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Understanding the mechanisms of zinc bacitracin and avilamycin on animal production: linking gut microbiota and growth performance in chickens

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Abstract Unravelling the mechanisms of how antibiotics influence growth performance through changes in gut microbiota can lead to the identification of highly productive microbiota in animal production. Here we investigated the effect of zinc bacitracin and avilamycin on growth performance and caecal microbiota in chickens and analysed associations between individual bacteria and growth performance. Two trials were undertaken; each used 96 individually caged 15-day-old Cobb broilers. Trial 1 had a control group (n = 48) and a zinc bacitracin (50 ppm) treatment group (n = 48). Trial 2 had a control group (n = 48) and an avilamycin (15 ppm) treatment group (n = 48). Chicken growth performance was evaluated over a 10-day period, and caecal microbiota was characterised by sequencing of bacterial 16S rRNA gene amplicons. Avilamycin produced no effect on growth performance and exhibited little significant disturbance of the microbiota structure. However, zinc bacitracin reduced the feed conversion ratio (FCR) in treated birds, changed the composition and increased the diversity of their caecal microbiota by reducing dominant species. Avilamycin only produced minor reductions in the abundance of two microbial taxa, whereas zinc bacitracin produced relatively large shifts in a number of taxa, primarily *Lactobacillus* species. Also, a number of phylotypes closely related to lactobacilli species were positively or negatively correlated with FCR values, suggesting contrasting effects of *Lactobacillus* spp. on chicken growth performance. By harnessing such bacteria, it may be possible to develop high-productivity strategies in poultry that rely on the use of probiotics and less on in-feed antibiotics.

Keywords Antibiotics · Microbiota · Caecum · Gastrointestinal tract · Productivity

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Introduction

Antibiotics have been routinely included in the diets of commercially bred poultry in many parts of the global industry. It is generally believed that feeding poultry with antibiotics improves their weight gain and feed efficiency, inhibits pathogen growth and reduces mortalities (Gaskins et al. 2002; Miles et al. 2006; Pfaller 2006). Despite the wide acceptance of the beneficial effects of antibiotics, the mechanisms driving these benefits remain largely unknown (Lin et al. 2013). Antibiotics have an impact on the intestinal microbiota, yet it is unclear whether they increase growth performance by reducing the energetic cost of the intestinal system through reductions of

total gut microbiota (Collier et al. 2003; Gaskins et al. 2002) and/or by modifying the gut microbial structure to a bacterial community more conducive to host growth (Dibner and Richards 2005). Hence, it is of value to understand the mode of action of antibiotics in the gut microbiota. Although there is a strong move away from the use of antibiotics in animal feed, it is still of value to understand how changes in gut microbiota associated with antibiotic use influence productivity. If the key attributes of highly productive microbiota can be understood, then it may be possible to develop alternative strategies to in-feed antibiotic usage in commercial practice.

The microbiota present in the gastrointestinal tract of animals plays a key role in the health of the host. A number of studies in chickens have shown the importance of gut microbiota in some vital functions related to gut development, mucosal immunity and digestion of feed and nutrient absorption by the host (Gong et al. 2007; Stanley et al. 2014; Stanley et al. 2016). A comprehensive understanding of how the gut microbiota influences the efficiency of energy utilisation by chickens would provide valuable knowledge for the poultry industry. Chickens are the most efficient farm animals in converting energy into meat (Choct 2009) and are an excellent model for studying methods to increase growth performance. Chicken performance is generally expressed by measures including feed conversion ratio (FCR), body weight gain (BWG) and amount of feed eaten (FE). A large number of studies have analysed the beneficial effects that antibiotics have on these performance measures. However, there are comparatively few studies in chickens that have investigated the impact of antibiotics on the bacterial communities in the intestine (Gong et al. 2008; Pedroso et al. 2006; Zhou et al. 2007). For instance, research has shown that two antibiotic treatments (bacitracin/virginiamycin or monensin) promoted the growth of *Clostridia* and reduced the microbiota diversity in the ileum of broiler chickens (Lu et al. 2008). Another study on chickens found that virginiamycin produced a stronger effect on the caecal microbiota, relative to bacitracin methylene disalicylate, manifested primarily via an enrichment in the genus *Faecalibacterium* (Neumann and Suen 2015). Not only few studies have attempted to understand how gut microbiota is affected by antibiotics, but also even fewer have analysed the relationship between gut microbiota and growth performance (Stanley et al. 2016; Torok et al. 2011b).

This work compared how avilamycin and zinc bacitracin influence the intestinal bacterial community. These antibiotics are two of the most frequently used in the poultry industry internationally (Sarmah et al. 2006). Both avilamycin and zinc bacitracin are considered growth promoters and have a spectrum of activity against gramme-positive organisms (Marshall and Levy 2011). Avilamycin is an oligosaccharide antimicrobial substance classified as an orthosomysin (Witte 2000), whereas zinc bacitracin is a mixture of high molecular weight polypeptides, produced by *Bacillus licheniformis* (Phillips 1999). To our knowledge, only one study has investigated the effect of both avilamycin and zinc bacitracin on chickens (Torok et al. 2011a). The authors of this study used terminal restriction fragment length polymorphism (T-RFLP) to analyse the development of gut microbiota during the first 17 days after hatching; however, the recent development of molecular tools, such as high-throughput DNA sequencing, allows greater in-depth analyses of gut microbiota. These tools provide an unprecedented opportunity to analyse microbial communities. In this study, two separate animal trials were conducted to investigate the impact of the two antibiotics on growth performance and gut microbiota using sequenced samples from 192 birds. This approach enabled us to obtain information on gut bacteria correlated with performance measures.

Materials and methods

Animal trials

Two animal trials (each with n = 96 birds) were performed consecutively. Briefly, one-day-old male Cobb 500 broiler chickens, from the Baiada Hatchery, Willaston, South Australia, Australia, were transferred to a chick rearing pen in a temperature-controlled experimental animal facility. Feed and water were supplied ad libitum throughout the experiment. The same batch of commercially prepared starter crumbles, based on wheat as the main cereal component (Ridley Agriproducts, Murray Bridge, SA), was used in both trials as the control and was stored under controlled cool and dry conditions for the duration of the trials. All birds within each trial were housed together and fed the control diet for the first 13 days of life to ensure microbiota exchange through typical bird behaviour, including coprophagy.

The experimental diets were introduced from day 15 in both trials. For trial 1 were (1) control and (2) control with zinc bacitracin, 50 ppm active ingredient, and for trial 2 were (1) control and (2) control with avilamycin, 15 ppm active ingredient.

For the purpose of accurate performance assessment, on day 13, chicks were transferred in pairs to 48 metabolism cages in a temperature controlled room (23–25 °C). Initial placement in metabolism cages in pairs was done to minimise stress and allow the birds to adjust to cages. At day 15, birds were moved into individual cages. Individual caging allowed the precise assessment of individual feed intake, energy in feed and unused energy from the feed appearing in excreta. Single bird caging and individual measurements and sampling were implemented in order to avoid competition for feed and to allow direct correlation of microbiota structure and productivity measurements on a bird by bird basis. Birds were euthanised and necropsied on day 25, and caecal contents

were collected from each bird. Samples from all birds from the two trials were analysed.

Feed conversion ratio was calculated as a ratio of feed eaten and weight gained. Thus, birds with low FCR, which needed less feed per kilogramme of weight gain, were the most efficient in converting feed to body mass. Gross energy was measured in feed and in faeces of each individual bird using a Parr isoperibol bomb calorimeter (Parr Instrument Company, Moline, IL). Apparent metabolisable energy (AME) in megajoule per kilogramme dry matter was calculated as (AME_{diet} = [(GE_{diet} × feed eaten) × ($GE_{excreta}$ × dry excreta)] / feed eaten / dry diet content). Gain rate was calculated as [weight gain (g) / start weight (g)], and feed eaten was total amount of feed eaten during the 10-day measurement period. All of the above measurements were taken from day 15 to day 25, during the time when birds were housed individually in metabolism cages.

DNA preparation, PCR amplification of 16S rRNA gene sequences and bioinformatics analysis

Briefly, DNA was isolated using the method of Yu and Morrison (Yu and Morrison 2004), and the V1-V3 region of the 16S ribosomal RNA (rRNA) gene was amplified (forward primer (Lane 1991), 5′ AGAGTTTGATCCTGG 3′; reverse primer W31 (Snell-Castro et al. 2005), 5′ TTACCGCG GCTGCT 3′) using the high-fidelity Q5 DNA polymerase (New England Biolabs). Pyrosequencing was performed using a Roche/454 FLX+ instrument and Titanium chemistry according to the manufacturer's instructions. Sff file processing was done using PyroBayes (Quinlan et al. 2008) and inspected for chimeric sequences with Pintail (Ashelford et al. 2005) and errors using Acacia (Bragg et al. 2012). Further trimming was done in QIIME (version 1.8; QIIME development team [http://qiime.org/]) (Caporaso et al. 2010) with sequence length 300–600 bases, no ambiguous sequences, minimum average quality score of 25 and maximum of 6 bases in homopolymer runs. OTU picking was done using Uclust with 97% similarity (Edgar 2010). Taxonomy was assigned using Blast against the GreenGenes database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (DeSantis et al. 2006) and QIIME defaults. Additional taxonomic assignments for OTUs of interest were performed using EzTaxon database (http://www.ezbiocloud.net/eztaxon) (Chun et al. 2007). All samples represented by less than 1000 sequences were removed from the analysis during rarefaction, leaving 191 samples used in the analysis. The dataset with the sequencing quality assessment is publically available in MG-RAST database (http://metagenomics.anl.gov/) under accession number mgl538409.

Permutational analysis of variance (PERMANOVA) is a statistical routine that calculates distance matrices among sources of variation to perform permutation tests for univariate or multivariate analysis of variance. This routine generates Pseudo-F statistics ratios to obtain P values (Anderson 2001). PERMANOVA was used to test for differences between groups on performance measures (AME, FCR, BWG and FE) and also for differences in microbiota composition. The distance matrices for the growth performance measures were based on Euclidean distance and for microbiota abundance data on both weighted and unweighted Unifrac distances (Lozupone and Knight 2005). Whenever a significant effect was found, pairwise tests were conducted. The permutational analysis of multivariate dispersions (PERMDISP) routine was applied to ensure the homogeneity of multivariate dispersions among groups (Anderson 2006). Canonical analysis of principal coordinates (CAP) was used to visualise between-group differences in microbiota composition (Anderson and Willis 2003). Analysis of variance (ANOVA) was used to test for differences in community diversity between groups using four diversity measures (i.e. richness, evenness, Shannon's and inverted Simpson's indices). ANOVA was also used to test for between-group differences in the abundance of individual microbial taxa. Redundancy discrimination analysis (RDA) was used to analyse the associations between each of the performance measures and the caecal microbiota composition of chickens in both trials. Also, Pearson's correlation tests were conducted to analyse the associations between individual microbial taxa and each performance indicator. A 'heat map' was created to visualise these correlations. For all ANOVA, RDA and correlation analyses, a square root transformation was applied to the normalised microbiota abundance data to reduce variance heterogeneity and increase predictive power. PERMANOVA, PERMDISP and CAP analyses were carried out using PRIMER software with the PERMANOVA+ add-on (version 6.1.16; Massey University [http://www.primer-e.com/]). RDA, Pearson's correlation tests and analyses of community diversity and taxonomic structure were performed in Zakrzewski et al. (2016).

Results

Growth performance measures

Comparison of the performance of the birds in trial 1 to that seen in trial 2 showed that the trials differed significantly in each of the four performance measures. Differences in FCR were highly statistically significant between trials ($P \leq 0.001$) (trial 1 1.64 ± 0.08 [from now on, average ± standard error]; trial 2 1.51 ± 0.05). However, the 'trial × treatment' factor was also statistically significant ($P \leq 0.001$). This interaction was produced because zinc bacitracin had a significant effect on the FCR in trial 1 ($P \leq 0.001$), resulting in lower values in treated birds

(1.61 ± 0.01) than in untreated birds (1.67 ± 0.01) (Fig. 1), but avilamycin did not have any effect on the FCR in trial 2 (P = 0.104) (treated 1.51 ± 0.01; untreated 1.50 ± 0.01) (Fig. 1). BWG was significantly higher in trial 2 (906 ± 8.4 g) than in trial 1 (858 ± 6.8 g) ($P \leq 0.001$), but did not vary between treated and untreated birds ($P > 0.050$). Similarly, FE only differed significantly between trials (P = 0.009), with trial 2 (1363 ± 12.7 g) showing lower values than trial 1 (1405 ± 9.9 g). The same pattern was found for AME values, only showing statistically different values between trials (P = 0.029) (trial 1 14.23 ± 0.07; trial 2 14.40 ± 0.04). PERMDISP results confirmed that all the above-mentioned significant results did not occur by heterogeneity of dispersion among groups ($P \leq 0.05$).

Structure of the caecal microbial community

A visual inspection of the microbiota profiles at the genus level in each group indicated high differences between trials, since trial 1 was dominated by *Lactobacillus* (Fig. 2a), whereas trial 2 was dominated by *Bacteroides* and *Lactobacillus* (Fig. 2b).

Diversity measures varied significantly between both trials at most taxonomic levels (i.e. OTU, genus, family, order and phylum levels). Intra-trial comparisons showed that in trial 1, all four diversity measures were significantly higher in zinc bacitracin-treated compared to untreated birds at the OTU level (richness P = 0.007, inverted Simpson P = 0.004, evenness P = 0.003 and Shannon P = 0.002) (Fig. 3). Additionally, both evenness and Shannon indices were significantly higher in treated compared to untreated birds at the genus level (P = 0.04 and P = 0.05, respectively) and also at the family level (P = 0.018 and P = 0.031, respectively). These results indicate that zinc bacitracin increased richness and evenness by controlling the dominance. No changes were found in any of the four diversity indices at either order or phylum levels ($P > 0.05$). In trial 2, there were no significant differences in diversity at any taxonomic level between avilamycin-treated and untreated control birds ($P > 0.05$) (Fig. 3).

At an OTU level, microbial community composition of the chickens differed significantly between trials using either unweighted (from now on UW) or weighted (from now on W) Unifrac distances (UW P = 0.001; W P = 0.001). The trial × treatment interaction was significant using both distances (UW P = 0.002; W P = 0.023). This significant interaction occurred because birds in trial 1 were affected by zinc bacitracin (UW P = 0.008; W P = 0.002) whereas those in the trial 2 were not affected by avilamycin (UW P = 0.093; W P = 0.49) (Fig. 4). PERMDISP results showed that all of the above-mentioned significant results were not produced by dispersion among groups ($P > 0.05$).

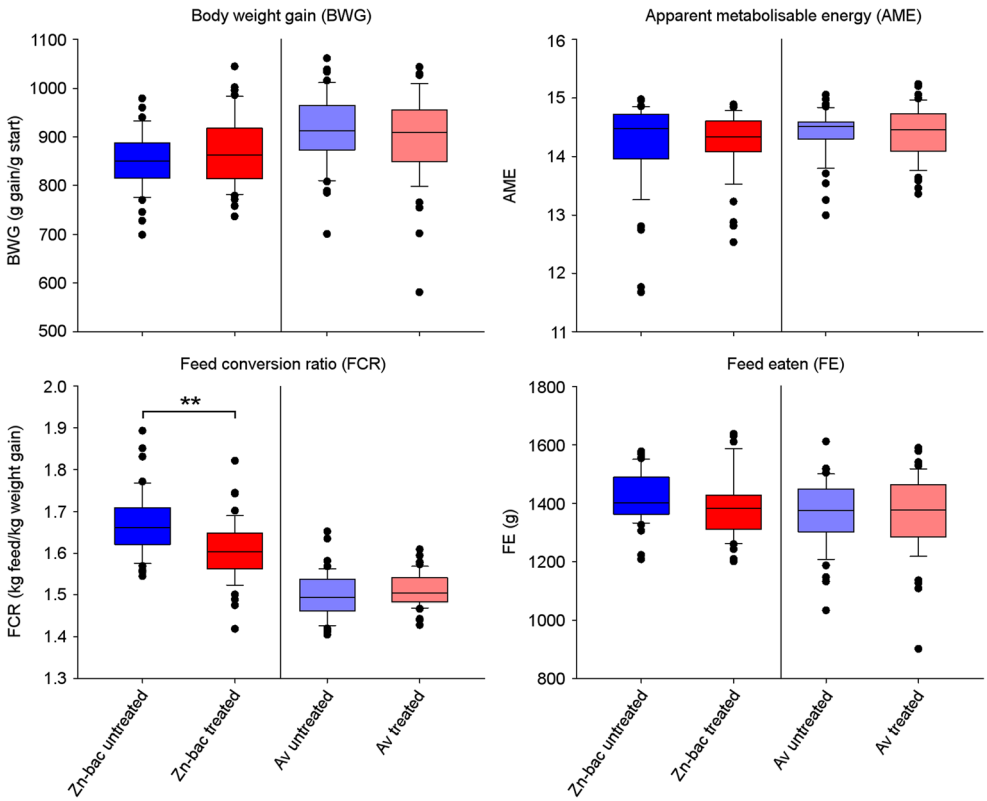

Fig. 1 Boxplots showing differences in performance measures between groups of birds. Antibiotic supplemented groups are coloured *red* and controls are presented in *blue* with zinc bacitracin trial presented in *darker shades* than avilamycin trial. *Zn-bac* zinc bacitracin, *Av* avilamycin. Significant differences between groups are indicated (**$P \leq 0.01$) (colour figure online)

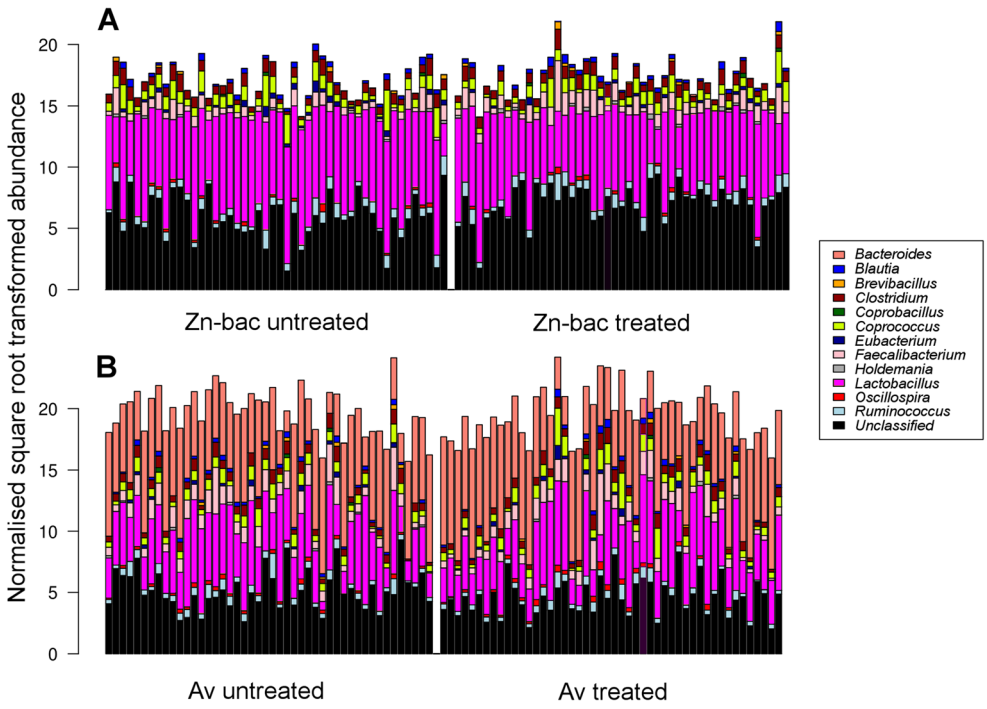

Fig. 2 Microbiota profiles showing normalised square root transformed abundances of the genera in each group of birds. **a** Birds in trial 1 and **b** those in trial 2. *Zn-bac* zinc bacitracin, Av avilamycin

Changes in microbial taxa

In trial 1, zinc bacitracin-treated chickens had significant changes (relatively to the untreated chickens) in the abundance of 16.9% of all OTUs (n = 95 OTUs) at a significance level of $P \leq 0.05$ and of 2.5% of all OTUs (n = 14 OTUs) at $P \leq 0.001$. Out of the 10 most abundant OTUs (by order of abundance) showing antibiotic-driven significant

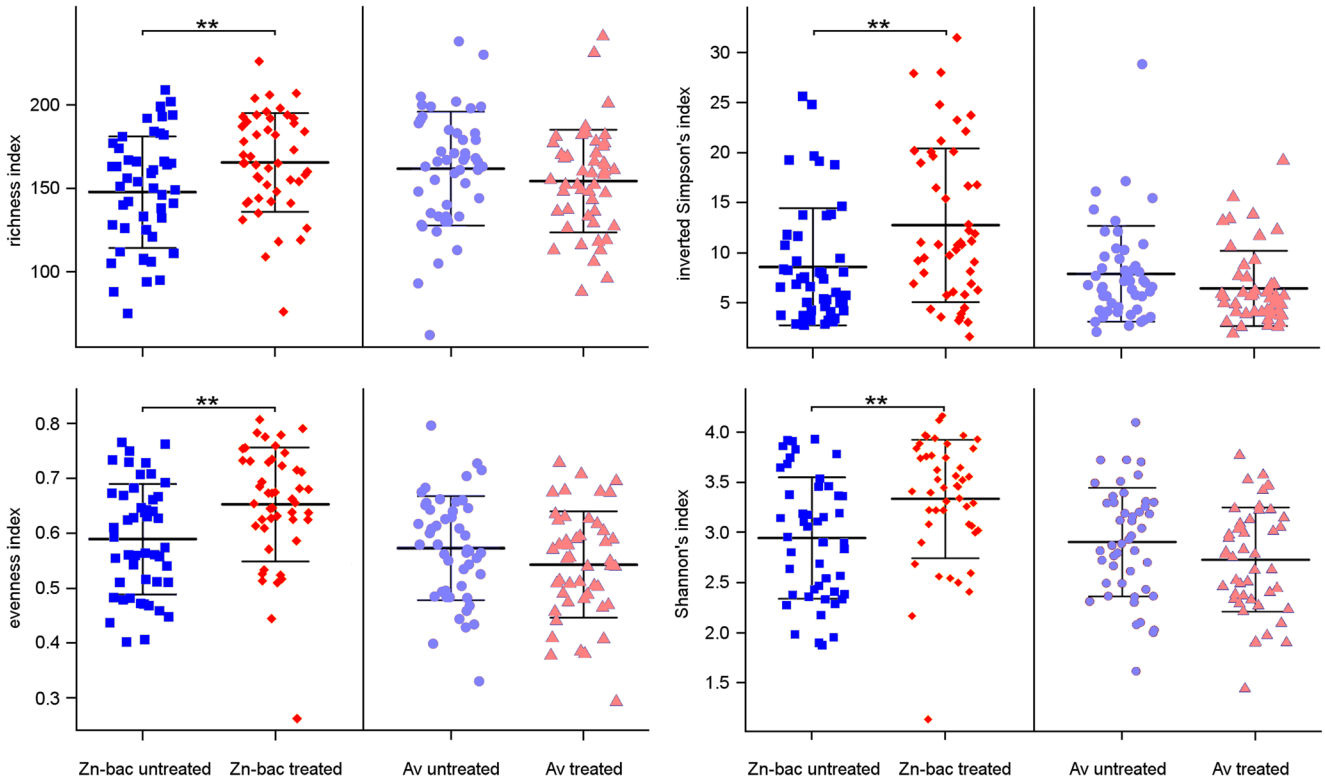

Fig. 3 Strip charts showing differences in four diversity measures (i.e. richness, evenness, inverted Simpson's and Shannon's indices) between groups at the OTU level. *Zn-bac* zinc bacitracin, *Av* avilamycin. Statistically significant differences are indicated (**$P \leq 0.01$)

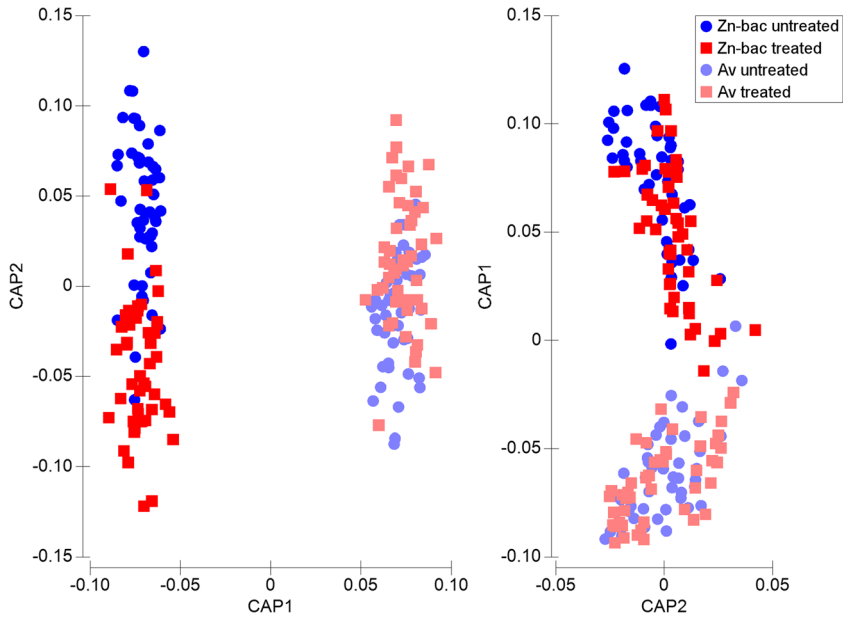

Fig. 4 Canonical analysis of principal coordinates (CAP) showing a constrained ordination of microbial taxa composition at the OTU level by trial and treatment. *Left plot* is based on unweighted Unifrac distance, and right plot is based on Weighted Unifrac distance. *Zn-bac* zinc bacitracin, *Av* avilamycin

shifts, two assigned to the *Lactobacillus* genus showed reductions, whereas the next five OTUs, assigned to the order *Clostridiales*, showed increases in zinc bacitracin-treated birds. At the genus level, zinc bacitracin-treated chickens exhibited significant reductions in the genus *Lactobacillus* ($P = 0.002$) and *Eubacterium* ($P = 0.016$) (Fig. 5a). The decrease in the abundance of *Lactobacillus* was partly caused by significant reductions of a phylotype related to *Lactobacillus salivarius* (Fig. 5b). Its identity was based on 50 OTUs, all assigned to *L. salivarius* ATCC 11741(T) strains with an average similarity (avg. sim. from now on) of 89.37%. Additionally, zinc bacitracin-treated birds also showed significant increases in the genus *Faecalibacterium* ($P = 0.004$) and in OTUs unclassified at the genus level ($P = 0.001$) (Fig. 5a). Also, at the species level, there were significant increases in a *Ruminococcus torques* phylotype ($n = 5$ OTUs, assigned to strain ATCC 27756(T), avg. sim. = 95.13%) in treated birds ($P = 0.046$) (Fig. 5b). Decreased numbers of the *Lactobacillus* genus resulted in similar significant reductions (at the order level) in *Lactobacillales* ($P = 0.002$), whereas increases in the *R. torques* phylotype and *Faecalibacterium* partly contributed to significant increases in *Clostridiales* ($P = 0.002$) (Fig. 5c). No significant changes were observed at any taxonomic levels beyond the order level. In trial 2, avilamycin produced significant changes in the abundance of 5.9% of all OTUs ($n = 36$ OTUs) at a level of significance of $P \leq 0.05$, but none were found at $P \leq 0.001$. Out of the 10 most abundant OTUs showing significantly different abundances between treatments, four were assigned to the order *Clostridiales* and two assigned to the genus *Lactobacillus* showed lower abundance in avilamycin-treated birds. At the species level, when compared with untreated birds, avilamycin-treated birds showed significantly lower numbers of a phylotype related to *Clostridium spiroforme* ($n = 3$ OTUs, strain DSM 1552(T), avg. sim. = 92.84%) ($P = 0.04$) and also of the family *Catabacteriaceae* ($P = 0.03$) (Fig. 5d). No other significant differences were found at any other taxonomic level ($P > 0.05$).

Associations between microbiota and performance measures in both trials

RDA showed significant associations between microbial community composition and three of the four selected performance measures (AME, FCR and BWG) at the OTU level (all three measures $P \leq 0.001$) and also at the genus level ($P = 0.03$, $P = 0.001$ and $P = 0.026$, respectively). However, FE was not significantly associated with the microbial community composition at either OTU or genus level ($P = 0.221$ and $P = 0.906$, respectively). Negative correlations with the FCR (OTUs in higher abundance in the birds with lower FCR values) and FE and the positive correlations with BWG or AME are considered desirable in chicken performance. Individual associations between each of the performance measures and the abundance of individual bacterium at the genus level are shown in a heat map (Fig. 6). In particular, two genera showed strong associations with a number of the performance measures. *Bacteroides* showed significant negative correlations with FCR ($P \leq 0.001$, $r = -0.65$) and FE ($P = 0.004$, $r = -0.207$) and a significant positive correlation with BWG ($P \leq 0.001$, $r = 0.241$). Exhibiting different trends, *Lactobacillus* was positively associated with FCR ($P \leq 0.001$, $r = 0.443$) and FE ($P \leq 0.001$, $r = -0.207$) and negatively associated with AME ($P = 0.006$, $r = -0.198$). Additionally,

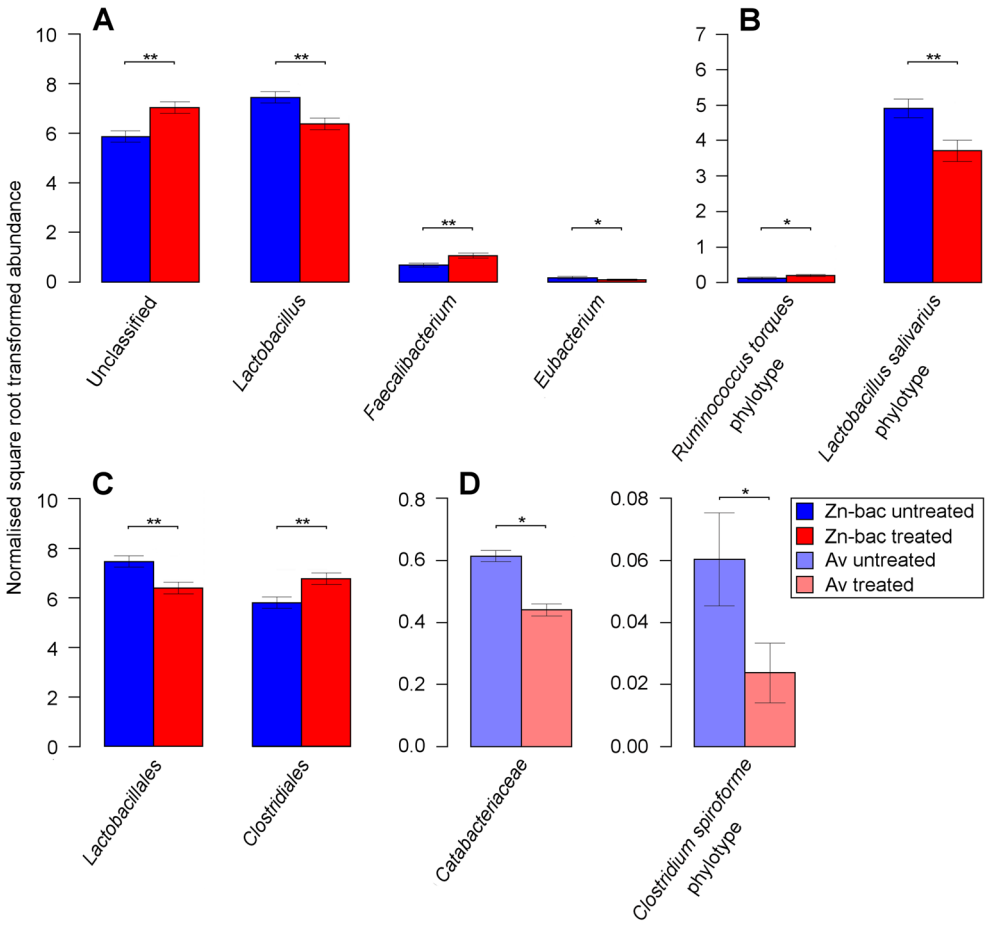

Fig. 5 Microbial taxa showing significant differences in normalised square root transformed abundance between groups. **a**, **b** Taxa at, respectively, the genus and species level in trial 1. **c** Taxa at the order level in trial 1. **d** Taxa at both family and species levels in trial 2. *Zn-bac* zinc bacitracin, *Av* avilamycin. Statistically significant differences are indicated (**$P \leq 0.01$; *$P \leq 0.05$). All species were identified based on OTU level at high similarity levels using the EzTaxon database (http://www.ezbiocloud.net/): *Ruminococcus torques* phylotype was assigned to 5 OTUs ($n = 5$ OTUs), all ATCC 27756(T) strains, with an averaged similarity (avg. sim.) of 95.13%; *Lactobacillus salivarius* phylotype ($n = 50$ OTUs), all ATCC 11741(T) strains (avg. sim. = 89.37%); and *Clostridium spiroforme* phylotype ($n = 3$ OTUs), all DSM 1552(T) strains (avg. sim. = 92.84%)

a number of other taxa showed relatively weaker correlations. *Ruminococcus* had a negative correlation with AME ($P = 0.042$, $r = -0.148$). Additionally, a number of other genera showed significant associations with FCR. Both *Faecalibacterium* and *Oscillospira* showed significant negative associations with FCR ($P = 0.002$, $r = -0.218$ and

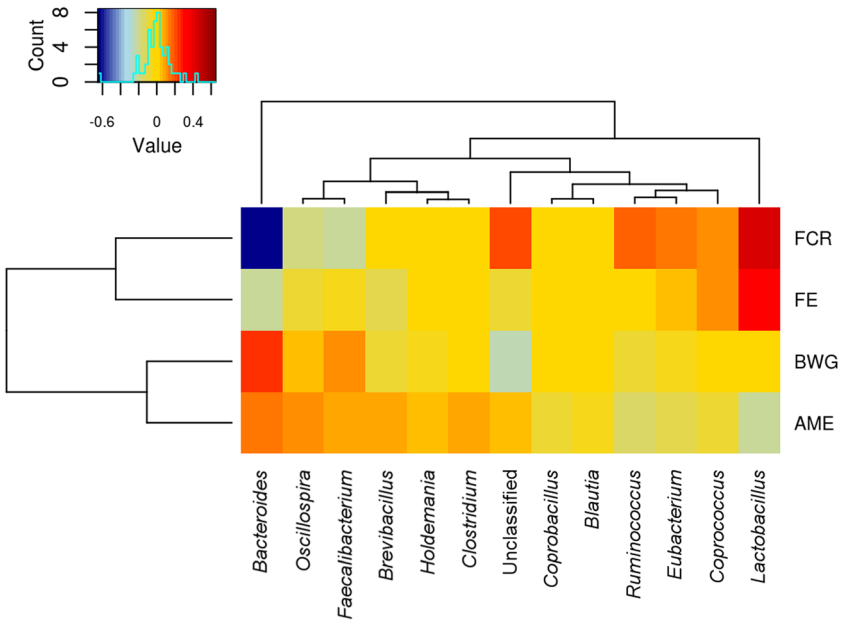

Fig. 6 Heat map showing correlations (based on Pearson's test) between the normalised square root transformed abundance of microbiota genera and the performance measures. Positive or negative correlations (r^2 values) are represented by shades of *red* or *blue* respectively, as indicated in the histogram (colour figure online)

$P = 0.007$, $r = -0.194$, respectively), whereas *Eubacterium* showed a significant positive correlation with FCR ($P = 0.028$, $r = 0.16$) (Fig. 6).

The associations between performance measures and taxa were also studied at the species levels to investigate whether there were any differences between members of the same genus. The only significant correlations were found in the *L. salivarius* phylotype, the most abundant lactobacilli, which was strongly positively associated with FCR ($P \leq 0.001$, $r = 0.642$) and negatively associated with AME ($P = 0.013$, $r = -0.181$) and BWG ($P = 0.007$, $r = -0.195$) (Fig. 7). Similar trends were followed by phylotypes closest to *Lactobacillus agilis* (n = 11 OTUs, strain DSM 20509(T), avg. sim. = 92.73%) (FCR $P \leq 0.001$, $r = 0.523$; AME $P \leq 0.001$, $r = -0.313$) and *Lactobacillus saerimneri* (n = 2 OTUs, strain DSM 16049(T), avg. sim. = 91.91%) (FCR $P \leq 0.001$, $r = 0.273$; AME $P = 0.034$, $r = -0.154$). Nevertheless, three other *Lactobacillus* species showed opposite patterns. The *Lactobacillus* phylotype closely related to *Lactobacillus crispatus* (n = 32, strain DSM 20584(T), avg. sim. = 91.76%), the second most abundant species in the genus, showed a highly negative association with FCR ($P \leq 0.001$, $r = -0.559$) and a positive association with BWG ($P \leq 0.001$, $r = 0.326$) (Fig. 7). Similarly, phylotypes related to *Lactobacillus reuteri* (n = 9 OTUs, strain JCM 1112 (T), avg. sim. = 93.43%) and *Lactobacillus vaginalis* (n = 7 OTUs, strain ATCC 49540(T), avg. sim. = 95.40%) showed negative correlations with FCR ($P \leq 0.001$, $r = -0.368$ and $P \leq 0.001$, $r = -0.366$, respectively) and positive correlations with BWG ($P = 0.021$, $r = 0.168$ and $P \leq 0.001$, $r = 0.259$, respectively).

Discussion

Inter- and intra-trial comparisons of microbiota composition and growth performance

Considering the widespread use of antibiotics in the poultry industry, it is important to discern their impact on gut microbiota and growth performance in order to understand their mode of action and enable the development of alternative production and treatment strategies for reduced reliance on antibiotic feed enhancers. An understanding of how changes in microbiota contribute to the productivity gains sometimes observed with antibiotic use may facilitate the design of alternative methods to achieve similar outcomes. Based on the microbiota analysis of two animal trials, this study showed high inter- and intra-trial differences in performance measures

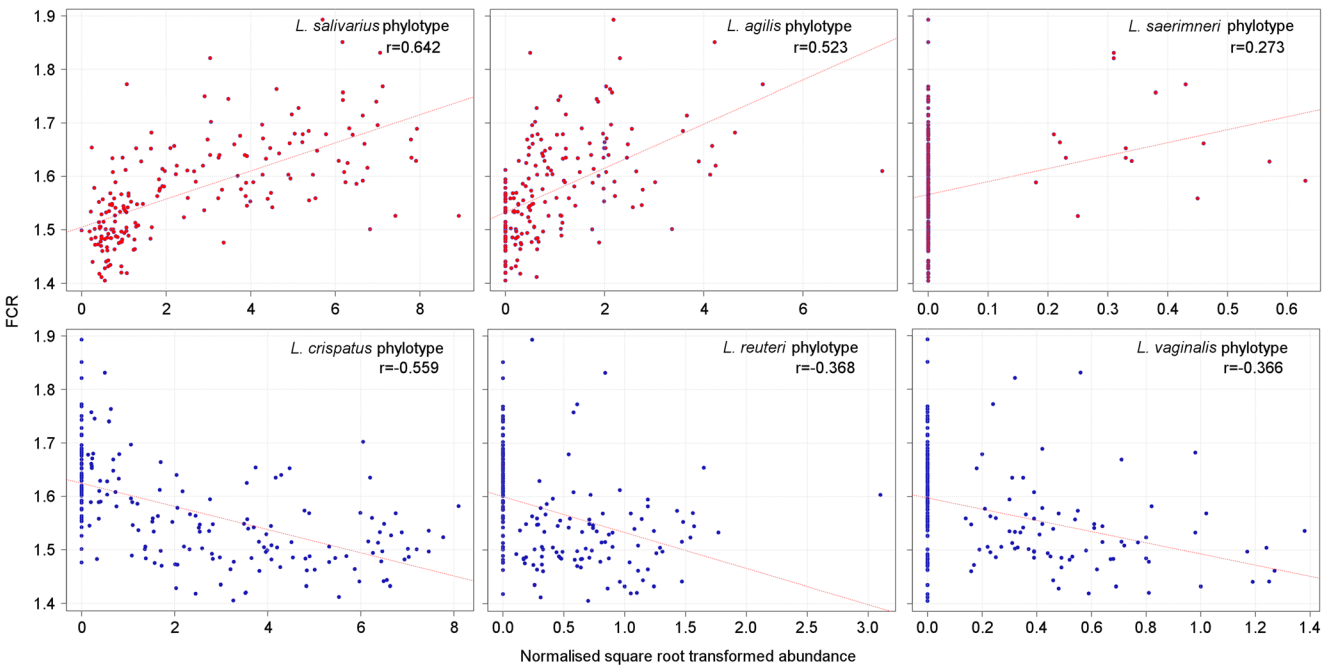

Fig. 7 Scatter plots showing individual correlations between normalised square root transformed abundances of six phylotypes associated to different species of *Lactobacillus* and FCR values. *Red colour* indicates positive correlations whereas *blue colour* indicates negative correlations. All six correlations were highly significantly correlated ($P \leq 0.001$). All phylotypes were identified based on OTU level using the EzTaxon database (http://www.ezbiocloud.net/): *L. salivarius* phylotype identity was assigned to 50 OTUs (n = 50 OTUs), all with closest culturable isolates of ATCC 11741(T) strains, with an averaged similarity (avg. sim.) of 89.37%; *L. agilis* phylotype (n = 11 OTUs, all DSM 20509(T) strains, avg. sim. = 92.73%); *L. saerimneri* phylotype (n = 2 OTUs, both DSM 16049(T) strains, avg. sim. = 91.91%); *L. crispatus* phylotype (n = 32 OTUs, all DSM 20584(T), avg. sim. = 91.76%); *L. reuteri* phylotype (n = 9 OTUs, all JCM 1112(T) strains, avg. sim. = 93.43%); and *L. vaginalis* phylotype (n = 7 OTUs, all ATCC 49540(T) strains, avg. sim. = 95.40%)

and microbiota composition, with each antibiotic (avilamycin and zinc bacitracin) showing contrasting results.

Birds in each trial showed distinctly different growth performance and microbiota composition, corroborating our previous findings in independent experiments (Stanley et al. 2013b; Stanley et al. 2016). These earlier studies found that chickens from the same breed, fed with identical diets, show differences in energy extraction and/or in transformation of feed into body weight (Hughes 2003; Stanley et al. 2016). Microbial profiles differed between trials, one comprised mainly of *Firmicutes* (93.0%, trial 1) and the other one with high abundance of *Bacteroidetes* (44.2%, trial 2). It has been established that the *Firmicutes*/*Bacteroidetes* ratio plays a role in human weight management, with high values in obese people and low values in lean people (Ley et al. 2006). Although this is a generalisation and it is strongly influenced by specific host species, it is likely that inter-trial differences in this ratio contributed to differences in productivity outcomes seen in the current trials.

The two antibiotics produced different results in growth performance and modification of the caecal microbiota of chickens. Compared to avilamycin, which had no influence on any of the performance measures, zinc bacitracin improved FCR (i.e. low FCR values) of chickens. In agreement with these findings, Ao and Choct (2013) showed FCR improvement in bacitracin-fed chickens, compared with control chickens. In contrast to our results, two studies showed that zinc bacitracin (at either equal or lower concentration levels than in this study) fed to chickens did not produce changes in either BWG or FCR relative to control chickens (Geier et al. 2009; Torok et al. 2011a). Another study reported that zinc bacitracin (at 20 ppm) in combination with salinomycin significantly increased BWG but did not influence FCR performance of treated chickens relative to untreated chickens (Engberg et al. 2000). In a similar fashion, our results from trial 2 diverge from previous research showing that either lower (Kim et al. 2011), higher or equal concentrations of avilamycin, relative to those used in our study (Chowdhury et al. 2009; Kim et al. 2012; Pedroso et al. 2006), produced higher BWG values (Kim et al. 2012; Kim et al. 2011; Chowdhury et al. 2009) and/or FCR performance (Kim et al. 2012) in treated chickens than in untreated chickens.

At the caecal microbial community level, avilamycin caused minor changes, as it did not affect the diversity or composition of the microbial community. Our results differ from other studies, where avilamycin produced changes in the composition of the gut microbial community of chickens (Kim et al. 2011; Pedroso et al. 2006). In this study, avilamycin produced significant changes in the abundance of only 6% of all OTUs, reducing the abundance of two taxa, *Catabacteriaceae* and a phylotype closest to *C. spiroforme* (92.84%). The latter taxon is a pathogenic rod-shaped bacterium that employs a binary protein mechanism to inflame the gastrointestinal tract of some animals (Stiles et al. 2014), inducing diarrhoea and colitis in some animals (Borriello and Carman 1983). Possibly, potential poultry pathogens related to *C. spiroforme* were susceptible to the antibiotic effect of avilamycin, which may have reduced their harmful effects on chickens. Also, avilamycin treatment resulted in reductions in the numbers of the relatively unknown *Catabacteriaceae* family. This taxa, which has been previously recorded in polluted water (Codony et al. 2009), has been previously found to be in higher abundance in chickens showing poor growth performance (i.e. high FCR values), compared with chickens showing good growth performance (i.e. low FCR values) (Stanley et al. 2016). Despite the reductions in the latter two taxa, all growth performance measures remained similar between avilamycin-treated and avilamycin-untreated control chickens, suggesting inferior performance of this antibiotic as a growth promoter in comparison to zinc bacitracin, under the conditions used here. Compared with avilamycin, zinc bacitracin had a much stronger impact on the gut microbiota, not only just varying the composition of the microbiota community but also increasing its diversity. Previous studies reported significant influences of bacitracin on gut microbiota composition (Engberg et al. 2000; Geier et al. 2009; Gong et al. 2008; Pedroso et al. 2006), but none of these found bacitracin-induced increases in microbial community diversity. This suggests that, in general, antibiotic treatment affects the composition, but not richness and/or evenness of the gut microbial community.

However, all the above-mentioned studies analysing the effects of these two antibiotics on growth performance and gut microbiota were conducted on chickens of different ages than in our study, and also most of them differed in the duration of the treatments. A number of studies have already indicated that the effect of antibiotics on the microbiota composition and growth performance of chicken is age- and dose-dependent (Gong et al. 2008; Zhou et al. 2007). We would also speculate that differences in response to antibiotics could be influenced by the profile of existing microbiota which can vary significantly (Stanley et al. 2013b). If a microbiota is relatively resistant to an antibiotic supplement, then there is unlikely to be much change in performance whereas it would be expected that a more responsive microbiota may result in greater productivity changes; this is essentially what we have observed here. Clearly, more research is necessary to understand the effects resulting from contrasting concentrations of antibiotics, particularly by looking at the possible interactions between the concentration, the age of the birds, the dietary treatment period and the pre-existing microbiota. Previous research has shown that growth conditions can alter the impact of antibiotics on microbiota composition and performance measures (Pedroso et al. 2006). A recent review highlighted the importance of experimental factors when designing experiments aimed at understanding and interpreting the effects of gut microbiota (Moore and Stanley 2016).

It is necessary to stress that each microbial community can respond to any treatment differently and that previous research showed that gut microbiota in different chicken flocks can differ significantly, even at a phylum level, having low levels of shared OTUs between the flocks (Stanley et al. 2013b). This study showed that zinc bacitracin-induced increase in microbiota diversity was associated with reductions in the abundance of the *Lactobacillus* genus, the most dominant taxa in trial 1. Our results suggest that a lower abundance of the highly dominant genus *Lactobacillus* could have promoted the growth of other less-dominant taxa, increasing the diversity and shifting the composition of the gut microbiota in treated chickens.

Associations between microbiota and performance measures

This study found strong associations between gut microbiota and the growth performance of the birds, reinforcing previous findings (Geier et al. 2009; Stanley et al. 2016; Torok et al. 2008). Particularly, some members of the *Lactobacillus* genus showed associations with FCR values. Similar to our results, it was previously found that antibiotics tend to reduce lactobacilli populations and also have linked these reductions with improved growth performance (Torok et al. 2011a). *Lactobacillus* is a genus associated with the production of the bile salt hydrolase enzyme (BHS) (Begley et al. 2006; Ridlon et al. 2006). Recently, it was suggested that reduced numbers of lactobacilli in antibiotic-treated chickens can reduce BHS activity, increasing the abundance of conjugated bile salts and ultimately leading to increased growth performance (Lin et al. 2013).

In accordance with the latter hypothesis, our results, and those reported by Engberg et al. (2000), found that the supplementation of zinc bacitracin to chickens reduced the abundance of a *Lactobacillus* sp.; in the latter study, reductions of *L. salivarius* were reported. In our study, a large cluster of OTUs assigned to a *L. salivarius* phylotype was reduced by zinc bacitracin. This phylotype was identified with strong positive correlations to FCR values, indicating that reductions of this taxon could have contributed to a better growth performance in zinc bacitracin-treated chickens. It is also possible that reduction of *Lactobacillus* dominance helped increase richness and evenness of the community and contributed to better health and performance via diversity enrichment as previously noted (Blaut and Clavel 2007). Additionally, other species of *Lactobacillus* were also correlated with the growth performance of chickens, yet their numbers were not affected by the antibiotics.

There were contrasting differences in *Lactobacillus* associations with performance measures. On one hand, some *Lactobacillus* phylotypes showed a similar pattern to the *L. salivarius* phylotype, with high abundance linked to poor growth performance. In contrast to these negative effects, our study also found other *Lactobacillus* phylotypes associated with improved growth performance (i.e. low FCR values) in chickens. In agreement with these results, another study found that *L. crispatus*-related OTUs were significantly positively correlated with AME values and that other *Lactobacillus* species were correlated with reduced performance, possibly by increasing bird appetite and feed eaten that were not matched with proportional weight gain (Stanley et al. 2016). In the current study, percentage similarity of OTUs associated with improved or inferior performance to known strains in the database was often not >97%, commonly considered as a species level. However, even complete 16S rRNA gene sequence with high sequence similarity (>97%) does not necessarily allow confident assignment to a species. Instead, more detailed analyses including DNA-DNA hybridisation, phenotyping, sugar fermentation and plasmid profiling, restriction enzyme analysis and pulsed field gel electrophoresis are required to confidently assign species level (Morelli 2013). Our data, and that of many other studies, indicates that the genus *Lactobacillus* and its relatives are not yet fully explored and there are likely to be many other species present in the gut, which affect productivity, which have not been cultured or characterised. This suggests that there are many unexplored strains present in the gut that have real potential for development as productivity enhancing probiotics.

In light of the somewhat opposing results related to the role of *Lactobacillus* in poultry performance, it is important to highlight that some studies have found striking differences in the way that either the same bacterial species or even different strains of the same species can act (Fåk and Bäckhed 2012). Thus, the effect of each of the above-mentioned bacteria on the growth performance of the chicken cannot be taken independently from the rest of the microbial community, as is often done in the literature. Although lactobacilli are generally considered as probiotics, there is currently a discussion on whether they are truly beneficial or not (Binnendijk and Rijkers 2013; Kechagia et al. 2013). Our results support the need for more discussion by indicating that some *Lactobacillus* species might have contrasting consequences for the health of the host expressed as growth performance.

In addition to the lactobacilli, the genus *Bacteroides* was also associated with improved growth (low FCR values) in chickens. *Bacteroides* is considered to have one of the highest hydrolytic activities among all known genera, being recognised as effective degraders of non-digestible carbohydrates such as cellulose and resistant starch (Al-Sheikhly and Al-Saieg 1980) and short-chain fatty acid producers (Collier et al. 2008). Our results are supported by previous studies indicating that some *Bacteroides* species were positively correlated with an improved ability to extract energy in broiler chickens (Stanley et al. 2013a). In general, our study showed striking differences in the way that some taxa can affect

growth performance of chickens. Further research is necessary to investigate, at a community level, the interactions between antibiotic administration and *Lactobacillus* and *Bacteroides* species in order to identify the underlying mechanisms leading to improved growth performance.

Overall, this study found that zinc bacitracin may enhance growth performance and even increase caecal microbiota diversity in some communities associated with reductions in the abundance of dominant species. Additionally, we found that 15 ppm avilamycin did not improve either chicken growth performance or microbial diversity under the conditions used here. Currently, there is considerable debate on whether these antibiotics pose risks to human health (Marshall and Levy 2011; Phillips et al. 2004), and several have been withdrawn from Europe and/or USA (Marshall and Levy 2011). Therefore, there is an increasing demand for alternatives to use within the poultry industry to achieve similar health and productivity outcomes (Janardhana et al. 2009).

A number of alternatives to antibiotics have been proposed. Particularly, probiotics and organic acids have been shown to produce similar beneficial effects than a number of antibiotics in intestinal microbiota and growth performance in chickens (Gunal et al. 2006; Kim et al. 2011; Stanley et al. 2016). By analysing the mechanisms driving the growth-promoting effects of antibiotics, this study identified a number of microbial taxa, mostly lactobacilli, which were positively or negatively associated with growth performance. Further research needs to be directed towards elucidating how these bacteria influence bird productivity. Such research is likely to require a better understanding of the physiology and culturing of what appears to be a large number of novel, previously uncultured, species of *Lactobacillus* that are influencing productivity.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The Animal Ethics Committees of the University of Adelaide (approval no. S-2011-218) and the Department of Primary Industries and Resources, South Australia (approval no. 25/11) approved this study.

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