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Effect of oxytetracycline as well as an acid-based feed supplement on the prevalence and abundance of selected antibiotic resistance genes in weaned piglets

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Abstract

The development of antibiotic resistance in livestock species is discussed as major threat to modern medicine. One potential strategy to battle resistance in animal production might be the application of feed additives with antimicrobial properties, such as organic acids. The aim of this study was to evaluate the impact of an acid-based feed additive on antimicrobial resistance traits of faecal microbiota in weaning piglets and to compare the results to antibiotic intervention with oxytetracycline. Therefore, 24 pigs were allocated to three treatment groups, fed a basal diet and a basal diet supplemented with either the antibiotic or the feed additive. Quantitative PCR was used to monitor the prevalence and quantity of frequently associated genes encoding resistance to tetracycline, streptomycin, and sulfonamide. The number of positive tested samples for *tet*B and *sul*1 decreased over time, with differences due to treatment. For all analysed genes, a decrease in $\log_{10}$ mean antibiotic resistance gene copy numbers was observed in all groups on day 10 of the experiment. The mean *tet*A and *tet*B gene levels were significantly higher in the oxytetracycline group than in the other groups indicating selection for these antibiotic resistance genes. No effects of the applied feed additive on the quantity of selected antibiotic resistance genes in faeces were detected. In contrast, antibiotic treatment clearly effected antibiotic resistance gene level and prevalence. Results implicate that, in the long run, novel feed additives have the potential to minimise the role of livestock as a reservoir for antimicrobial resistance.

Introduction

In the last decades, livestock production has intensified dramatically to meet the rising demand of animal derived foods. In the light of economic production, the use of antibiotics has become an integrated part of the production system. Following the ban of antibiotic growth promoters in the European Union, due to the quick rise of antibiotic resistances, antibiotics are specifically used as a metaphylactic or therapeutic treatment. Strict rules exist how to apply antibiotics in animal production [1]. However, each antibiotic usage might contribute to the risk of antibiotic resistance transfer and dissemination [2].

In pig farming, antibiotic treatment is often applied in young piglets [3] because mortality is high in critical periods of growth, such as the first week of life and immediately after weaning. The post-weaning stress and the related immunocompromised state promote the development of bacterial infections such as *E. coli* associated diarrhea [4,5]. Chlortetracycline and oxytetracycline are among the most commonly used antibiotics in pig production in the European Union and the United States [6,7]. Therefore, it is not surprising that tetracycline resistance represents the most commonly detected resistance among bacteria. Resistance occurs mainly due to acquisition of *tet* genes that are involved in the active efflux of the drug or by protecting the ribosome from its action [8]. One main concern is the co-selection of other important resistances. Association of resistance genes with mobile genetic elements allows the accumulation of a large number of different resistance genes or other types of genes, which might be kept in the bacterial communities even without selective pressure. Among Gram-negative *tet* genes, the widespread *tet*A and *tet*B genes have been shown to be linked to other resistance genes. They are frequently associated with genes conferring resistance to streptomycin (*str*A, *str*B) and/or sulfamethoxazole (*sul*1, *sul*2) [9].

Due to the risk of gene transfer, antibiotic resistance in livestock species is discussed as major risk to public health. Besides improvement of biosecurity and instalment of vaccination programs, the use of novel feed additives could help to reduce antibiotics in livestock production and at the same time maintain animal health and productivity [10]. Organic acids were reported to stimulate the digestive system of pigs and to improve pig performance because of their antimicrobial activity [11-13]. Several studies show that certain organic acids can inhibit growth of multi-resistant bacteria and that they can act against bacterial biofilms, which cannot be achieved by antibiotics [14,15]. The impact of acid-based feed supplements on antimicrobial resistance traits has not been investigated in pigs so far. The aim of our study was to compare the effect of additionally feeding oxytetracycline and

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an acid-based feed additive on the development of resistance gene levels in weaned piglets. Quantitative PCR (qPCR) was used to assess the prevalence and quantity of frequently linked genes conferring resistance to tetracyclines, aminoglycosides and sulfonamides in faeces samples.

Materials and methods

Animals and housing

The experiment was conducted in the experimental centre of farm animals at the Slovak University of Agriculture in Nitra. All procedures related to the experiment were carried out following the European Guidelines for the Care and Use of Animals for Research Purpose [16]. The animal experiment was approved by the State Veterinary and Food Administration of the Slovak Republic (project number 1485/15-221).

After weaning, 24 Large White piglets (mixed sex, four weeks of age) were randomly distributed to three treatment groups (n=8), taking into account the body weight, sex, and mother sow. Animals were housed pairwise in pens on concrete floor under controlled environmental conditions and had free access to water during the whole duration of the trial. Piglets as well as their dams had no history of antibiotic usage.

Dietary treatment and sampling

Pigs received one of three different dietary treatments in dry form *ad libitum*: a basal diet serving as control group (CON; without supplements), a basal diet supplemented with the antibiotic oxytetracycline (OTC; "Chevita", Chevita GmbH, Germany) or a basal diet supplemented with a commercial feed additive consisting of a blend of propionic, formic and acetic acid, cinnamaldehyde and a permeabilizing complex (FA; BIOTRONIC TOP3, BIOMIN Holding GmbH, Austria, 2 kg/t). The antibiotic was administered directly after weaning for 10 days in a dose recommended by the manufacturer (40 mg oxytetracycline hydrochloride/kg body weight/day). Individual faecal samples were collected from the rectum at day 0, 10 and 28, respectively. Samples were placed into individual tubes, snap frozen on dry ice and stored at -40 °C until further usage.

Bacterial community DNA extraction of faeces

Total bacterial community DNA was extracted from 200 mg of frozen faecal samples using the QIAamp DNA Stool Mini Kit (QIAgen, USA). After addition of 1.4 mL buffer ASL, a mechanical lysis step using the homogenizer Precellys 24 (Bertin, USA) at 6800 g for 15 s was included in the protocol before heating the suspension for 5 min at 95 °C. Further steps were performed according to the manufacturer's instructions.

Quantification of the total bacterial load and antibiotic resistance genes

The above-prepared DNA was quantified and adjusted to 4 ng/µl for subsequent qPCR analysis of antibiotic resistance genes encoding resistance to tetracycline (*tet*A, *tet*B), streptomycin (*str*A, *str*B) and sulfonamides (*sul*1, *sul*2). To measure the total bacterial load, the 16S ribosomal RNA (*rRNA*) gene was quantified.

All qPCR assays were performed on a Mastercycler Realplex 2S (Eppendorf, Germany) using SYBR Green detection chemistry. Details on primers and thermal cycling conditions are given in (Table 1). Each reaction was carried out in a total volume of 15 µL, containing 2 µL of DNA template, 1.5 µL of each primer (1.5 pmol/µL), 7.5 µL of the 2x Kapa SYBR Fast master mix (Biosystems, USA) and 2.5 µL of nuclease-free water. The thermal profile consisted of initial activation at 95 °C for 3 min followed by 40 cycles of denaturation at 95°C for 15s, annealing at primer specific temperature for 20 s and extension at 72 °C for 20 s.

Standard curves were generated with known quantities of target genes cloned into pGEM-T easy plasmids (Promega Corporation, USA). The gene copy number was calculated as described previously [17] and expressed as gene copies per gram faeces.

All samples, standards and controls (non-template control and positive control) were run in triplicates.

Standard curves were further used to define the limit of quantification (LOQ) and limit of detection (LOD) for each individual qPCR assay. The LOQ was defined as the most diluted DNA standard before cycle threshold (Ct) values deviated from the linear range of the standard curve. The LOD was set as the lowest concentration in the standard serial dilution where at least one of the triplicates was positive or before the Ct deviated from the average Ct of a false positive.

Data Management and Statistical Analysis

Faecal samples with resistance gene quantities below the specified LOQ and above the LOD were assigned half the LOQ. Sample concentrations below the LOD were reported as not detected and not included in analysis.

All statistical tests were performed using the software IBM SPSS statistics, Version 22.0 (IBM Corp., USA). The individual pig was used as experimental unit to test the influence of dietary treatment and the sampling time on antibiotic resistance gene quantification. Gene quantities were $\log_{10}$ transformed to achieve normal distribution. Levene's test was performed to verify homogeneity of variances. A one-way analysis of variance (ANOVA) was used to estimate overall differences between the group means, which were further compared using Bonferroni test. In the case of variance inhomogeneity, Welch's robust test was used to compare group means followed by Tamhane post-hoc test. Differences at $P<0.05$ were considered significant.

Results

Quantification of the 16S *rRNA* gene as measure of the total bacterial load revealed a stable microbial population in the piglets of the trial (Table 2). At the start of the experiment mean log 16S rRNA gene copy numbers in faecal samples ranged from 10.82 to 10.88 in the individual treatment groups followed by a minor decrease on day 10 (ranging from 10.75 to 10.78) and increase on day 28 (10.92 to 10.98). A significant difference was found in the CON group between day 10 and day 28 ($P=0.012$). Since trends descrizbed for the gene copy estimates did not change after normalisation with the 16S *rRNA* gene, only the absolute quantification was taken for further analysis.

As not all targeted antibiotic resistance genes were detected in each sample over the entire trial period, prevalence of antibiotic resistance genes was also taken into account (Table 3). Among the analysed resistance genes, *tet*A, *sul*2, *str*A and *str*B were detected in faecal samples from all animals, except two where *strA* and one where *strB* could not be detected. *Tet*B and *sul*1 genes were found in all samples before treatment. The number of *tet*B and *sul*1- positive samples decreased during the experiment, revealing differences based on treatment. Compared to the CON and the FA group, the number of *tet*B- positive samples was higher in OTC-fed piglets on day 10 and 28. The *sul*1 gene was detected in all samples of the treatment groups on day 0 and day 10. At the last time point, it was found just in two samples of the FA group, whereas six and seven samples of the CON and OTC group were positive, for *sul*1.

Table 1. Primer characteristics targeting bacterial 16S *rRNA* and selected antibiotic resistance genes.

Gene	Primer	Primer sequence (5´-3´)	Amplicon size (bp)	Annealing temperature (°C)	Reference
16S rRNA	16S-F	ACTCCTACGGGAGGCAGCAGT	180	58	[38]
	16S-R	TATTACCGCGGCTGCTGGC			
tetA	tetA-F	GCTACATCCTGCTTGCCTTC	210	64	[39]
	tetA-R	CATAGATCGCCGTGAAGAGG			
tetB	tetB-F	TACGTGAATTTATTGCTTCGG	206	62	[40]
	tetB-R	ATACAGCATCCAAAGCGCAC			
sul1	sul1-F	CGCACCGGAAACATCGCTGCAC	163	66	[17]
	sul1-R	TGAAGTTCCGCCGCAAGGCTCG			
sul2	sul2-F	TCCGGTGGAGGCCGTATCTGG	191	66	[17]
	sul2-R	CGGGAATGCCATCTGCCTTGAG			
strA	strA-F	CCTGGTGATAACGGCAATTC	546	66	[41]
	strA-R	CCAATCGCAGATAGAAGGC			
strB	strB-F	ATCGTCAAGGGATTGAAACC	509	60	[41]
	strB-R	GGATCGTAGAACATATTGGC			

Table 2. Log_{10} gene copies of 16S *rRNA* gene in faecal samples from weaned piglets receiving a basal diet (CON) and a basal diet supplemented with oxytetracycline (OTC) or an acid-based feed additive (FA). Mean values with standard deviation (in parentheses) are shown.

Gene	Treatment	Mean log_{10} gene copies/gram		
		day 0	day 10	day 28
16S rRNA	CON	10.82 (0.10)	10.78 (0.10)	10.98 (0.13)
	OTC	10.84 (0.13)	10.75 (0.19)	10.92 (0.12)
	FA	10.88 (0.22)	10.75 (0.13)	10.96 (0.09)

Table 3. Prevalence of selected antibiotic resistance genes detected by qPCR from total community DNA of faecal samples from weaned piglets receiving a basal diet (CON) and a basal diet supplemented with oxytetracycline (OTC) or an acid-based feed additive (FA).

Gene	Treatment	Number of positive samples/total samples tested		
Days		day 0	day 10	day 28
tetA	CON	8/8	8/8	8/8
	OTC	8/8	8/8	8/8
	FA	8/8	8/8	8/8
tetB	CON	8/8	2/8	5/8
	OTC	8/8	8/8	6/8
	FA	8/8	6/8	2/8
***str*A**	CON	8/8	7/8	8/8
	OTC	8/8	8/8	8/8
	FA	8/8	8/8	7/8
***str*B**	CON	8/8	8/8	8/8
	OTC	8/8	8/8	8/8
	FA	8/8	8/8	7/8
***sul*1**	CON	8/8	8/8	6/8
	OTC	8/8	8/8	7/8
	FA	8/8	8/8	2/8
***sul*2**	CON	8/8	8/8	8/8
	OTC	8/8	8/8	8/8
	FA	8/8	8/8	8/8

Figure 1 displays the mean log_{10} copy number of *tet*A, *tet*B, *str*A, *str*B, *sul*1 and *sul*2 genes over time (days 0, 10, 28) in response to treatments. At start of the experiment, the mean log_{10} copies of individual antibiotic resistance genes were in a similar range for the CON, OTC and FA group. On day 10, a decrease in all copy numbers was found, respectively, for all groups. ANOVA analysis revealed a statistically significant time-dependent effect. The levels of *tet*A and *tet*B genes turned out to be significantly reduced in faeces of the CON (*tet*A P=0.033, *tet*B P=0.001) and the FA group piglets (*tet*A P=0.007; tetB P=0.011). For *str*A and *str*B a significant time-dependent decrease was found in faeces of the CON (strA P=0.02, strB P=0.001) and the OTC group piglets (*str*A P<0.001, strB P<0.001). Over time, there was a significant reduction of *sul*1 only in samples from the FA group (P=0.046), whereas *sul*2 gene copies were significantly decreased in CON and OTC samples *(*P=0.001, P<0.001).

Looking at the effect of the treatments, the mean *tet*A and *tet*B gene quantities turned out to be significantly higher in OTC group samples compared to those of the other groups on both day 10 (towards CON P=0.015, P<0.001; towards FA P=0.007, P=0.035) and day 28 (CON P=0.001, P=0.025 and FA P<0.001). On day 28, significant differences were obtained only between the OTC and CON group samples (P=0.025). Streptomycin resistance gene quantities were also affected, with higher *str*B levels in piglets of the FA compared to the OTC group on day 28 (P=0.034).

Discussion

Recent publications highlight the importance to evaluate positive and negative impacts of various alternatives to antibiotics on the antimicrobial resistance in animal production [18-20]. Assessment of antibiotic resistance in various environments has long relied on culture-based approaches focusing on specific cultivable species. Because a significant proportion (60-80%) of the faecal microbiota is not cultivable [21,22], we used PCR amplification to investigate the prevalence and persistence of antimicrobial resistance genes. This strategy might provide more accurate information on the actual magnitude of antibiotic resistance in the microbial population. The genetic determinants of resistance that were targeted within this experiment consisted of those being exclusively or at least commonly reported in Gram-negative enteric bacteria. The most common resistance mechanism in Gram-negative bacteria is the energy-dependent efflux pump system, which is encoded by diverse *tet* genes, with *tet*A and *tet*B being the most frequently described [23]. These genes are predominantly found in tetracycline resistant *Enterobacteriaceae*, including members of non-pathogenic microbiota as well as pathogens. They are usually located on large conjugative plasmids, which also carry other antibiotic determinants [24]. Various combinations with antibiotic resistance genes for streptomycin (*str*A, *str*B) and sulfonamides (*sul*1, *sul*2) are reported and they often cluster on plasmids, integrons or transposons [25,26] .

In all faecal samples, 16S *rRNA* gene concentrations remained stable over the entire experiment, irrespective of pig age and dietary treatment. The quantification of the 16S *rRNA* gene has been used to normalise the abundance of resistance genes to the bacterial population

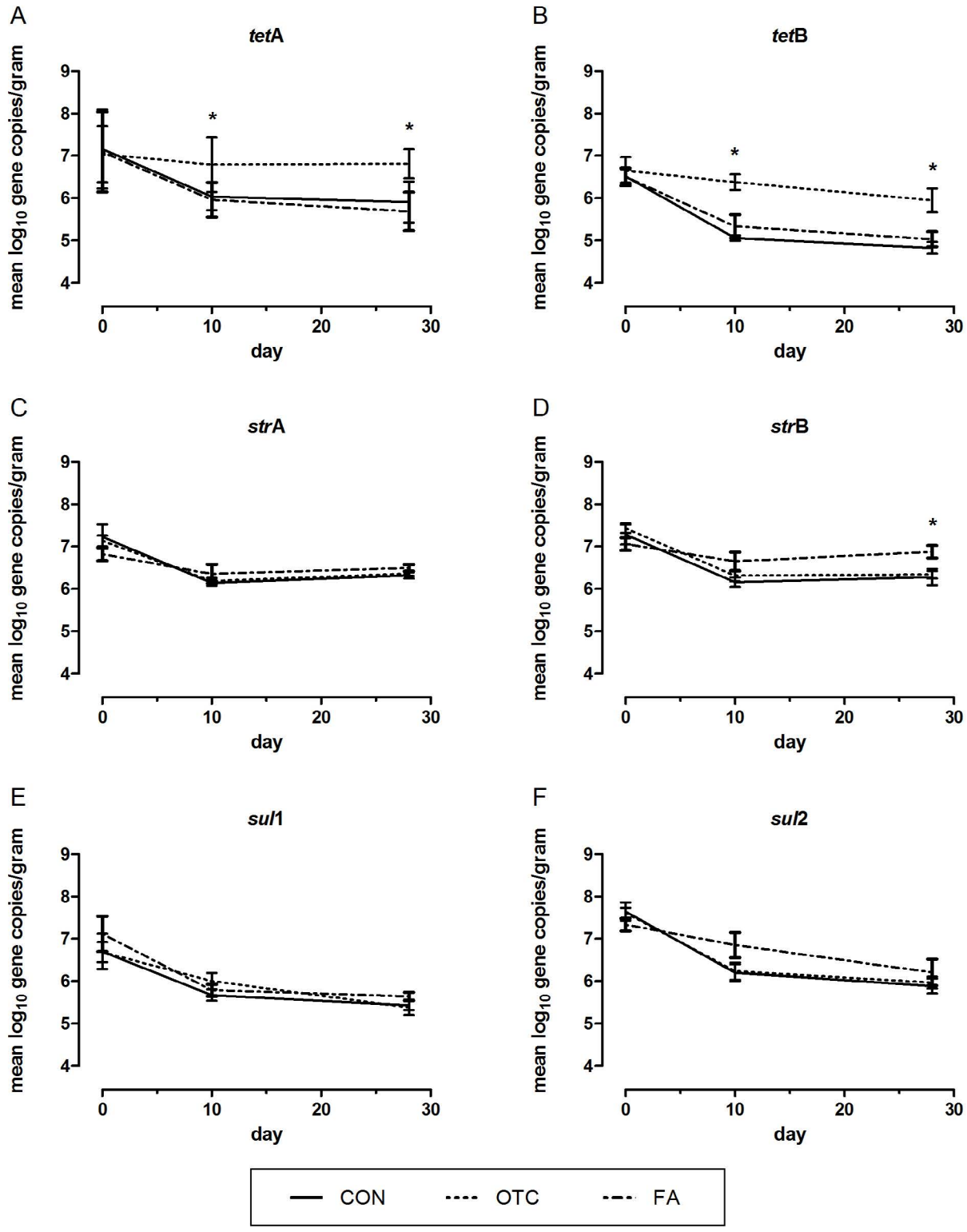

Figure 1. Non-standardized log10 copies of antibiotic resistance genes resistance genes (A) tetA, (B) tetB, (C) strA, (D) strB, (E) sul1 and (D) sul2, per gram of fecal sample from weaning piglets receiving a basal diet (CON) and a basal diet supplemented with oxytetracycline (OTC) or an acid-based feed additive (FA). Mean values with standard deviation are shown. * Superscripts indicate treatment specific differences ($P<0.05$).

and to account for variations in DNA extraction efficiency [27]. Because in this experiment no differences based on normalisation were obtained, data of the absolute quantification are reported. In the beginning of the study, all faecal samples were positive for the selected resistance genes, although the piglets as well as their dams were never exposed to any antibiotics. A similar situation has been found in previous studies [28,29], which also reported the presence of background resistances in the absence of any antibiotic selective pressure. In our study, resistance gene copy numbers were higher in the pigs as in the sows (unpublished results) but decreased with time which might reflect age related differences in the microbiota. Faecal bacterial communities are in general dominated by two major phyla, *Firmicutes* and *Bacteroidetes*. As pigs age, the proportion of Gram-negative bacteria is decreasing and a shift towards a Gram-positive microbiota is reported [30]. Thus, it might be speculated that the declining trend in the level of resistance genes might be a result of the expansion of Gram-positive bacteria in the underlying bacterial population. The levels of *tet* genes were not reduced in faeces of piglets additionally administered oxytetracycline, which was indicated by stable *tet*A and *tet*B $\log_{10}$ gene copy numbers at both time points (d10, d28), compared to declining ones in non-antibiotic treatments. Previous studies reported elevated levels of resistant bacteria or resistance genes under selective pressure, revealing a clear association between antibiotic consumption and antibiotic resistance [31-33]. Interestingly, shedding of tetracycline resistant bacteria in faecal samples continued after withdrawal of the antibiotic, indicating a long-lasting effect of antibiotic supplementation.

Also, when the occurrence of antibiotic resistance genes in individual samples instead of gene quantity was analysed, a clear effect of oxytetracycline supplementation on faecal prevalence of *tet*B was detected, at both time points (d10, d28). Although *tet*B is known to have the widest host range in Gram-negative bacteria among the different *tet* genes [24], having no selective pressure, a clear reduction of positive samples was observed for CON and FA group piglets. In contrast, *tet*A remained stable showing presence in 100% of the samples over the entire trial period. In enterobacterial isolates, a strong negative correlation of *tet*A and *tet*B was observed for the distribution of these genes, indicating a potential plasmid incompatibility [34]. Although both genes encode for an efflux pump in the lipid bilayer of the bacteria, *tet*B might be associated with higher levels of resistance, presence of multiple resistance genes and intensive farming conditions [35]. Lee and coworkers [36] even report higher minimal inhibitory concentrations towards tetracycline associated with *tet*B. Yet, in the present study, the OTC feed supplementation seems to have favoured *tet*A over *tet*B, which was also reported in a previous study published by Agga *et al.* [28].

No treatment specific differences were observed for the gene quantity of *sul*1. However, a decreased faecal prevalence in the FA, and to a lesser extent in the CON group, might indicate a correlation of *tet*B and *sul*1 for a subset of the resistant bacterial population. Genetic linkage of selected antibiotic resistance genes is common. For *E. coli,* respectively, association of *tet*A and *sul*1 is more frequently reported [9].

Interestingly, *strB* was the only gene, with increased levels in the FA group, only on day 28, when comparing its $\log_{10}$ gene copy numbers to those of the OTC group piglets. Both streptomycin resistance determinants, *str*A and *str*B, are widely distributed in Gram-negative bacteria and were found to be linked together on the same transcriptional unit [37]. Yet, quantity of *str*A was not affected by the acid-based feed supplement. Applied qPCR analyses are not suitable to make conclusions on the bacterial level, though correlation of individual assays might indicate specific patterns of multi- and co-resistance, but do not constitute a standalone approach in this regard. However, as a total community-based approach qPCR gives an overall picture of the resistance level and constitutes a reliable tool to easily check for antibiotic resistance genes in environmental samples and might be used for evaluating the impact of growth promoters in livestock farming.

Conclusion

Clear effects of the antibiotic treatment on the resistance gene prevalence and quantity were observed. An obvious reduction in the quantity of selected antibiotic resistance genes by the acid-based feed additive was not detected in the present study. Still, antimicrobial activities of organic acids are well known, and therefore, their potential to reduce bacterial antimicrobial resistance should be further investigated. Upcoming experiments should more thoroughly study the role of the gut microbiota and/or mobile genetic elements such as plasmids, transposons and integrons in the dissemination of resistance genes in pigs over time and the effect of feed supplements on this dissemination.

Conflict of interests

BIOMIN Holding GmbH provided support in the form of salaries for authors GW, VK and VN, but did not have any additional role in the experimental design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflict of interest.

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