Journal of Hazardous Materials, 143*, 541–548.

Huyghebaert, G., Ducatelle, R., & Van Immerseel, F. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal, 187*, 182–188.

Henriques, I. S., Fonseca, F., Alves, A., Saavedra, M. J., & Correia, A. (2006). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Research in Microbiology, 157*, 938–947.

La-Ongkhum, O., Pungsungvorn, N., Amornthewaphat, N., & Nitisinprasert, S. (2011). Effect of the antibiotic avilamycin on the structure of the microbial community in the jejunal intestinal tract of broiler chickens. *Poultry Science, 90*, 1532–1538.

Lai, X. L., Wu, P. X., Ruan, B., Liu, J., Liu, Z. H., Zhu, N. W., & Dang, Z. (2019). Inhibition effect of kaolinite on the development of antibiotic resistance genes in

https://doi.org/10.1007/s42860-021-00118-9 Published online by Cambridge University Press

Escherichia coli induced by sublethal ampicillin and its molecular mechanism. *Environmental Chemistry, 16*, 347–359.

Levy, S. B., McMurry, L. M., Barbosa, T. M., Burdett, V., Courvalin, P., Hillen, W., Roberts, M. C., Rood, J. I., & Taylor, D. E. (1999). Nomenclature for new tetracycline resistance determinants. *Antimicrobial Agents and Chemotherapy, 43*, 1523–1524.

Luo, Y., Mao, D., Rysz, M., Zhou, Q., Zhang, H., Xu, L., & Alvarez, P. J. J. (2010). Trends in antibiotic resistance genes occurrence in the Haihe river, China. *Environmental Science & Technology, 44*, 7220–7225.

Mamber, S. W., & Katz, S. E. (1985). Effects of antimicrobial agents fed to chickens on some gram-negative enteric bacilli. *Applied and Environmental Microbiology, 50*, 638–648.

Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: Impacts on human health. *Clinical Microbiology Reviews, 24*, 718–733.

Mazel, D. (2006). Integrons: Agents of bacterial evolution. *Nature Reviews Microbiology, 4*, 608–620.

Mead, G. C. (1997). Bacteria in the gastrointestinal tract of birds. *Gastrointestinal Microbiology, 2*, 216–240.

Mendes, R. E., Castanheira, M., Toleman, M. A., Sader, H. S., Jones, R. N., & Walsh, T. R. (2007). Characterization of an integron carrying bla_{IMP-1} and a new aminoglycoside resistance gene, *aac(6')-31*, and its dissemination among genetically unrelated clinical isolates in a brazilian hospital. *Antimicrobial Agents and Chemotherapy, 51*, 2611–2614.

Moghadam, M.M., Amiri, M., & Riabi, H.R.A. (2016). Evaluation of antibiotic residues in pasteurized and raw milk distributed in the south of Khorasan-e Razavi province, Iran. *Journal of Clinical and Diagnostic Research*, *10*, FC31-FC35.

National Research Council. (1994). *Nutrient Requirements of Poultry:* Ninth edition (revised). National Academies Press, USA.

Ng, L. K., Martin, I., Alfa, M., & Mulvey, M. (2001). Multiplex PCR for the detection of tetracycline resistant genes. *Molecular and Cellular Probes, 15*, 209–215.

Oakley, B. B., & Kogut, M. H. (2016). Spatial and temporal changes in the broiler chicken cecal and fecal microbiomes and correlations of bacterial taxa with cytokine gene expression. *Frontiers in Veterinary Science, 3*, 11.

Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature, 405*, 299–304.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research, 29*, e45.

Pruden, A., Pei, R. T., Storteboom, H., & Carlson, K. H. (2006). Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. *Environmental Science & Technology, 40*, 7445–7450.

Qian, X., Sun, W., Gu, J., Wang, X. J., Zhang, Y. J., Duan, M. L., Li, H. C., & Zhang, R. R. (2016). Reducing antibiotic resistance genes, integrons, and pathogens in dairy manure by continuous thermophilic composting. *Bioresource Technology, 220*, 425–432.

Qu, H. M., Cheng, Y. F., Chen, Y. P., Li, J., Zhao, Y. R., & Zhou, Y. M. (2019). Effects of dietary zeolite supplementation as an antibiotic alternative on growth performance, intestinal integrity, and cecal antibiotic resistance genes abundance of broilers. *Animals, 9*, 909.

Roberts, M. C. (2003). Tetracycline therapy: Update. *Clinical Infectious Diseases, 36*, 462–467.

Slamova, R., Trckova, M., Vondruskova, H., Zraly, Z., & Pavlik, I. (2011). Clay minerals in animal nutrition. *Applied Clay Science, 51*, 395–398.

Speer, B. S., Bedzyk, L., & Salyers, A. A. (1991). Evidence that a novel tetracycline resistance gene found on 2 *Bacteroides* transposons encodes an NADP-requiring oxidoreductase. *Journal of Bacteriology, 173*, 176–183.

Stanley, D., Hughes, R. J., & Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Applied Microbiology and Biotechnology, 98*, 4301–4310.

Wang, F. H., Qiao, M., Lv, Z. E., Guo, G. X., Jia, Y., Su, Y. H., & Zhu, Y. G. (2014). Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China. *Environmental Pollution, 184*, 247–253.

Zaid, M. R. B., Hasan, M., & Khan, A. A. (1995). Attapulgite in the treatment of acute diarrhoea: A double-blind placebo-controlled study. *Journal of Diarrhoeal Diseases Research, 13*, 44–46.

Zhang, J. M., Lv, Y. F., Tang, C. H., & Wang, X. Q. (2013). Effects of dietary supplementation with palygorskite on intestinal integrity in weaned piglets. *Applied Clay Science, 86*, 185–189.

Zhang, J. Y., Chen, M. X., Sui, Q. W., Tong, J., Jiang, C., Lu, X. T., Zhang, Y. X., & Wei, Y. S. (2016a). Impacts of addition of natural zeolite or a nitrification inhibitor on antibiotic resistance genes during sludge composting. *Water Research, 91*, 339–349.

Zhang, J. Y., Chen, M. X., Sui, Q. W., Wang, R., Tong, J., & Wei, Y. S. (2016b). Fate of antibiotic resistance genes and its drivers during anaerobic co-digestion of food waste and sewage sludge based on microwave pretreatment. *Bioresource Technology, 217*, 28–36.

Zhang, Y. J., Li, H. C., Gu, J., Qian, X., Yin, Y. N., Li, Y., Zhang, R. R., & Wang, X. J. (2016c). Effects of adding different surfactants on antibiotic resistance genes and IntI1 during chicken manure composting. *Bioresource Technology, 219*, 545–551.

Zhao, L., Dong, Y. H., & Wang, H. (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of the Total Environment, 408*, 1069–1075.

Zhou, C. H., Zhao, L. Z., Wang, A. Q., Chen, T. H., & He, H. P. (2016). Current fundamental and applied research into clay minerals in China. *Applied Clay Science, 119*, 3–7.

(Received 10 August 2020; revised 17 February 2021; AE: Chun Hui Zhou)