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ORIGINAL ARTICLE

Continuous feeding of antimicrobial growth promoters to commercial swine during the growing/finishing phase does not modify faecal community erythromycin resistance or community structure

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Abstract

Aims: To investigate the effect of continuous feeding of antimicrobial growth promoters (tylosin or virginiamycin) on the swine faecal community.
Methods and Results: The study consisted of two separate on-farm feeding trials. Swine were fed rations containing tylosin (44 or 88 mg kg^{-1} of feed) or virginiamycin (11 or 22 mg kg^{-1} of feed) continuously over the growing/finishing phases. The temporal impact of continuous antimicrobial feeding on the faecal community was assessed and compared to nondosed control animals through anaerobic cultivation, the analysis of community 16S rRNA gene libraries and faecal volatile fatty acid content. Feeding either antimicrobial had no detectable effect on the faecal community.
Conclusions: Erythromycin methylase genes encoding resistance to the macrolide–lincosamide–streptogramin B (MLS_B) antimicrobials are present at a high level within the faecal community of intensively raised swine. Continuous antimicrobial feeding over the entire growing/finishing phase had no effect on community *erm*-methylase gene copy numbers or faecal community structure.
Significance and Impact of the Study: Antimicrobial growth promoters are believed to function by altering gut bacterial communities. However, widespread MLS_B resistance within the faecal community of intensively raised swine likely negates any potential effects that these antimicrobials might have on altering the faecal community. These findings suggest that if AGP-mediated alterations to gut communities are an important mechanism for growth promotion, it is unlikely that these would be associated with the colonic community.

Introduction

In North America, the inclusion of low doses of antimicrobials in swine feed has been practiced for decades as a means to improve growth and feed utilization (Shryock and Page 2006). Two antimicrobial growth promoters (AGPs) commonly used in swine production are virginiamycin and tylosin. Virginiamycin is a mixture of streptogramin A and B (Butaye *et al.* 2003), whereas tylosin is a macrolide (Giguere 2006). In Canada, tylosin is allowed to be fed at levels up to 44 mg kg^{-1} feed depending on the growth phase (44 mg kg^{-1} starters, 22 mg kg^{-1} growers and 11 mg Kg^{-1} in finishers), whereas virginiamycin can be fed at levels of 11 mg kg^{-1} independent of the growth phase (www.inspection.gc.ca). Bacterial resistance to either tylosin or virginiamycin can result from the

transfer of genes conferring the ability to chemically modify these compounds, encode efflux pumps or methylate the 23S rRNA. In anaerobes, resistance occurs through the *erm*-methylase genes; a family of rRNA methylases that confer resistance to macrolide–lincosamide–streptogramin type B antimicrobials (MLS_B: Roberts 2003).

Antimicrobial growth promoters do not promote growth in germ-free animals, which supports that these agents function by targeting the gut-associated microbial community. A wide variety of mechanisms have been proposed to explain their effectiveness, for example, AGPs may reduce microbial loads, thereby decreasing host/bacteria competition for dietary nutrients (see Shryock and Page 2006). There is evidence supporting that short-term feeding of AGPs can impact and change swine gut communities. Reductions in ileal bacterial loads in response to different AGPs used in rotation have been observed in barrow pigs (Collier *et al.* 2003) and in ileal *Lactobacillus* spp. in swine fed a standard diet containing virginiamycin (Agudelo *et al.* 2007). More recently, Rettedal *et al.* (2009) found significant community composition shifts in both the ileal mucosal and lumen communities of weaned piglets fed chlortetracycline over a 2-week period. Much less is known concerning the specific affects of AGPs on the caecal, colonic and faecal communities. However, AGPs have been observed to decrease intestinal lactobacilli and alter volatile fatty acid profiles in both swine caecal *in vitro* fermentations as well as lumen samples taken from the colon of swine fed ionophores (Vervaeke *et al.* 1979; Wuethrich *et al.* 1998). Virginiamycin has also been reported to increase transit time in swine fed high fibre-containing diets, improving nutrient utilization thereby increasing dietary energy (Ravindran *et al.* 1984).

A perplexing and troubling aspect of the long-term continuous use of AGPs is the impact on acquired antimicrobial resistance. At some point, antimicrobial resistance genes would be expected to become so widely disseminated within swine gut bacterial communities that this should eventually reduce the efficacy of many AGPs. While previous studies examining the impacts of AGPs on swine faecal communities have focused primarily on their effect on the prevalence of resistance in a limited number of readily cultivated faecal genera, it is clear that the incidence of antimicrobial resistance within these genera is higher in swine from farms where AGPs are routinely used (Rood *et al.* 1978; Gellin *et al.* 1989; Jackson *et al.* 2004). Studies using a variety of molecular approaches to assess community resistance in both faecal and manure samples from intensively raised swine also support this (Jindal *et al.* 2006; Patterson *et al.* 2007; Zhou *et al.* 2009). For example, Jindal *et al.* (2006) found that 80% of the manure community 23S rRNA from a farm where AGPs were routinely used was methylated at the position that confers resistance to MLS_B antimicrobials. Despite evidence supporting high indigenous antimicrobial resistance in the gut communities of intensively raised swine, these agents remain effective as growth promoters (Cromwell 2004) although an alternative mechanism of action for AGPs has recently been proposed (Niewold 2007).

While AGPs are recognized as a valuable production tool, concerns regarding the transfer of resistance into clinically important bacteria have resulted in their use being banned in many jurisdictions, and it is likely that this will also occur in Canada. To design rational and practical alternatives to AGPs, it is important to better understand how these agents affect the swine gut community. In this study, the impact of continuous feeding of two AGPs on both the abundance of faecal community erythromycin methylase genes and community structure in swine was determined.

Materials and methods

Swine feeding trials

Swine faecal samples were obtained from a swine farm in Quebec, Canada, where AGPs are routinely used. Animals were initially divided into three pools of 100 pigs each, consisting of nondosed controls and two groups of dosed animals fed rations supplemented with either tylosin or virginiamycin over the growing/finishing phase. All treatment groups were housed within the same barn but into separate sections of pens. In trial I, animals were dosed continuously at 11 mg kg^{-1} feed with virginiamycin and at 44 mg kg^{-1} tylosin, then at double these levels (22 and 88 mg kg^{-1}) for trial II. Specific biosecurity measures were applied during the trial: farm personnel were advised to change footwear when moving from one area to another to avoid carrying over bacteria from one group to another. Any pig that required therapeutic antimicrobial treatment during the feeding trials was withdrawn from the group prior to treatment and put in a specific area, separated from the other groups.

Fresh faecal samples were collected at the initiation of feeding (day-0) and at weeks 5, 7, 9, 11 and 15 for trial I, and at weeks 3, 5, 7, 9, 11 and 15 for trial II. Two pooled samples were collected from each treatment group. Pooled samples were collected as follows: approximately 10 g of faecal matter was collected in five different locations within each pen of a given treatment. There were 10 pens per treatment. In total, 50 g was collected in each pen for a total amount of 500 g per treatment. Pooled faecal samples were stored on ice, shipped by courier and processed immediately on arrival.

Anaerobic cultivation of the faecal community

Anaerobic cultivation was carried out using a 1 g sample from each pooled treatment (control, tylosin-fed, virginiamycin-fed). Samples were transferred to 50-ml preweighed falcon tubes to determine the precise weight, then transferred to an anaerobic chamber (90:10, CO_2/H_2, v/v). For trial I, faecal samples were serially diluted and plated onto L-10 medium (Caldwell and Bryant 1966) containing either 2 μg ml^{-1} tylosin or virginiamycin (Sigma Chemical Company, Toronto, Ontario, Canada). For trial II, 5 μg ml^{-1} of each antimicrobial was used. All antibiotic plates were prepared fresh 1 day prior to use. Inoculated plates were incubated at 38°C for 48 h. Following incubation, colonies were enumerated, and 10 random picks for further characterization were made from the plate containing 30–300 colonies.

Single picks were regrown in 5-ml L-10 liquid medium then restreaked onto L-10 agar following 24-h incubation. A single isolated colony was picked from each plate and regrown in 10 ml of L-10 liquid medium overnight. From each culture, 5 ml was frozen down as a stock culture, and the remainder used for the isolation of genomic DNA as previously described (Brooks *et al.* 2009).

Direct faecal microscopic counts

Approximately 1·0 g of faeces was transferred to a 50-ml preweighed falcon tube, and the precise weight of the faeces determined. Nine millilitres of Tris-buffered saline was added along with 1·0 g of 5-mm sterile glass beads. Cells were resuspended by vigorous vortexing (2 min, maximum speed), placed into ice to allow settling of large insoluble material (20 min) then serially diluted to 10^{-4} in TBS. Samples (0·5 ml) were stained using ethidium bromide (5 μg ml^{-1}) for a fifteen-minute period, then filtered onto 0·2-μm membranes (Whatman Nucleopor Track-Etch; Anachemia Science, Mississauga, Ontario, Canada) and manually counted under fluorescent illumination.

Identification of bacteria

Initial differentiation of individual isolates was carried out by comparing the relative mobility of PCR amplicon(s) generated from the V2–V3 region (position 338 to 534 relative to the *Escherichia coli* 16S rRNA gene) of the 16S-rRNA genes, using primers and denaturing gradient gel electrophoresis (DGGE) electrophoresis conditions as previously described (Muyzer *et al.* 1993; Walter *et al.* 2000). Isolates that could not be differentiated on the basis of the V2–V3 region alone were further characterized by comparison of the ribosomal intergenic spacer region as previously described (Jan-Roblero *et al.* 2004).

Near full-length 16S rRNA genes were amplified and cloned from each unique ribotype using primers, PCR conditions and cloning conditions as previously reported (Brooks *et al.* 2009). Speciation of each isolate was determined by comparison of the near full-length rRNA gene sequence using the classifier available at the Ribosomal Database Project (RDP) website (Wang *et al.* 2007: http://rdp.cme.msu.edu/classifier/classifier.jsp).

Identification of rRNA methylase genes

Primers and PCR conditions for detection of *erm*B, *erm*F and *erm*T were as previously described (Chen *et al.* 2007), as were those for *erm*G (Wang *et al.* 2005) and *erm*Q (http://faculty.washington.edu/marilynr). Amplicons generated from each reaction using faecal community DNA were cloned, sequenced to confirm identity and the resulting clones used to prepare standards for gel comparisons and for use as standards in Q-PCR determinations. Isolates were screened using these primer sets only, and once a gene was identified in a given isolate, no further screening was carried out. PCR products were resolved electophoretically in 0·8% (w/v) agarose in parallel with a DNA ladder and *erm*-gene standards.

Volatile fatty acid analysis

Faecal volatile fatty acids (VFA) for samples from trial II were analysed by the method of Weaver *et al.* (1997) using a Hewlett-Packard 5880A Series Gas chromatograph with a Nukol fused silica capillary column (60 × 0·25 mm × 0·25 μm film thickness coated with nitroterephthalic acid–modified polyethylene glycol polymer; Supelco, Bellefonte, PA, USA). Duplicate samples of approximately 0·5 g were weighed into 14-ml round-bottom polystyrene tubes to which 25 μl of 50% sulfuric acid, 0·25 ml 2-ethylbutryic acid (internal standard) and 2·0 ml of water were added. Samples were homogenized using a hand-held Pro200 homogenizer, centrifuged at 1500 ***g*** for 20 min and the supernatant filtered through a 0·45-μm PTFE syringe filter. A 1-μl aliquot of the filtrate was injected into the chromatograph equipped with a splitter (samples were split at 1 : 50). The column was ramped from 50°C (initial temperature) to 200°C at a rate of 8°C per min. VFAs were detected by flame ionization detector.

Faecal dry weight determinations

Duplicate faecal samples (0·5 g) from each pooled faecal sample were weighed into preweighed open plastic

scintillation vials and the precise weight of the samples determined. Faecal samples were freeze dried for 3 days. Dry weights were determined by immediately weighing the vials after removing the samples from the freeze-dryer. Plate counts and direct cell counts were corrected based on the dry weight determinations (CFU or cells g^{-1} dry weight faeces).

16S rRNA community analysis

Community faecal DNA was prepared by grinding faeces in liquid nitrogen and sand, and the genomic DNA purified as previously described (Brooks *et al.* 2009). In all cases, DNA quality was assessed by gel electrophoresis (0·8% agarose), the concentration determined with a spectrophotometer and the DNA stored at −20°C. Temporal changes in the faecal communities under each diet for trial II was assessed by DGGE. The variable V2–V3 region of the 16S rRNA community genes were amplified by PCR and resolved by DGGE as previously described (Brooks *et al.* 2009). Band cross-sectional areas were expressed as relative intensity (a fraction of the total area within lanes) and analysed by cluster analysis using city block distances (Beals 1984) followed by nonmetric multidimensional scaling analysis (NMS; Fromin *et al.* 2002) using a Windows-based statistics program (Statistica, Tulsa, OK, USA).

Near full-length 16S rRNA libraries were prepared from faecal samples from both trials I and II as previously described (Brooks *et al.* 2009). Libraries corresponding to day-0, and week-15 tylosin-fed, virginiamycin-fed and control (no AGP) were prepared for each feeding trial as previously described (Brooks *et al.* 2009). Near full-length 16S rDNA clones were initially screened for chimeras using Chimera check (Cole *et al.* 2003), with suspect sequences removed from further analysis. Sequences were imported, aligned and edited using the ARB database (Ludwig *et al.* 2004). Operational taxonomic units (OTUs) were defined at a 3% sequence divergence cut-off as determined using DOTUR (Schloss and Handelsman 2005). Sequence homology to previously identified species of bacteria or previously reported phylotypes was determined using the SeqMatch program available through the RDP (Wang *et al.* 2007).

Quantitative PCR determinations

Quantitative PCR (Q-PCR) was carried out using a Stratagene model MX 3005P cycle engine (Cedar Creek, TX, USA). The total faecal bacterial load (16S rRNA gene copy number) was estimated using the universal primers HDA1/HDA2 targeting the variable 2-3 region of the 16S rRNA gene (Walter *et al.* 2000), as previously described (Brooks *et al.* 2009). Quantification of *erm*F, *erm*B and *erm*T gene copy numbers were carried out using previously described primer sets and Q-PCR conditions (Chen *et al.* 2007). Previously described primer sets were utilized for quantification of *erm*G and *erm*Q (http://faculty.washington.edu/marilynr). Reaction mixtures for *erm*G and *erm*Q determinations consisted of 1× Master Mix (Stratagene, La Jolla, CA, USA), 500 nmol l^{-1} of each primer, 30 nmol l^{-1} of the ROX reference dye and faecal community DNA in a final volume of 50 μl. Q-PCRs were subjected to an initial denaturation step at 95°C for 10 min followed by 39 additional cycles at 95°C for 30 s, a 1-min extension at the annealing temperature (64 and 60°C for *erm*G and *erm*Q, respectively) and a final 1-min extension at 72°C. Standard curves for all primer sets were prepared using 10-fold serial dilutions of pCR2·1-TOPO plasmids containing each cloned target sequence. Values for the standard curve were expressed in terms of gene copy number ng^{-1} target DNA. For estimation of targets in community faecal DNA samples, serial dilutions were prepared for each time point with subsequent calculation of copy number based on threshold values (CT) values falling within the mid-range of the standard curve.

Three independent community DNA samples were prepared from each faecal sample. Each copy number determination was performed in triplicate. Melting temperature analysis of all PCR products was performed following amplification to confirm the specificity of the reaction by slow heating at 0·5°C $cycle^{-1}$ increment from 55 to 95·5°C, with continuous fluorescence monitoring. In each case, with the exception of the HDA1/HDA2, primer specificity was confirmed by cloning and sequencing randomly selected clones prepared from amplicons generated from the faecal community DNA.

Statistical testing

Analysis of *erm*-gene abundance was assessed using a 3-factor ANOVA (trial, treatment, time). Prior to ANOVA, data were checked for a potential correlation between means and SD. When a correlation was observed, the data were transformed using the Box–Cox formula: $T(Y) = (Y^{\lambda} - 1)/\lambda$, where Y is the response variable and λ is the transformation parameter (Onishi 2002). Values of λ were chosen to minimize the mean-square error using STATISTICA (Statsoft, Tulsa, OK, USA). Post hoc analyses, when warranted, were performed by Tukey's Honestly Significant Difference test.

Statistical comparisons among 16S rDNA clone libraries were performed using ∫-Libshuff using a Bonferroni correction (Schloss *et al.* 2004) and a distance matrix generated by ARB (Ludwig *et al.* 2004). TREECLIMBER (Schloss and Handelsman 2006) was used to identify

lineages contributing to community differences among clone libraries. Matrices required for TreeClimber were generated using ARB.

For cluster analysis, OTU frequency distributions were first calculated for each experimental condition then subjected to cluster analysis using Statistica (Statsoft). Differences in plate counts, direct cell counts and volatile fatty acids outputs were assessed by anova followed by a Tukey's post hoc test.

Nucleotide accession numbers

Nucleotide sequences have been deposited in GenBank under the following accession numbers: HQ716065–HQ716717.

Results

Temporal change in the cultivable faecal communities

Cultivable resistant faecal bacteria from swine fed either AGP or no antimicrobials (control) over the time course of both feeding trials were determined by anaerobic plate counting. For trial I, the plating medium contained 2 μg ml^{-1} tylosin or virginiamycin, based on previously reported minimum breakpoints for *Enterococcus faecalis* (Aarestrup *et al.* 2000). However, control counts determined on medium containing no antibiotics at the mid-point and end of trial I were identical to those determined in the presence of antimicrobials (results not shown), suggesting that the readily cultivable community was entirely resistant to either AGP. Initially, we mistakenly assumed that this may have been a reflection of too low a concentration of tylosin or virginiamycin used for plating. For trial II, the level for each antimicrobial was increased to 5 μg ml^{-1} for all plating determinations. Within each trial, the total numbers of resistant bacteria in faeces from the controls or AGP-fed swine were not significantly different, although there was a high degree of variability in total numbers at each sampling point ($1{\cdot}64 \times 10^{10} \pm 1{\cdot}19 \times 10^{10}$ and $6{\cdot}22 \times 10^{9} \pm 6{\cdot}26 \times 10^{9}$ CFU g dry weight faeces^{-1} averages for trials I and II, respectively). Direct faecal microscopic counts were stable over time ($4{\cdot}88 \times 10^{10} \pm 1{\cdot}78 \times 10^{10}$ cells g dry weight faeces^{-1}). Faecal dry weight adjusted plate counts represented 34 and 13% of the direct microscopic faecal counts for trials I and II, respectively.

Cultivable species distribution

The identity and occurrence of the isolates cultivated in both trials I and II are listed in Table 1. In all cases, samples were dominated by facultative anaerobes, predominantly *Streptococcus hyointestinalis*, *Enterococcus faecalis*, *Enterococcus durans* and *Lactobacillus reuteri* and a low proportion of obligate anaerobes. We found no differences in community composition between samples from swine fed APGs and their respective controls in either feeding trial. In trial I, a higher number of species were isolated on the plates containing tylosin, whereas in trial II, a greater number of species were isolated from the virginiamycin-containing plates. Overall, species composition in trial I was somewhat different than found in trial II, although a similar range of genera were isolated (Table 1).

Identification of rRNA methylase genes in the cultivated faecal community

Using previously described primer sets, we identified an *erm*-methylase gene in the majority of isolates from both feeding trials (Table 1). In trial I, all of the isolates were found to carry an *erm*B gene. This was also true for the majority of isolates from trial II, although additional species which carried *erm*F, *erm*G, *erm*Q and *erm*T were also isolated. In several isolates from trial II, we were unable to identify a specific *erm*-methylase gene (Table 2), although the isolates were only screened against primer sets that gave a positive result using faecal community DNA. Isolates of *Mitsoukella* sp. and *Prevotella* sp. from trial II were found to contain a greater diversity of *erm*-resistance genes than the remainder of the cultivable community (Table 2).

Quantification of community erythromycin methylase genes

To determine whether continuous AGP feeding had any impact on *erm*-gene abundance in the whole faecal community, gene copy numbers for *erm*B, *erm*F, *erm*G, *erm*Q and *erm*T within each treatment group (day 0, week 7 and week 15) in both trials were determined by Q-PCR. Initial analysis of the pooled data from both trials identified significant interactions between trial, treatment and sampling time in direct comparisons of copy numbers for both methylase and 16S rRNA genes. Normalization of the entire data set by expressing *erm*-gene copy numbers as a percentage of community 16S rRNA gene copy numbers for each respective sample reduced the number of significant differences among samples within each feeding trial. The mean relative abundance for each *erm*-gene under each treatment in each feeding trial is shown in Table 2. Despite normalization of the data, there were single treatments at single sampling times within each feeding trial that remained significantly different (Table 2). While *erm*B was the dominant marker in the cultivated communities, *erm*G was the dominant marker

Table 1 Identity, numbers of isolates, occurrence and resistance determinant from isolates from both trials I and II. The total number of isolates evaluated was 772

		Trial I		Trial II		
Identity	Isolates	Tylosin	Virginiamycin	Tylosin	Virginiamycin	*erm*-gene
Escherichia coli	13	+	+	+	+	B
Proteus mirabilis	3				+	B
Prevotella sp.	13	+		+	+	B, F, G
Streptococcus hyointestinalis	260	+	+	+	+	B
Other *Streptococcus* sp.	2			+	+	B
Enteroccocus durans	47	+	+	+	+	B
Enterococcus faecalis	187	+	+	+	+	B
Enterococcus asini	22			+	+	B
Other *Enterococcus* sp.	4	+	+		+	B
Vagococcus sp.	8			+	+	T
Pediococcus urina	8	+		+	+	B, ND*
Lactobacillus animalis	21	+		+	+	B
Lactobacillus ruminus	5	+	+			B
Lactobacillus reuterii	40	+	+	+	+	B
Lactobacillus plantarum	4			+	+	B
Lactobacillus johnsonii	6	+	+	+		B
Lactobacillus salivarius	2	+	+			B
Mitsoukella sp.	14	+	+	+	+	B, F, T, Q, ND
Selenomonas sp.	8	+	+		+	B, ND
Megasphaera sp.	6	+	+		+	B
Sporomusa sp.	4	+	+		+	B
Clostridium sp.	11	+	+	+	+	B, T, ND
Clostridium perfringens	1	+				B
Eubacterium sp.	32	+	+		+	B
Dorea longicatena	4			+	+	B, ND
Ruminococcus sp.	2				+	B, ND

*ND, identity of resistance gene not determined.

Table 2 Abundance of *erm*-methylase genes (% of the total 16S rRNA gene copy number) under each treatment in trials I and II. Values represent the mean and accompanying standard deviation for values determined at day 0, week 7 and week 15. Individual sample sets having significant increased or decreased gene copy numbers compared to the respective control set are indicated

		Proportion (%) of 16S rRNA copy number				
Feeding trial	Treatment	*erm*G	*erm*F	*erm*B	*erm*Q	*erm*T
I	Control	22·39 ± 12·03	5·43 ± 4·90	3·35 ± 1·56	0·34 ± 0·23	0·28 ± 0·20
	Tylosin (44 mg kg^{-1})	24·31 ± 3·94	5·92 ± 4·76	3·41 ± 1·28	0·27 ± 0·16*	1·33 ± 1·25†
	Virginiamycin (11 mg kg^{-1})	25·37 ± 17·72	4·77 ± 2·10	2·98 ± 1·50	0·24 ± 0·12	0·93 ± 0·66‡
II	Control	13·54 ± 4·93	2·42 ± 1·63	1·72 ± 0·45	0·28 ± 0·24	0·30 ± 0·25
	Tylosin (88 mg kg^{-1})	19·11 ± 5·95§	3·79 ± 2·09¶	1·46 ± 0·07	0·27 ± 0·11	0·24 ± 0·17**
	Virginiamycin (22 mg kg^{-1})	13·04 ± 4·79††	1·40 ± 0·69‡‡	1·64 ± 1·18	0·19 ± 0·10	0·39 ± 0·17§§

*Week-15 tylosin-fed ($P = 0·001$).
†Week-7 tylosin-fed ($P < 0·001$).
‡Week-7 virginiamycin-fed ($P < 0·001$).
§Week-7 tylosin-fed ($P = 0·023$).
¶Day-0 tylosin-fed ($P < 0·001$).
**Week-7 and -15 tylosin-fed ($P < 0·001$).
††Week-15 virginiamycin-fed ($P = 0·005$).
‡‡Week-15 virginiamycin-fed ($P = 0·004$).
§§Week-7 virginiamycin-fed ($P < 0·001$).

within the entire faecal communities of both trials I and II. There were no differences in the community resistance associated with AGP feeding at either level and their respective controls within each feeding trial.

Faecal community analysis

To assess the impact of either AGP on the dominant faecal community, we examined temporal change in both faecal VFA content and community DGGE profiles in faecal samples from trial II. We reasoned that community changes would likely be more apparent under the highest AGP concentration. Total VFA content for trial II ranged from 200 to 350 μg g^{-1} wet weight faeces over the course of the feeding (Fig. 1). In general, there were no significant differences in total faecal VFA content between the controls and those fed AGPs. Under all treatments, a spike in VFA content occurred at week 3, which was predominantly acetic acid (results not shown) and likely results from initial adaptation to the rations. Total faecal VFA content were slightly lower in the faeces from tylosin-fed swine, particularly at the latter stages of the feeding trial, although these differences were not significant ($P > 0{\cdot}05$). Analysis of individual fatty acids showed a decrease in acetic, butyric, propionic and valeric acid levels over time under all dietary regimes. Total concentrations of iso-butyric and iso-valeric acid remained stable over the initial 12 weeks and slightly increased nearing the termination of sampling (results not shown). Levels of butyric, propionic and valeric acid were slightly lower in the tylosin-fed swine, although these differences were not significant ($P > 0{\cdot}05$).

Temporal change in the dominant faecal communities from trial II (88 mg kg^{-1} tylosin or 44 mg kg^{-1} virginiamycin and control) was initially assessed through the analysis of DGGE 'fingerprint' profiles generated from the variable V2–V3 region of community 16S rRNA genes. A NMS of these fingerprints is shown in Fig. 2a. The day-0 communities from each treatment grouped into a single cluster, indicating that the pooled samples were initially very similar. However, over the duration of feeding, no clustering with respect to treatment was observed. While there was a considerable spread among community profiles over time, in NMS analysis these distances are relative and the scattering of the individual points likely represents sample to sample variability rather than significant alterations to any community resulting from the feeding of either AGP.

16S rRNA community analysis

To provide a more complete assessment of the impact of AGP feeding on community structure, 16S rRNA gene libraries were prepared from faecal samples from both trials I and II. Four libraries were constructed covering each feeding trial including day-0 and week-15 (no AGPs), week-15 tylosin-fed and virginiamycin-fed faecal samples. Total clones per library ranged from 64 to 89, for a total

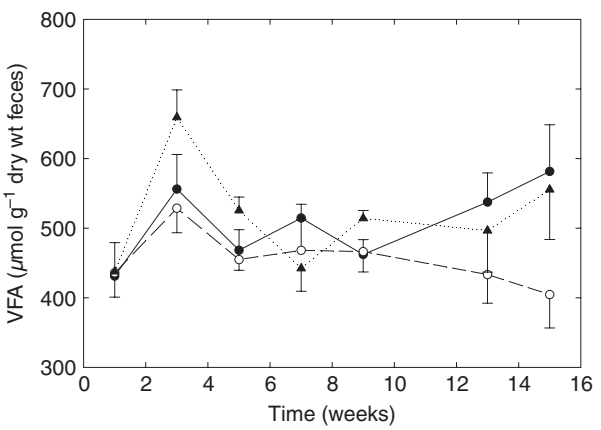

Figure 1 Change in volatile fatty acid content (μg g^{-1} wet weight faeces) over the course of trial II in swine fed 88 mg kg^{-1} tylosin, 22 mg kg^{-1} virginiamycin and control (no antimicrobial growth promoters). —●—, Control; –○–, Tylosin; ···▲···, Virginiamycin.

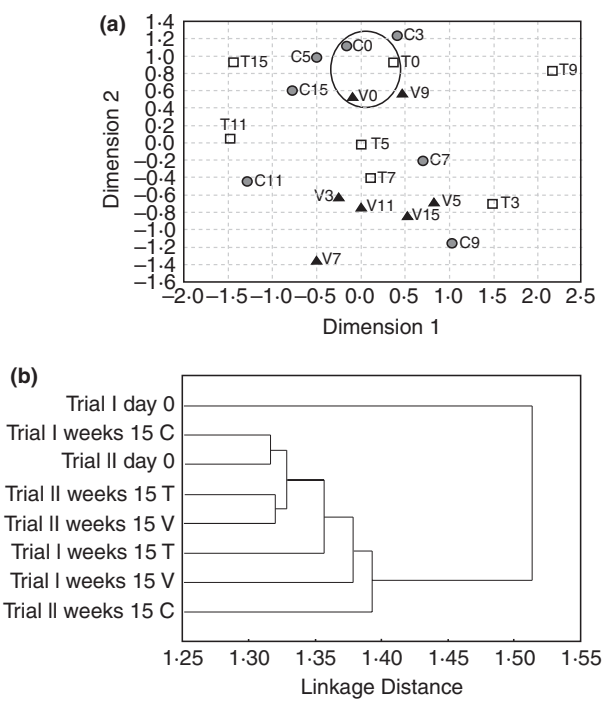

Figure 2 (a) Nonmetric multidimensional scaling analysis analysis of denaturing gradient gel electrophoresis fingerprints from each control and treatment pool over the course of trial II. Points are labelled corresponding to treatment: (C) no antimicrobial growth promoters (T) tylosin at 88 mg kg^{-1} (V) virginiamycin at 22 mg kg^{-1}. The sample week is indicated on each point. (b) Dendrogram cluster comparing phylotype composition among clone libraries from trials I and II.

of 654 clones, of which 45% were homologous with previously reported phylotypes and 17% homologous to previously cultivated species of bacteria (<3% sequence divergence).

Clone libraries from each feeding trial were initially compared using ∫-Libshuff, a statistically based test that determines whether the phylogenetic structure of two or more communities are the same (Schloss *et al.* 2004). In trial 1, the control community on day 0 was significantly different from the week-15 control and AGP-fed communities ($P < 0{\cdot}0043$). Analysis of the trial I using TreeClimber (Schloss and Handelsman 2006), a parsimony-based statistical test, which can identify the lineages primarily responsible for these differences, indicated that the phylum *Firmicutes* was responsible for this significant difference, although no single familial lineage was wholly responsible (results not shown). In trial II, no significant differences in community phylogenetic structure were observed ($P > 0{\cdot}0043$ as determined by ∫-Libshuff) yet significant differences were found between trials I and II (results not shown), likely reflecting the community variability between the two different groups of swine.

Differences in phylotype composition among all clone libraries were assessed by cluster analysis using City-block (Manhattan) distances (Fig. 2b). The day-0 library in trial I was compositionally very different from all other clone libraries, consistent with the results from ∫-Libshuff. The remaining libraries grouped into a single cluster but were not delineated by trial or treatment and compositionally they were all quite different. A heat plot illustrating the distribution of clones within each library resolved to the level of family and phylum is shown in Fig. 3. Overall, faecal community structure (i.e. the distribution of clones at the familial level) was similar between the faecal communities of trial I and II as well as between each treatment. All of these faecal communities were compositionally richer than indicated by the cultivation-based analysis. In common with other mammalian faecal communities, these were dominated by the phyla *Firmicutes* and *Bacteriodetes* (Ley *et al.* 2008). The majority of clones aligned into a limited number of familial lineages including the *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae*, *Streptococcaceae*, *Porphyromonadaceae* and *Prevotellaceae*.

Discussion

Continuous feeding of swine with feed medicated with tylosin or virginiamycin had no effect on altering the number of resistant faecal bacteria over the duration of each feeding trial. In fact, swine initially entering either feeding trial contained high numbers of faecal bacteria resistant to both AGPs, a finding consistent with previous studies examining resistance among isolates from intensively managed swine farms (Rood *et al.* 1978; Gellin *et al.* 1989; Jackson *et al.* 2004; Chapin *et al.* 2005; Jindal *et al.* 2006; Patterson *et al.* 2007). The cultivable communities were dominated by lactic acid bacteria having a species distribution similar to that found in previous studies examining swine faeces, particularly with respect to the high proportion of Gram-positive cocci (Salanitro *et al.* 1977; Russell 1979). While there were some minor differences in the genera and species isolated between each feeding trial, this study found no evidence to suggest that continuous feeding of either antimicrobial had any substantive effect on altering the composition of the cultivable faecal communities compared with their respective controls.

The majority of isolates carried the *erm*B gene, although there was a difference in the occurrence and distribution of additional *erm*-methylase genes in isolates from trial II. In contrast to that found for the cultivable communities, *erm*G represented the dominant gene in the faecal communities from both trials. This methylase gene is widely distributed among gut bacteria, having been reported in various clostridia and lactic acid bacteria, *Bacteroides*, *Porphyromonas* and *Prevotella* (Wang *et al.* 2005; Roberts 2003).

Similar to that found for the cultivable communities, continuous AGP feeding had no effect on the relative abundance of *erm*-genes. While significant variability was associated with the determination of community *erm*-gene copy numbers, expression of *erm*-gene abundance relative to the *rrn* copy number for each individual sample set eliminated most of the temporal variability in our Q-PCR determinations within each trial. The high degree of variability likely results from the use of whole faeces and may reflect differences in the relative proportions of DNA from sloughed off host gut epithelial cells, the bacterial community and undigested dietary material in each faecal community DNA sample. Despite this, there remained individual treatment samples having significantly higher or lower gene copy numbers compared to the corresponding control (Table 2). We are not certain whether these differences could reflect daily variations in community composition, the variability associated with using a pooled faecal sample taken from a subset of animals within each treatment group at each sampling time or the inherently high variability associated with these determinations (Chen *et al.* 2007).

Collectively, *erm*-gene copy numbers represented 34–25% of the *rrn* copy numbers for trials I and II, respectively. An important question is how these relative determinations actually reflect the distribution of these genes among genera throughout the entire faecal community. This is somewhat difficult to assess with certainty as not only do copy numbers for *rrn* genes vary from species

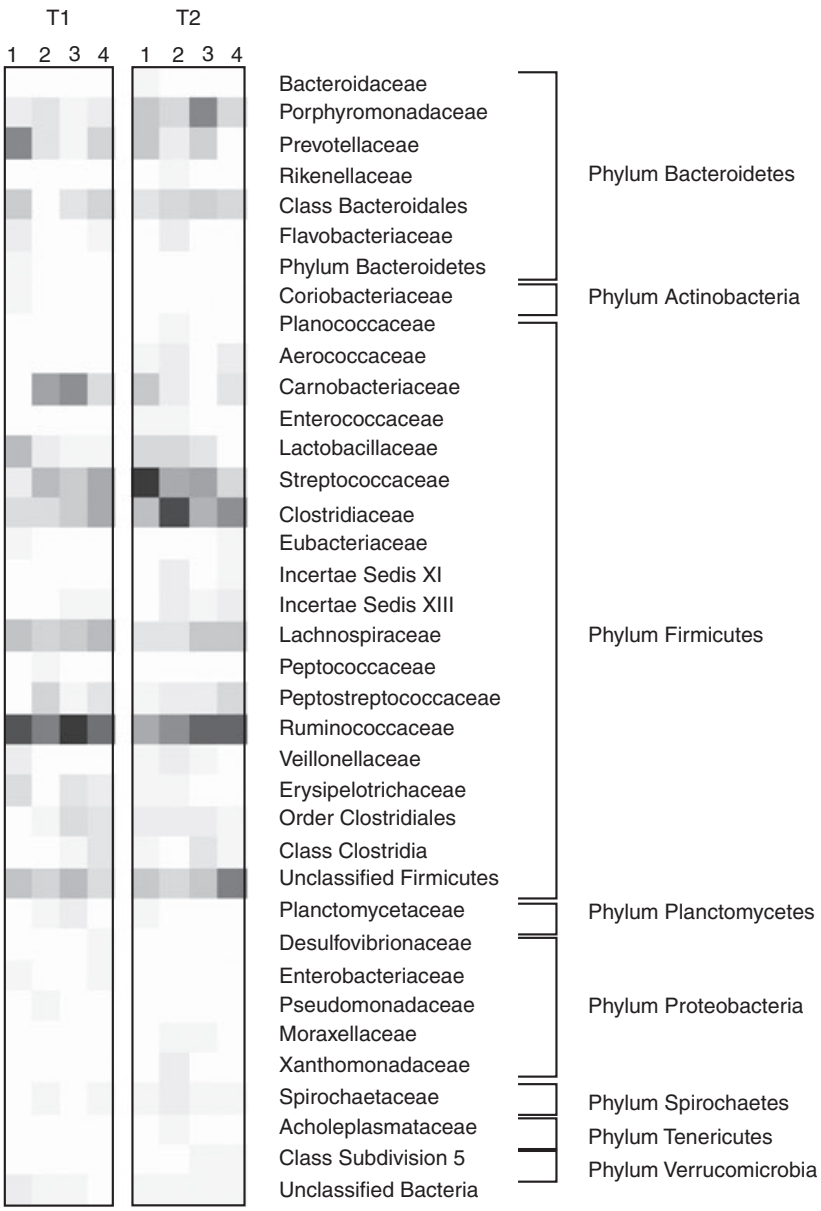

Figure 3 Heat plot illustrating the distribution of clones within each 16S rRNA clone library in both trials I and II resolved at the family and phylum level. Lane 1: day-0 faecal community, lane 2: week-15 control faecal community, lane 3: week-15 tylosin-fed faecal community, lane 4: week-15 virginiamycin-fed faecal community.

to species (Klappenbach *et al.* 2001), but community carriage would also be affected by the extent to which the *erm*-genes are encoded on multicopy plasmids or represent single genomic transposon insertions or by how much of the community carries multiple *erm*-markers. However, a conservative estimate based on the average *rrn* copy number for bacteria (4: http://ribosome.mmg.msu.edu) would indicate that collectively *erm*-gene copy numbers across each feeding trial approach or exceed the total number of bacteria present. Differences in total *erm*-gene abundance observed between trials I and II may also reflect differences in the distribution of genes across each of these communities.

Our estimates of MLS_B resistance in swine faeces are similar to previously reported determination for both faeces and manure. Patterson *et al.* (2005) determined the relative abundance of community *erm*-methylase genes in faecal community DNA samples from intensive swine operations using a DNA macroarray. While *erm*-methylase gene abundance was somewhat different than what we observed (*erm*B > *erm*F > *erm*G > *erm*X), collectively *erm*-genes represented 11·4–20·7% of *rrn* abundance across three faecal samples from intensively raised swine where 30–90% of corresponding cultivable communities were resistant to erythromycin. A previous quantitative PCR estimate in manure for intensively raised swine

(Chen *et al.* 2007) found the *erm*-gene pool represented 8% of *rrn* abundance (*erm*B > *erm*T > *erm*F > *erm*X > *erm*A). While both the *erm*-gene content and relative abundance was different than reported here, our pooled estimates for *erm*B, *erm*F and *erm*T are similar accounting for approximately 9 and 5% of the *rrn* in the total pool from trial I and II, respectively. An additional approach using oligonucleotide probes to determine the extent of the *erm*-methylase-mediated 23S rRNA modification by membrane hybridization (Jindal *et al.* 2006) or fluorescent *in situ* labelling (Zhou *et al.* 2009) also found that up to 80% of the community 23S was methylated in a manure sample where approximately 80% of the cultivable community was resistant to tylosin. Both the high level of resistance within the cultivable faecal community and abundance of faecal *erm*-genes indicate that these swine are not substantively different in terms of community faecal resistance than found with other intensively raised swine.

Feeding AGPs has been shown to impact swine ileal communities (Collier *et al.* 2003; Agudelo *et al.* 2007; Rettedal *et al.* 2009) although much less is known regarding their potential impacts on swine faecal communities. While changes in the faecal community induced by feeding of AGPs might be expected to be reflected in differences in the collective faecal metabolic activity and/or community structure, we found no evidence to suggest that either antimicrobial, even when fed at twice the recommended dose, had any significant impact on the faecal community from these animals. This is consistent with a previous molecular-based study in poultry where the effects of virginiamycin were found to decrease as sampling moved more distally along the gut tract (Dumonceaux *et al.* 2006). However, these findings are in marked contrast to studies investigating the impacts of AGPs on the faecal community using rodent models (Perrin-Guyomard *et al.* 2001; Patterson *et al.* 2005; Brooks *et al.* 2009), where AGPs not only altered the cultivable faecal community, but could also significantly affect both composition and community structure of faecal 16S rDNA gene libraries (Brooks *et al.* 2009). This distinction between rodent models and intensively raised animals likely reflects differences in the indigenous levels of resistance associated with each of these animals.

In summary, consistent with previous studies, the present study found high levels of resistance to the MSL_B antimicrobials in the cultivable faecal community and a correspondingly high abundance of faecal community *erm*-methylase genes in intensively raised swine. If antimicrobial-mediated shifts in gut microbial communities represent an important mechanism for growth promotion, given these very high levels of resistance, it would be unlikely that such changes would be associated with the colon. While these findings indirectly support previous suggestions that changes in swine ileal communities may be responsible for the beneficial effects of AGPs, there is an obvious need to further examine how long-term AGP use has impacted community resistance in swine ileal communities.

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