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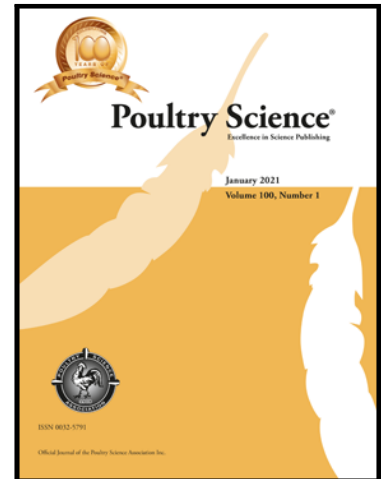
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PERACETIC ACID AS ANTIMICROBIAL ALTERNATIVE

Precursor-derived in-water peracetic acid impacts on broiler performance, gut microbiota and antimicrobial resistance genes

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Conflict of interest: LC, FDM, KF and AF are employees of Gama Healthcare Ltd and Aga Nanotech Ltd, the manufacturers of the precursor formulations used in this study. Gama Healthcare Ltd and Aga Nanotech Ltd provided the PAA precursors used in this work. As co-authors, LC, FDM, KF and AF had influence over the study design and analysis, however they had a smaller role to play in the decision to publish or preparation of the manuscript.

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ABSTRACT

Past antimicrobial misuse has led to the spread of antimicrobial resistance amongst pathogens, reportedly a major public health threat. Attempts to reduce the spread of antimicrobial resistant (AMR) bacteria are in place worldwide, amongst which finding alternatives to antimicrobials has a pivotal role. Such molecules could be used as "green alternatives" to reduce the bacterial load either by targeting specific bacterial groups or more generically, functioning as biocides when delivered *in vivo*. In this study, the effect of in-water peracetic acid as a broad-spectrum antibiotic alternative for broilers was assessed via hydrolysis of precursors sodium percarbonate and tetraacetylethylenediamine. Six equidistant peracetic acid levels were tested from 0 to 50ppm using four pens per treatment and 4 birds per pen (i.e., 16 birds per treatment and 96 in total). Peracetic acid was administered daily from day 7 to 14 of age whilst measuring performance parameters and end-point bacterial concentration (qPCR) in crop, jejunum and caeca, as well as crop 16S sequencing. PAA treatment, especially at 20, 30 and 40 ppm, increased body weight at day 14, and feed intake during PAA exposure compared to control ($P<0.05$). PAA decreased bacterial concentration in the crop only ($P<0.05$), which was correlated to better performance ($P<0.05$). Although no differences in alpha- and beta-diversity were found, it was observed a reduction of *Lactobacillus* ($P<0.05$) and *Flectobacillus* ($P<0.05$) in most treatments compared to control, together with an increased abundance of predicted 4-aminobutanoate degradation (V) pathway. The analysis of the AMR genes did not point towards any systematic differences in gene abundance due to treatment administration. This, together with the rest of our observations could indicate that proximal gut microbiota modulation could result in performance amelioration. Thus, peracetic acid may be a valid antimicrobial alternative that could also positively affect performance.

Key words: Peracetic acid, Microbiota, Antimicrobial Resistance, Antimicrobial Alternative, Broiler

INTRODUCTION

Chicken gut microbiota consists of a multitude of microbial symbionts longitudinally colonizing the gastrointestinal tract, whose interactions with the host affect well-being and performance at

several levels, including nutritional, immunological and physiological (Diaz Carrasco et al., 2019). Host colonization is thought to start soon after hatching, with low parental contribution, and presents both temporal and longitudinal fluctuations, which primarily depend on environmental factors (Stanley et al., 2014). Due to the well documented interactions between microbiota and host, manipulation of the microbial communities through probiotics (Memon et al., 2021), prebiotics (Ricke, 2021) and for several decades also via antimicrobial growth promoters (AGP) (Collignon, 2004; Costa et al., 2017) has been applied to impact broiler production. However, past AGP misuse has led to an increased antimicrobial resistance (AMR) amongst pathogens, characterized as a major health threat for both animals and humans alike (Munita and Arias, 2016).

The issue of AMR associated with poultry production has reached concerning levels (Nhung et al., 2017; Agyare et al., 2019), thus alternative to antibiotics, such as for example probiotics, prebiotics, organic acids and plant extracts have been proposed (Lewith and Jacob, 2005). Other type of alternatives may also exert antimicrobial activity, include enzymes, organic acids, immunostimulants, bacteriocins, bacteriophages, phytogenic feed additives, phytoncides, nanoparticles and essential oils (Mehdi et al., 2018). Peracetic acid (PAA) is an emerging biocide widely used in contexts beyond poultry production, e.g., wastewater treatment (Zhang et al., 2020) or poultry processing with proven inhibitory effect on pathogens such as *Campylobacter* (Micciche et al., 2019). Here, we propose and test the possible *in vivo* effect of PAA in broiler birds administered through water, on gastrointestinal microbial communities, main AMR gene relative abundance and performance. The generic antimicrobial activity of PAA action We tested the broad-spectrum antimicrobial activity of different PAA levels of inclusion at the end of the starter phase, for seven days, on young birds during a 14-day trial without interfering with the normal microbiota colonization dynamics through the first week, hence the choice of administering PAA only from day 7 to day 14. We observed positive effects on performance, correlated to a reduction of bacterial concentration in the crop and specifically to a significant reduction of *Firmicutes* at phylum level and

Lactobacillus at genus level. Our findings indicate both the possible use of in-water PAA as an antimicrobial alternative and the importance of the upper gut microbiota in broiler performance in young broiler birds.

MATERIALS AND METHODS

Animal Study

The animal study was carried out at the Allermuir Avian Innovation and Skills Centre (AISC), SRUC. Study design and protocol were approved by SRUC Animal Welfare and Review Body (POU AE 20-2019). A total of 96 day-old Ross 308 male broilers were placed in 24 pens within two rooms, with 4 birds per pen and 4 pens per treatment (stocking density at day 14: ~1.9Kg/m^2) , with a total of six treatments (Table *1*) between day 7 and day 14 of age, which was the last day of the trial. Chickens were fed a standard commercial wheat, soybean meal based diet formulated to have 20.8% crude protein, 1.21% d-lysine and 12.8% apparent metabolizable energy. Feed was offered as starter diet (mash, *ad libitum*) throughout the study designed to meet standard nutrient requirements (crude protein: ~23%, 2800 Kcal metabolizable energy/Kg). At day 0 all the birds were wing-tagged and allocated to one of the 24 pens following a randomised complete block design, where treatments were also randomly allocated within each of four blocks. Number of birds and replicate was based on a number of previous dose-response studies with a similar design, in which 4 replicates and a set of orthogonal contrast statements were used to assess linear and quadratic effects of equidistant treatment levels (Smith et al., 2013).

Treatment Preparation

Peracetic acid (PAA) was produced in water by hydrolysis of precursors sodium percarbonate (SP) and tetraacetylethylenediamine (TAED) (AGA Nanotech, Hemel Hempstead, UK). In addition, disodium ethylenediaminetetraacetic acid (EDTA) was used as a stabilizer to prevent PAA degradation and citric acid was added to counterbalance the effect of SP and TAED on pH (Table *1*). Water treatments were freshly prepared and administered to the chicken daily. The control at 0ppm

of PAA was prepared by addition of EDTA only as vehicle control. The precise precursor ratio needed to obtain the different PAA levels of inclusion (Table 1) were established *in vitro* prior to the animal study via measuring the PAA concentrations at each level using the using the free and total chlorine AccuVac (HACH, Loveland, Colorado, United States) method as described below. Permachem N,N-diethyl-p-phenylenediamine total Chlorine reagent (HACH, product number 2105628) was added to 10mL of TAED, SP, EDTA and citric acid solution. Therefore, the absorbance at 530nm was read via using the HACH spectrophotometer (DR6000 UV-VIS, HACH, Loveland, USA). Thus, PAA concentration was calculated from Chlorine values via multiplying Cl_2 output of the spectrophotometer reads by 1.07 (i.e., scaling factor between chlorine and PAA concentration) and the dilution factor.

Performance Analysis

Individual body weight (BW), feed issued, and feed refusals were measured at day 0, day 7, day 10 and day 14 to allow measurement of bird-level BW, bird-level body weight gain (BWG), pen-level feed intake (FI) and pen-level feed conversion ratio (FCR). Mortality correction for the latter was not required as there was no mortality before or during the experimental phases.

Sampling

At day 14, all the birds were humanely culled via cervical dislocation, and content from crop, jejunum, caeca and colon were pooled by pen and gut segment. pH was measured in the colon content (1111105 2-star benchtop pH meter, Thermo Scientific, Waltham, USA) whilst the content of the other segments was snap frozen at ~-78°C (dry ice) before being transferred to an ultra-low-temperature freezer (-80°C) pending further analysis.

DNA Isolation

Approximately 0.25g of gut content were transferred in the PowerBead tubes of the DNeasy PowerSoil Kit whilst being mixed with 60μL of solution C1 of the same kit (Part no. 12888-100, QIAGEN, Hilden, Germany). The tubes were placed in a FastPrep-24TM 5G homogenizer

(116005500, MP Biomedicals, Irvine, USA) for 55 seconds at 5.5m/s. Afterwards, QIAGEN 12888-100 manufacturer instructions were followed to isolate total DNA, which was immediately stored at -80ºC until further analysis.

Bacterial Absolute Quantification

Standard Curve Preparation.

Standard curve for absolute qPCR quantification was built through nine ten-fold serial dilutions of linear plasmid (Hou et al., 2010) containing qPCR target as insert. The latter was amplified from the isolated DNA through PCR reaction and separated via 1.5% agarose gel after electrophoresis at 100V for 80 minutes. The 25μL-reaction mix included 1X of KAPA Taq ReadyMix with dye (Kapa Biosystems), 0.2μM of each primer (Table 2) and nuclease-free water. PCR conditions were 95 °C for 3 min, 35 cycles including 95 °C, 60ºC both for 30 seconds and 72ºC for 1 min, followed by a final elongation at 72°C for 10 min.

The amplicons were excised from gel, purified following the protocol from Wizard® SV Gel and PCR Clean-Up System (Promega) and cloned into a pCR2.1 plasmid vector (TA CloningTM Kit, Thermo Fisher Scientific), prior to transformation of ligase reaction into chemically competent One shot® INVαF' *E. coli* cells (Thermo Fisher Scientific) by heat shock.

The plasmid was isolated from liquid Luria-Bertani cultures inoculated with positive-X-gal-transformed colonies through the QIAprep Miniprep kit as per manufacturer instructions (27104, QIAGEN, Hilden, Germany). Insert presence was verified both by EcoRI (R3101S, New England BioLabs, Ipswich, USA) restriction enzyme digestion and by Sanger sequencing (DNA Sequencing and Services, Medical Sciences Institute, School of Life Sciences, University of Dundee). Finally, plasmids were linearized using 5 units of HindIII (R3104S, New England BioLabs, Ipswich, USA) and 1X of CutSmart® buffer (B7204, New England BioLabs, Ipswich, USA) in 50μL total volume.

Linear plasmid-copy number (CN) concentration was calculated from NanoDrop spectrophotometer ng/µL reads (TM 1000, Thermo Fisher Scientific, Waltham, USA) and further used through the qPCR reactions.

Absolute qPCR Quantification.

Absolute qPCR quantification was carried to quantify total number of bacteria by targeting the V3 region of the 16S rRNA gene (Table 2). All reactions were carried out in 20µL containing 1X of Takyon qPCR MasterMix with Low Rox (UF-LSMT-B0701, Eurogentec, Seraing, Belgium), 50nM of each primer (Table 2), 10ng of DNA template and nuclease-free water (129114, QIAGEN, Hilden, Germany). Cycling conditions (Mx3000P thermocycler, Agilent Technologies, Santa Clara, USA) were 95°C for 3 min followed by 40 cycles at 95°C for 5 s and 65°C for 35 s, at the end of which fluorescence was detected. Qualitative template control was performed through melting curve analysis.

All the reactions were run in triplicate. Excellent reaction efficiency metrics were detected throughout the analysis, based on R^2, slope and efficiency of the standard curve, whose average values were calculated as ~0.99, ~-3.3 and ~100%, respectively.

qPCR Data Analysis.

Copy number (CN) per reaction for each sample was calculated based on the linear regression model fitted with standard curve fluorescence and cycle threshold (Stratagene Mx3000P software, Agilent technologies).

Therefore, CN per reaction was first converted into bacterial cells per reaction (BC_r) by normalizing CN to 5.2 average copy number of 16S gene per bacterial cell at the time of writing (Stoddard et al., 2015). Finally, BC per gram of sample was calculated using equation (1) below (Singh et al., 2014).

$$\frac{BC_r \cdot C \cdot D}{S \cdot V}. \quad (1)$$

Where, C and D were concentration and dilution volume of the extracted DNA, respectively, whilst S was the amount of DNA subjected to qPCR and V was the amount of sample used to isolate DNA (Singh et al., 2014).

Antimicrobial Resistance Gene Analysis

Table 2 depicts primers and annealing conditions applied through the relative qPCR quantification of the six AMR poultry-relevant genes selected. Each reaction (20µl) was run in triplicate and included 1X Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (600882, Agilent technologies, Santa Clara, USA), 1ng of crop content gDNA template and nuclease-free water (129114, QIAGEN, Hilden, Germany), 250nM of each primer for *tetW, vanC, aadA* and 350nM for *vanA, tetQ* and *mecA*. Each array included a non-template control and the samples from the control group to eliminate inter-run bias whilst melting curve analysis assessed reaction quality. Amplification conditions (Mx3000P thermocycler, Agilent Technologies, Santa Clara, USA) were 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 20 s annealing as per Table 2.

Fold-change relative abundance (i.e., $2^{-\Delta\Delta Ct}$) as per protocol used by other authors (Walsh et al., 2011; Juricova et al., 2021) was calculated as the Ct difference between the AMR genes and the 16S rRNA as a normalizer (ΔCt) between treatments and controls (ΔΔCt).

16S rRNA Gene Sequencing

Library Preparation.

16S rRNA gene sequencing on crop-content gDNA was carried out by Omega Bioservices (Norcross, USA) targeting the V4 region of the bacterial 16S rRNA gene (F515$_b$ (Parada et al., 2016): 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3'; R806$_b$ (Apprill et al., 2015): 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAA-3').

Amplicon PCR (total volume of 25µL) components (final concentration) were, 12.5 ng of template DNA,1x KAPA HiFi HotStart ReadyMix (KK3604, Kapa Biosystems, Wilmington, USA) and

0.2μM of each primer, whereas amplification conditions were 95°C for 3 min (initial denaturation) followed by 25 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 30 s), and a final elongation of 5 min at 72°C. PCR product clean-up was carried out using Mag-Bind RxnPure Plus magnetic beads (M1378-01, Omega Bio-tek, Norcross, USA). A second index PCR amplification, used to incorporate barcodes and sequencing adapters was performed maintaining the component concentrations as described above. Cycling conditions were 95°C for 3 min, followed by 8 cycles of 95°C, 55°C and 72°C, each held for 30 s, thus a final 5-minute elongation step at 72°C. Finally, the libraries ~600 bases in size were checked using a 2200 TapeStation (5067, Agilent technologies, Santa Clara, USA) and quantified using QuantiFluor dsDNA System (E2671, Promega, Madison, USA) before normalization, pooling and sequencing (2 x 300bp paired end read setting) on the MiSeq (SY-410-1003, Illumina, San Diego, USA).

Bioinformatic Analysis

A total of 5,480,595 FASTQ paired end demultiplexed reads (~ 230,000x2 reads/sample) were imported and analyzed in QIIME2 v2022.2 (Bolyen et al., 2019), through which ~105,000 reads/sample were retained after being joined via VSEARCH (Rognes et al., 2016), with quality score of ~40 throughout the sequence length both before and after quality-filtering with minimum Phred score of 20 (McKinney, 2010; Bokulich et al., 2013). Therefore, Deblur was used to denoise, with sequence trimming set at 290bp (Amir et al., 2017) and taxonomy was assigned using the q2-feature-classifier plugin via applying a Naïve Bayes classifier, trained based on the $F515_b/R806_b$ primers and the last release of the Silva data base (138, 99% of similarities) (Pruesse et al., 2007; Pedregosa et al., 2011; Bokulich et al., 2018). Diversity analysis was carried out on even sequence depth of 3,790, retaining 90,960 (48.61%) features in 24 (100%) samples, allowing calculation of α-diversity through richness and Shannon's diversity index (Anderson, 2001; Kim et al., 2017), testing for significance through the Kruskal-Wallis test (Kruskal and Wallis, 1952; Benjamini and Hochberg, 1995). Moreover, β-diversity was measured through the Bray-Curtis dissimilarities and

the Jaccard similarity index (Jaccard, 1908; Bray and Curtis, 1957), followed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001). Functional gene prediction based on 16S data was carried out via using PICRUSt2 (Langille et al., 2013). Differential abundance analysis was carried out via Microbiome Multivariable Association with Linear Models 2 (MaAsLin2, Mallick et al., 2021) in R v4.1.2 (R Core Team, 2021). This included the use of a negative binomial distribution (NEGBIN, Zhang et al., 2017) for differential microbial abundance analysis on cumulative sum scaling (CSS) normalized taxonomical data in order to reduce the bias introduced by differences in sampling depth (Pereira et al., 2018). NEGBIN was also used on trimmed mean of M values (TMM) for metabolic pathways prediction output of PICRUSt2 to account for the homological content of transcriptomic data (Robinson and Oshlack, 2010).

The package qiime2R (Bisanz, 2018) was used to graphically represent QIIME2 outputs produced through the analysis.

Statistical Analysis

Linear mixed model (LMM) was carried out to assess whether treatments had a significant impact on the variables analyzed, fit with linear and quadratic treatment terms allowing the assessment of eventual dose response effect. All the analyses were carried out in R (R Core Team, 2021), were the LMM was fitted using "*lmer*" function from the *lme4* package (Bates et al., 2015), thus "*lmerTest*" was applied to calculate the *p* value for the *t* tests output of *lme4* through using Satterthwaite's method (Kuznetsova et al., 2017). In addition, both linear and quadratic terms were incorporated with a contrast analysis within "*lmer*" allowing the identification of a possible optimum level. Metabolic pathways predicted via PICRUSt2 pipeline were analyzed through alpha- and beta-diversity via the Vegan package in R and differential abundance analysis was carried out through NEGBIN on TMM normalized data via Maaslin2.

Treatment and time, for longitudinal data, were both input as fixed effects in LMM, whereas the hierarchy of "Room/Block/Pen/Bird" represented the random effects, the model was fitted with

random intercept. Finally, Tukey corrected post hoc comparison for significant regressions or interactions, was carried out through using the function "*emmeans*" (Lenth, 2021). Longitudinal BW was Log_{10} transformed prior to fitting the model.

RESULTS

Performance and Colonic pH

Results from the analysis of the different performance parameters and colonic pH are summarized in Table 3. As expected, there was a significant effect of time to the cumulative BW at bird-level ($F_{(2,180)}=8097.01$, $P<0.01$). At day 14, birds on the 20, 30 and 40 ppm PAA treatments were 10.6, 10.0 and 12.5% heavier ($P<0.05$) than the control birds. This effect was less pronounced for birds allocated to the 10 and 50 ppm PAA treatments, which were 7.3% ($P=0.14$) and 6.1% ($P=0.21$) heavier than the control birds, respectively). This concurred with a significant quadratic effect of PAA inclusion on both longitudinal BW ($F_{(2,92)}=4.5$, $P=0.01$) and day 14 BW ($F_{(2,92)}=4.10$, $P=0.02$).

Pen level FI analysis revealed increased FI from day 7 to day 14 ($F_{(5,17)}=2.17$, $P=0.107$), with a clear positive effect birds on the 20, 30 and 40 ppm PAA treatments, recording 0.15, 0.13 and 0.13 Kg more, respectively compared to the 1.37 kg for the control birds ($P<0.05$). Pen level FCR did not differ between PAA treatments, whilst colonic pH did not change amongst the experimental conditions.

Total Bacterial Quantification and Correlation with Performance

As expected, bacterial concentration differed between crop, jejunum and caecal content ($F_{(2,35.9)}=133.57$, $P<0.01$, Figure 1). However, PAA treatment impacted on bacterial abundance in the crop only. Bacterial concentration (mean $log_{10}\pm SD$) was reduced at PAA levels of 10ppm (8.56 ± 1.02; $P<0.05$), 20 ppm (8.89 ± 1.00; $P=0.103$), 40 ppm (8.87 ± 1.04; $P=0.09$) and 50ppm (8.61 ± 0.99; $P<0.05$) compared to the control (9.70 ± 0.08). Bacterial concentration in the jejunum and caecal content did not differ between PAA treatments and averaged 6.59 ± 0.87 and 9.78 ± 0.26,

respectively, though caecal bacterial concentration appeared greatest for the 40ppm and 50ppm treatments.

To explore possible relationships between bacterial load and performance, BW at day 14 was fitted in an additional LMM as a depended variable and crop bacterial concentration as a fixed effect, again with room/block representing the hierarchy of random effects, this revealed an inverse relationship between the two variables (F(1,22)=8.47, P<0.01, Figure 2), with better performance (greater BW at d14) for birds with lower crop bacterial concentration. Indeed, the model demonstrated that high bacterial concentrations as observed in the control birds concurred with reduced performance at endpoint, compared to birds on PAA treatments, which was associated with greater final BW and lower crop bacterial abundance.

Crop Antimicrobial resistance gene analysis

Six AMR genes were analyzed in total by relative quantification using the ΔΔCt method using the 16S rRNA gene as a normalizer and read-outs for the control birds as baseline (Table 4). Whilst there were no significant linear regressions between PAA level and any AMR gene relative abundance, a quadratic relationship was observed between PAA and tetQ relative abundance (F(2,21)=4.05, P=0.03), arising from a decrease associated with 10, 20 and 30 ppm PAA compared to the control, followed by an increased gene abundance at 40 and 50 ppm PAA. In addition, a tentative quadratic relationship was observed between PAA and mecA (F(2,18)=2.72, P=0.09), arising from an increased gene level at 20, 30 and 40 ppm PAA compared to control and 50 ppm PAA. Furthermore, although AMR relative abundance was associated with rather large variance throughout, compared to the control, relative abundance was greater for aadA at 50 ppm PAA, vanC at 40 ppm PAA and mecA at 20 ppm PAA (all P<0.05) and mecA at 40 ppm PAA at P=0.09.

Crop 16S rRNA Gene Sequencing

Both alpha- and beta-diversity of the crop microbial community, as measured through the richness index, Shannon index and Bray-Curtis dissimilarity index and the Jaccard distance index,

respectively, did not differ between PAA levels. Although especially richness appeared to decrease in a rather similar fashion in response to PAA level (Figure 3) when compared to the reduction in total bacterial concentration (qPCR), there were no linear (P=0.78) or quadratic (P=0.36) relationships observed.

The most abundant phylum across the treatments was Firmicutes (~50%), followed by Proteobacteria (~25%) and Cyanobacteria (~20%), less dominant phyla were Actinobacteriota and Bacteroidota representing only ~0.5% of the total population in all the samples (Table 5). Firmicutes decreased for all PAA levels compared to control (0ppm), though most pronounced for 10 ppm PAA (P<0.05) and 40 ppm PAA (P=0.10). Similarly, albeit Cyanobacteria and Proteobacteria reads were smaller for all PAA levels compared to the control, this was significant for the 10 ppm group only (P<0.01). The 10 most predominant genera (Table 6) were *Lactobacillus* accounting for almost 50% of the reads followed by Cyanobacteria (unassigned) and *Rickettsiales* (unassigned), each covering ~20% of the total community, *Acinetobacter* (~1.5%) and *Erwiniaceae* (unassigned), *Pseudomonas, Escherichia-Shigella*, *Ruminococcus* (*torques* group), unassigned Enterobacteriaceae and Enterobacterales all less than 1% of the total. *Lactobacillus* normalized reads were reduced for all PAA levels tested compared to the control (P<0.05) apart from 50ppm. *Flectobacillus* was also reduced for all PAA levels tested, though significantly so for the 20 ppm PAA group (P<0.01). In contrast, whilst *Enterococcus* normalized reads were decreased for most PAA levels compared to the control (P<0.05), they were increased for the 20 ppm PAA level (P<0.05). Although the less dominant *Ruminococcus* (*torques* group) was found to be decreased for all PAA levels compared to the control (P<0.05) apart from 40ppm, *Subdoligranulum* reads were decreased for both 10 and 50 ppm PAA (P<0.05), though noticeably increased at 40 ppm PAA (P<0.05).

Figure 4 shows the NEGBIN calculated differential abundance of TMM normalized predicted pathway abundance for the different PAA levels tested. Diversity analysis did not reveal any statistically significant difference between the treatments apart from a tendency of 20 ppm PAA to

an increased Shannon index (P=0.05), pointing towards both qualitative and quantitative similarities of predicted metabolic pathways. The aminobutanoate degradation (V) pathway was noticeably less abundant in both control and for 10 ppm PAA compared to the rest of the treatments (P<0.05), whereas albeit the pathway of formaldehyde assimilation II (RuMP Cycle) was less abundant in almost all the treatments, significant reduction was observed only for 10 ppm PAA compared to control (P<0.01). Similarly, (aerobic) toluene degradation IV (via catechol) pathway was reduced in all treatments compared to control, although significant only for 50 ppm PAA (P<0.01).

DISCUSSION

Antimicrobial resistance poses a serious threat to animal and human health, mainly emphasized by a reduced treatment effectiveness towards bacterial infections (World Health Organization, 2014). Finding alternative molecules to antimicrobials is amongst the strategies to decrease the AMR spread rate (Ghosh et al., 2019) through environment (Furtula et al., 2013) and livestock (Thanner et al., 2016). Here, we have assessed for the first time the potential antimicrobial alternative PAA for its effect on broiler performance, bacterial communities, and gut pH.

In particular, we tested six different levels of inclusion of PAA prepared fresh and administered daily from day 7 to day 14 of a two-week trial. The absence of observable side effects and mortality during the treatment week pointed towards the safety of this therapeutical approach, whilst we recorded an increased BW at day 14 in all treatment levels, though with less noticeable effects for the 10 and 50 ppm groups with some effects already visible at day 10. In general, average day14-BW through the six experimental conditions, was 466.67g, thus 11.39% (60.3g) lower than the target at day 14 (527g) for the breed, whereas the average FI per bird throughout the experimental conditions between day 7 and day 14 was 367g, which was 5.66% lower than FI/bird from day 7 to day 14, according to performance objectives for the breed (389g).

Moreover, we found that incorporating the quadratic term in the mixed model was associated with a significant effect on both longitudinal BW and observations at day 14, indicating a possible

dose-response effect. Therefore, the analysis of the two model components possibly pointed towards a range of optimum levels of inclusion for PAA in-water administration, with an optimal response at 20ppm. This view was also supported by the observation that FI was most pronouncedly increased for 20, 30 and 40 ppm PAA.

The efficacy of the PAA treatment as a potential broad-spectrum antimicrobial alternative was confirmed by the observed reductions in crop bacterial concentration (Kitis, 2004), which reached a biologically relevant ~1 Log_{10} reduction (Mayr et al., 2010) as PAA concentration increased, and without evident changes throughout the other gut locations analyzed.

Whilst overall bacterial concentration was as per expectation throughout crop, jejunum, and caeca (Shang et al., 2018), these findings are consistent with the theoretical fast rate of PAA formation from its precursors, and its subsequent hydrolysis. Indeed, PAA reacts with water to form acetic acid and hydrogen peroxide (Zhao et al., 2007), thus it is assumable that without further encapsulating the precursors for further distal gut delivery (Chourasia and Jain, 2003), the likelihood of formation of active molecules is expected to be greater in the proximal gut. This concept represents the basis for further studies exploring alternative delivery methods of encapsulated PAA precursors to assess potential effect in the distal gut. Such proximal effect of PAA could also explain why colonic pH did not change, as PAA likely never reached the hind gut to modulate bacterial fermentation. However, it is worth to notice that colonic pH was found to be lower than the expected reported range of 7.0-8.0 (Ravindran, 2013; Skoufos et al., 2016). In addition, water acidification may improve performance, likely mediated through a reduction in pH of the gastrointestinal tract content (Hamid et al., 2018). The administration of PAA via precursors hydrolysis as presented here is not comparable with such approaches, as it does not cause variation of the gastrointestinal pH, which remains close to physiological levels.

Interestingly, it was noticed a monotonic inverse relationship between crop bacterial concentration and BW, with higher performance associated with lower bacterial load in the upper

gut. Numerous studies have explored the potential relationship between especially the ileal and caecal microbiota and performance (Tiihonen et al., 2010; Torok et al., 2011; Ovi et al., 2021), whilst the number of studies that evaluate the role of the upper gut microbiota (Rinttilä and Apajalahti, 2013) or specifically of the crop microbiota (Ren et al., 2019) is poorly represented. For example, *Lactobacillus* is a primary symbiont in the crop (Yadav and Jha, 2019), whose decreased abundance has been correlated to a decreased intestinal activity of bile salt hydrolase produced by this genus, therefore possibly leading to an increased host-lipid digestion and energy harvest (Chand et al., 2017), which in turn could potentially promote host lipid metabolism, energy harvesting and increased weight gain (Lin et al., 2013). This could likely explain the correlation between higher live weight and decreased *Lactobacillus* CSS normalized reads at increased PAA concentrations that was observed. On the other hand, *Lactobacillus* is traditionally recognized as a beneficial, probiotic strain for its action in the distal intestine, promoting performance, inhibiting pathogen growth by competition, and providing organic nutrients to the rest of the bacterial community (Ehrmann et al., 2002; Kabir, 2009; Pokorná et al., 2019; Sinha et al., 2020). Albeit *Lactobacillus* use as a probiotic has been validated by numerous studies focusing on the lower intestine, evidence provided here suggest that its reduction in the proximal gut could lead to better host-driven lipid digestion. Indeed, our findings not only indicate that microbiota modulation in the proximal tract is strictly correlated to performance amelioration but also suggest that the gastrointestinal section targeted should be taken into consideration when modulating specific genera whilst designing novel probiotic strategies.

We did not find any difference in crop microbial alpha- and beta-diversity due to treatment, which might indicate that the changes that we reported in terms of single phyla or genera did not affect the entropy of the general bacterial communities, whose variations where more quantitative than qualitative as indicated by the differences in bacterial concentration (qPCR) and marginally by the calculated OTU richness (16S sequencing).

We found that *Cyanobacteria* level was lower in treated birds than control, especially in the 10ppm PAA group. The role of cyanobacteria within the gastrointestinal tract is not fully elucidated, although this phylum seems to be connected to a series of developmental and metabolic host functions (di Rienzi et al., 2013; Hu and Rzymski, 2022). Nevertheless, it cannot be ignored that at sequencing level, reads from this phylum could potentially also include chloroplast from indigested plant material (Willson et al., 2018).

We observed a drastic reduction of *Flectobacillus* for all PAA levels. This genus is known to contribute to mucosal immunity and homeostasis in other host species, especially IgM mediated by producing sphingolipids (Sepahi et al., 2016; Jin Song et al., 2019). This does not seem to accord with increased performance observed, although this apparent contrast could have arisen from its low relative abundance, supported by absence of effects on diversity parameters, and therefore by its marginal role towards host interactions. It could also point towards a possible marginal sphingolipid role within the proximal gut, as opposite to the established positive roles known through the distal intestine (Vesper et al., 1999).

Enterococcus faecium has been described as a beneficial probiotic in Ross308 broilers, capable of improving performance whilst retaining carcass quality features (Gheisar et al., 2016), which could agree with our finding for *Enterococcus* genus CSS normalized reads increased in the 20ppm PAA group. However, since its abundance for the other PAA levels remained lower than the levels observed in the control, such role at crop level might not be biologically relevant. Care should be taken when considering this strain as a probiotic in relation to broiler performance due to its associated vancomycin resistance (Cetinkaya et al., 2000; Ahmed and Baptiste, 2018). However, in our data, only a slight fold change increase was found for vanC in the 50 ppm PAA group, also associated to a high degree of variation.

Finally, we observed that the abundance of the 4-aminobutanoate degradation (V) predicted pathway was increased through all treatments ≥20ppm, which has been correlated to increased

insulin secretion in humans (Sanna et al., 2019) and could therefore be associated to an increased level of host glucose metabolism. From the six AMR genes analyzed, aadA, vanC and mecA recorded sporadic higher relative abundance in some of the treatments, although the level of PAA at which this was observed was rather inconsistent. Whilst it could be argued that therefore a reduction in AMR relative abundance triggered by PAA cannot be excluded, these observations were associated with relatively large degree of variation. Moreover, the absence of linearity between PAA level and AMR-DNA relative abundance may suggest these may be more likely chance findings rather than a systematic response to PAA intervention. However, the significance or tendency observed for the quadratic regression for tetQ and mecA, respectively, could point towards a selection pressure at some of the levels of inclusions and the need to optimize PAA administration concentration accordingly.

In conclusion, this first study strongly supports the view of the role of PAA as a possible broad-spectrum antimicrobial alternative, when administered in water for a week to young birds up to the age of 14 days. Our results also suggest that the modulation of the upper gut (i.e., crop) microbiota of young birds could contribute to changes in the host capability to metabolize specific nutrients, such as lipids and glucose, possibly leading to ameliorated performance in young birds. Therefore, our study not only suggests that the microbiota inhabiting the proximal intestine should be considered as a target for host-interaction modulation but also indicates that the established antimicrobial action of PAA could be applied *in vivo* to young chickens.

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CONFLICT OF INTEREST

LC, FDM, KF and AF are employees of Gama Healthcare Ltd and Aga Nanotech Ltd, the manufacturers of the precursor formulations used in this study. Gama Healthcare Ltd and Aga Nanotech Ltd provided the PAA precursors used in this work. As co-authors, LC, FDM, KF and AF had influence over the study design and analysis, however they had a smaller role to play in the decision to publish or preparation of the manuscript.

The authors declared that no competing interests exist.

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Table 1. Water treatments from day 7 to day 14 of age. The different PAA levels of inclusion were obtained by mixing different concentrations of SP and TAED and by adding EDTA and citric acid as stabilizers. Control at 0 ppm was obtained by adding EDTA only

Treatment	PAA level of inclusion	TAED (g/l)	SP (g/l)	NaEDTA (g/l)	Citric acid (g/l)
1	0 ppm	0	0	0.05	0
2	10 ppm	0.035	0.065	0.05	0.04
3	20 ppm	0.055	0.095	0.05	0.05
4	30 ppm	0.07	0.13	0.05	0.07
5	40 ppm	0.09	0.17	0.05	0.09
6	50 ppm	0.10	0.20	0.05	0.1

PAA: peracetic acid; TAED: tetraacetylethylenediamine; SP: sodium percarbonate; NaEDTA:

ethylenedinitrilotetraacetic acid disodium salt.

Table 2. List of primers used in this study to amplify the AMR genes and the V3 region of the 16S rRNA gene

Resistance/target (class, gene)	Primers (5' → 3')	Annealing	Amplicon	Reference
Streptomycin, spectinomycin (Aminoglycoside, *aadA*)	Fw: GCAGCGCAATGACATTCTTG Rev: ATCCTTCGGCGCGATTTTG	60 °C	282 bp	(Esperón et al., 2018)
Vancomycin (glycopeptide, *vanA*)	Fw: GCCGGAAAAAGGCTCTGAA Rev: TTTTTTGCCGTTTCCTGTATCC	60 °C	90 bp	(He et al., 2020)

Vancomycin (glycopeptide, *vanC*)	Fw: CTTATGTTGGTTGCCATGTCG Rev: CGATTGTGGCAGGATCGTT	60 °C	138 bp	(Flipse et al., 2019)
Tetracycline (*tetW*)	Fw: AGCGACAGCGTGAGGTTAAA Rev: AAGTTGCGTAAGAGCGTCCA	60 °C	153 bp	(Juricova et al., 2021)
Tetracycline (*tetQ*)	Fw: AGAATCTGCTGTTTGCCAGTG Rev: CGGAGTGTCAATGATATTGCA	63 °C	167 bp	(Aminov et al., 2001)
Methicillin, penicillin (β-lactam, *mecA*)	Fw: AACCACCCAATTTGTCTGCC Rev: TGATGGTATGCAACAAGTCGTAAA	60 °C	135 bp	(Kelley et al., 2013)
V3 region 16S rDNA	341F: CCTACGGGAGGCAGCAG 518R: ATTACCGCGGCTGCTGG	60 °C	192 bp	(Muyzer et al., 1993)

Table 3. Performance and colonic pH data at different intervals through the treatment week. Means ± standard deviations are shown for each treatment according to the detailed number of observations

	Average body weight/period (Kg, day 14)	Average feed intake/bird/period (Kg, days 7-14)	Average FCR/bird/period (days 7-14)	Colonic pH (day 14)
0 ppm	0.432 ± 0.065[A] (n=16)	1.37±0.1[a] (n=4)	1.23±0.06 (n=4)	6.94±0.55 (n=4)
10 ppm	0.464 ± 0.067 (n=16)	1.45±0.08 (n=4)	1.24±0.08 (n=4)	7.32±0.58 (n=4)
20 ppm	0.478 ± 0.061[B] (n=16)	1.53±0.05[b] (n=4)	1.24±0.01 (n=4)	6.94±0.40 (n=4)
30 ppm	0.475 ± 0.056[B] (n=16)	1.51±0.11[b] (n=4)	1.30±0.14 (n=4)	6.88±0.88 (n=4)
40 ppm	0.486 ± 0.049[B] (n=16)	1.51±0.04[b] (n=4)	1.26±0.04 (n=4)	7.17±0.24 (n=4)
50 ppm	0.459 ± 0.066 (n=16)	1.43±0.13 (n=4)	1.25±0.05 (n=4)	7.34±0.43 (n=4)

Different superscripts in the same row indicate statistically significant differences (P<0.05 and

P<0.10 if upper or lowercase, respectively) output of the linear mixed model and Tukey corrected

post hoc comparison of the type III LMM analysis of variance with Satterthwaite's method.

Table 4. Mean fold change relative abundance (±standard deviation) of the six AMR genes analyzed through relative qPCR with the $2^{-\Delta\Delta Ct}$ method, using the 16S rRNA gene as normalizer and the 0ppm control for comparison

PAA	*aadA*	P value	*tetQ*	P value	*vanC*	P value
0ppm	1.36 ± 0.93[A]		1.74 ± 1.48		0.75 ± 0.22[A]	

10ppm	4.6 ± 4.88	0.12	0.12 ± 0.03	0.43	0.72 ± 0.1	0.99
20ppm	3.86 ± 2.73	0.23	0.35 ± 0.21	0.50	0.86 ± 0.07	0.95
30ppm	3.21 ± 2.96	0.37	0.32 ± 0.35	0.49	1.36 ± 1.36	0.76
40ppm	2.76 ± 2.81	0.50	2.04 ± 1.94	0.89	5.15 ± 5.57^B	0.03
50ppm	8.88 ± 9.04^B	<0.01	2.99 ± 3.84	0.54	1.48 ± 0.97	0.72
PAA	*tetW*	*P value*	*vanA*	*P value*	*mecA*	*P value*
0ppm	2.19 ± 2.74		1.54 ± 1.2		1.13 ± 0.58^{A,a}	
10ppm	1.89 ± 1.03	0.43	0.48 ± 0.21	0.61	1.29 ± 0.44	0.94
20ppm	1.82 ± 0.92	0.50	0.22 ± 0.12	0.52	5.4 ± 4.72^B	0.04
30ppm	2.45 ± 1.59	0.49	2.42 ± 3.72	0.67	3.45 ± 4.82	0.26
40ppm	4.67 ± 4.55	0.89	2.48 ± 2.9	0.65	4.64 ± 3.26^b	0.09
50ppm	1.64 ± 1.99	0.54	0.75 ± 0.57	0.70	0.93 ± 0.55	0.92

Different superscripts in the same column indicate statistically significant differences ($P<0.05$ and $P<0.10$ if upper or lowercase, respectively) based on the linear mixed model and Tukey corrected post hoc comparison of the type III LMM analysis of variance with Satterthwaite's method.

Table 5. Taxonomical composition at phylum level through the six different levels of inclusion of PAA administered through the study. The table depicts the calculated average of the relative abundance for each phylum at each treatment level ±SD calculated amongst the replicates

	0ppm (n=4)	10ppm (n=4)	20ppm (n=4)	30ppm (n=4)	40ppm (n=4)	50ppm (n=4)
Firmicutes	57.7 ± 10.1^{A,a}	58.9 ± 6.9^B	47.1 ± 7.5	62.2 ± 7.7	51.7 ± 11.9^b	55.6 ± 8.4
Cyanobacteria	18.4 ± 5.5	17.2 ± 6.7	21.7 ± 5.8	15.6 ± 5.1	19.3 ± 6.5	13.8 ± 3.9
Proteobacteria	23.5 ± 10.3^A	23.5 ± 10.2^B	31 ± 8.3	21.9 ± 3.1	28.7 ± 6.3	30.5 ± 5.2
Actinobacteriota	0.06 ± 0.06^A	0.01 ± 0.03^B	0.04 ± 0.05^B	0.02 ± 0.02^B	0.05 ± 0.07^B	0.02 ± 0.03^B
Bacteroidota	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0	0.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
Unassigned	0.01 ± 0.01^A	0 ± 0^B	0.01 ± 0.02^B	0.03 ± 0.02^B	0 ± 0.01^B	0.02 ± 0.01^B

Different superscripts in the same row indicate statistically significant differences ($P<0.05$ and $P<0.10$ if upper or lowercase, respectively) based on linear model analysis of the NEGBIN transformed reads.

Table 6. Taxonomical composition at genus level through the six different levels of inclusion of PAA administered through the study. The table depicts the calculated average of the relative abundance for each genus amongst the 10 most abundant ones at each treatment level ±SD calculated amongst the replicates.

	0ppm (n=4)	10ppm (n=4)	20ppm (n=4)	30ppm (n=4)	40ppm (n=4)	50ppm (n=4)
Lactobacillus	57.42 ± 10.06^A	58.88 ± 6.91^B	46.23 ± 7.48^B	62.07 ± 7.69^B	51.41 ± 12.14^B	55.5 ± 8.43
Cyanobacteria (Unassigned)	18.39 ± 5.46^a	17.23 ± 6.73^b	21.74 ± 5.83	15.63 ± 5.06	19.33 ± 6.46	13.76 ± 3.93

Rickettsiales (Unassigned)	20.77 ± 11.11	20.58 ± 9.6	27.74 ± 9.11	18.27 ± 2.68	25.88 ± 5.18	24.12 ± 8.23
Acinetobacter	1.61 ± 1.62	1.49 ± 1.26	1.15 ± 0.96	1.54 ± 1.22	0.84 ± 0.68	1.64 ± 1.25
Erwiniaceae (Unassigned)	0.25 ± 0.21^A	0.11 ± 0.07^B	0.37 ± 0.25	0.28 ± 0.19	0.44 ± 0.18	0.81 ± 0.87^B
Pseudomonas	0.13 ± 0.09	0.15 ± 0.05	0.38 ± 0.31	0.36 ± 0.5	0.32 ± 0.18	0.25 ± 0.19
Escherichia-Shigella	0.09 ± 0.1	0.45 ± 0.27	0.64 ± 0.27	0.39 ± 0.42	0.17 ± 0.17	0.13 ± 0.1
Ruminococcus (torques group)	0.06 ± 0.07	0.02 ± 0.02^B	0.07 ± 0.04^B	0.04 ± 0.05^B	0.06 ± 0.09	0.02 ± 0.02^B
Enterobacteriaceae (Unassigned)	0.27 ± 0.19^A	0.38 ± 0.16	0.33 ± 0.17	0.81 ± 0.77	0.5 ± 0.4	2.96 ± 2.68^B
Enterobacterales (Unassigned)	0.14 ± 0.22^A	0.13 ± 0.09	0.14 ± 0.06	0.08 ± 0.08	0.25 ± 0.09	0.33 ± 0.32^B
Others	0.89 ± 0.47	0.59 ± 0.25	1.21 ± 0.67	0.53 ± 0.24	0.8 ± 0.32	0.49 ± 0.21

Different superscripts in the same row indicate statistically significant differences ($P<0.05$ and $P<0.10$ if upper or lowercase, respectively) based on linear model analysis of the NEGBIN transformed reads.

Figure 1. Log10 bacterial concentration in the three different gut locations analyzed

Figure 2. Correlation between body weight gain and Log10 crop bacterial concentration. Each dot represents the bacterial abundance associated with the different treatments (in different colors), whereas the size of the dot indicates whether the measurement was associated with either of the rooms. Linear models for each treatment are represented by the colored lines, indicating the monotonic inverse relationship between the variables especially for treatments whose level of inclusion was higher than 20ppm

Figure 3. Microbial OTU alpha-diversity; richness calculated through the different levels of inclusion

Figure 4. NEGBIN calculated differential abundance of TMM normalized predicted pathway abundance