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Impact of oral amoxicillin and amoxicillin/clavulanic acid treatment on bacterial diversity and β-lactam resistance in the canine faecal microbiota

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Running title: Amoxicillin and clavulanic acid effects on faecal microbiota

Abstract

Background

Aminopenicillins with or without a β-lactamase inhibitor are widely used in both human and veterinary medicine. However, little is known about their differential impact on the gut microbiota and development of antimicrobial resistance.

Objectives

To investigate changes in the faecal microbiota of dogs treated with amoxicillin or amoxicillin/clavulanic acid.

Methods

Faeces collected from 42 dogs (21 per treatment group) immediately before, during and 1 week after termination of oral treatment with amoxicillin or amoxicillin/clavulanic acid were analysed by culture and 16S rRNA gene sequence analysis.

Results

In both groups, bacterial counts on ampicillin selective agar revealed an increase in the proportion of ampicillin-resistant Escherichia coli during treatment, and an increased occurrence and proportion of ampicillin-resistant enterococci during and after treatment. 16S rRNA gene analysis showed reductions in microbial richness and diversity during treatment followed by a return to pre-treatment conditions approximately 1 week after cessation of amoxicillin or amoxicillin/clavulanic acid treatment. While no significant differences were observed between the effects of amoxicillin and amoxicillin/clavulanic acid on microbial richness and diversity, treatment with amoxicillin/clavulanic acid reduced the abundance of taxa that are considered part of the beneficial microbiota (such as Roseburia, Dialister and Lachnospiraceae) and enriched Escherichia, although the latter result was not corroborated by phenotypic counts.

Conclusions

Our results suggest a limited effect of clavulanic acid on selection of antimicrobial resistance and microbial richness when administered orally in combination with amoxicillin. However, combination with this β-lactamase inhibitor appears to broaden the spectrum of amoxicillin, with potential negative consequences on gut health.

Introduction

Aminopenicillins such as amoxicillin alone or in combination with the β-lactamase inhibitor clavulanic acid are among the most widely used antimicrobials in both human and veterinary medicine. Although amoxicillin and amoxicillin/clavulanic acid are often used interchangeably, the addition of clavulanic acid is believed to broaden the antibacterial spectrum of amoxicillin.[1] It can be hypothesized that such a broader spectrum of amoxicillin/clavulanic acid would cause a higher risk of displacing the indigenous microbiota and favour development of antimicrobial resistance. Treatment with amoxicillin, amoxicillin/clavulanic acid or other β-lactams has been implicated in the selection of resistant bacteria of clinical relevance, such as Escherichia coli producing ESBLs in humans and various animal species.[2–6] Oral treatment with amoxicillin has been shown to change the composition and diversity of the human and animal gut microbiota in a drug-specific manner as compared with members of other antimicrobial classes.[7–16] However, comparative studies between closely related antimicrobial drugs are lacking. Such information is important for antimicrobial stewardship to make recommendations on how to prioritize drugs that belong to the same antimicrobial class and are used for the same indications.

The objective of this study was to investigate changes in the composition of the faecal microbiota caused by oral treatment with amoxicillin and amoxicillin/clavulanic acid using dogs as a model. The impact of the two drugs on selection of β-lactam-resistant *E. coli* and enterococci was determined by antimicrobial selective culture, and their influence on microbial richness and diversity was assessed by 16S rRNA gene sequence analysis.

Materials and methods

Dog recruitment and sample collection

Dogs were recruited from two companion animal practices in Denmark. Dogs were enrolled in the study if: (i) they were prescribed oral amoxicillin or amoxicillin/clavulanic acid; (ii) they had not received local or systemic antimicrobial treatment in the previous month; (iii) they did not present with diarrhoea; and (iv) a signed consent form was obtained from their owner. Faeces were collected from each dog on day 0 (before treatment), day 5 (during treatment) and days 12–14 (1 week after cessation of treatment). Fresh faecal deposits were collected by the owners in pre-labelled collection bags and transported to the laboratory by courier service. Information about sex, age, weight, health status, indication for antibiotic treatment and treatment duration was recorded for each dog.

Sample processing and storage

A 1 g sample of faeces was suspended in 9 mL of peptone water with 15% glycerol, homogenized using a stomacher at maximum speed for 2 min and frozen in triplicate at –80°C for later culture. Additionally, three samples of 1 g of faeces each were frozen at –80°C for microbiome analysis.

Culture-based analysis

E. coli and enterococci were used as bacterial indicators of β-lactam resistance due to their ubiquitous occurrence in faeces and their role as commensal opportunistic pathogens in both humans and animals. Frozen faecal dilutions were thawed to prepare 10-fold dilutions in physiological saline up to 10^{-6}. Three 20 µL drops of each dilution were inoculated onto five agar media (Oxoid, UK): MacConkey agar; MacConkey supplemented with ampicillin (32 mg/L); MacConkey supplemented with cefotaxime (1 mg/L); Slanetz–Bartley agar; and Slanetz–Bartley supplemented with ampicillin (16 mg/L). These five media provided counts of total *E. coli*, ampicillin-resistant *E. coli*, cefotaxime-resistant *E. coli*, total enterococci and ampicillin-resistant enterococci, respectively. MacConkey agar plates were incubated for 24 h at 37°C and Slanetz–Bartley agar plates at 42°C for 48 h. Lactose-positive colonies growing on MacConkey agar and presumptive enterococcal colonies on Slanetz–Bartley agar were quantified, and one representative colony from the highest dilution was identified using MALDI-TOF MS (BioMérieux, France). Only colonies growing on MacConkey and Slanetz–Bartley confirmed by MALDI-TOF MS were considered in the counts of total/resistant E. coli and enterococci. If more than one colony morphology was observed, quantification and species confirmation were performed separately.

Three types of analysis were performed on the data generated by culture:

(i) The prevalence of dogs carrying each β-lactam resistance indicator was compared between sampling times (dependent variable: Day) taking both treatment groups together and each treatment group separately; and between treatment groups on each day (dependent variable: Treatment). Secondly, the effect of the Treatment duration on the prevalence of positive dogs was tested by logistic analysis using the glm function (family=binomial) in R version 3.4.4 (15 March 2018).[17]

(ii) Counts of total and resistant bacteria were analysed for each indicator taking into account repeated measures by fitting a generalized linear mixed-effects model (GLMM) using the glmer function (family=Poisson) of the lme4 package in R.[18] Models were built for the counts of each bacterial indicator (independent variables: Total *E. coli, ampicillin-resistant E. coli*, *cefotaxime-resistant E. coli*, *Total enterococci* and *ampicillin-resistant enterococci*) adding dependent variables Dog and Day as a random effect in the format (1|Dog:Day) to specify the clustered nature of the data set; and Treatment and Treatment duration as fixed effects. The model that better fitted the data was selected based on the Akaike information criterion (AIC).

(iii) The proportions of resistant bacteria calculated as *ampicillin-resistant E. coli*/*Total E. coli*, *cefotaxime-resistant E. coli*/*Total E. coli* and *ampicillin-resistant enterococci*/*Total enterococci* were compared between days and between treatment groups using Wilcoxon test in R.

16S rRNA gene sequence analysis

Samples were analysed using 16S rRNA gene sequencing at GenoScreen (Lille, France). Details on the DNA extraction, sequencing protocols, computation of microbiota diversity indices, as well statistical treatment of the results are provided in Supplementary data.

Ethics

The local ethics and administrative committee at the University Hospital for Companion Animals (University of Copenhagen) approved the study protocol prior to commencement. According to the Danish Animal Experimentation Act §1.2., no further permission is required to collect faecal samples from dogs.

Results

Study population

Sixty-four dogs were enrolled in the study, of which 22 were excluded due to missing samples before or during treatment (n = 5), missing sample information (n = 5), administration of other antibiotics (n = 5), lack of compliance with antibiotic regimen (n = 3), concomitant treatment that may affect the antibiotic pharmacokinetics (n = 1), discontinuation of treatment (n = 1), sample arrival to the laboratory after 26 h (n = 1) or wrong sampling time (n = 1). The remaining 42 dogs received amoxicillin (n = 21) or amoxicillin/clavulanic acid

(n = 21) orally at a standard dosage of 10–20 mg/kg twice daily. Five dogs that had been treated beyond 7 days were not excluded but collection of the third sample was postponed to 7 days after cessation of treatment. Characteristics of the two treatment groups are shown in Table 1. Based on $\chi 2$ tests, the treatment groups had no significant differences in the composition of breed, sex, age, weight, treatment duration, day of collection of the second and third sample (during and after treatment, respectively), number of days between the cessation of treatment and the collection of the third sample or organ system involved in the diagnosis ($P \geq 1$). Details for each dog are shown in Table S1. In nine dogs (five in the amoxicillin group and four in the amoxicillin/clavulanic acid group), the third sample (after treatment) was not collected.

Prevalence and counts of β-lactam resistance indicators

E. coli was isolated from all samples except six (one sample before treatment and two during treatment in each group). Based on MALDI-TOF MS, other lactose-positive species isolated from MacConkey agar were identified as *Klebsiella pneumoniae* and *Buttiauxella agrestis*. *Enterococcus faecium* was the species most frequently isolated from Slanetz–Bartley agar (54%, 66% and 50% of all enterococci before, during and after treatment, respectively), followed by *Enterococcus faecalis* (40%, 26% and 31% before, during and after treatment, respectively). Other *Enterococcus* species identified at lower frequencies included *E. avium*, *E. hirae*, *E. raffinosus*, *E. casseliflavus*, *E. gallinarum* and *E. durans*. *E. faecium* was the major contributor to ampicillin resistance, accounting for 80%, 89% and 62% of all ampicillin-resistant enterococci before, during and after treatment, respectively.

Figure 1 shows the prevalence of dogs carrying ampicillin-resistant *E. coli*, cefotaxime-resistant *E. coli* and ampicillin-resistant enterococci on each day and in each treatment group. The prevalence of dogs carrying ampicillin-resistant enterococci increased significantly during antibiotic treatment taking both treatment groups together ($P = 4.35 \times 10^{-5}$, OR = 5.58, 95% CI = 2.5–13.62) and separately (amoxicillin: $P = 0.0053$, OR = 5.8, 95% CI = 1.8–24.9; amoxicillin/clavulanic acid: $P = 0.0029$, OR = 5.4, 95% CI = 1.9–18.64). There were no significant differences between treatment groups in the prevalence of dogs carrying any of the resistance indicators, and such prevalence was not significantly influenced by treatment duration.

Before treatment, the counts of total *E. coli* and enterococci ranged from 0 to 2.4×10^9 cfu/g (mean = 1.2×10^8, median = 4.7×10^6), and 0 to 5.2×10^7 cfu/g (mean = 6.7×10^6, median = 7.3×10^3), respectively. Figure 2 shows counts of total and resistant bacterial indicators in both treatment groups. Poisson regression analysis showed significantly higher counts of total enterococci in the amoxicillin/clavulanic acid group compared with the amoxicillin group ($P = 0.0164$) (Table 2). This difference between treatment groups was, however, observed on all three sampling days, meaning that it was likely to be a random effect not associated with treatment. The model that better fitted data on total *E. coli* counts included the variable *Treatment duration*; however, it did not have a significant effect. For all the remaining indicators, the models that better fit the data were those excluding *Day*, *Treatment* and *Treatment duration*, indicating a lack of effect of these variables (Table 2).

The proportion of ampicillin-resistant *E. coli* and ampicillin-resistant enterococci increased significantly during treatment. The increased proportion of resistance returned to initial values for ampicillin-resistant *E. coli*, while it remained significantly higher for ampicillin-resistant enterococci after the termination of treatment. The proportion of resistant counts was not significantly different between treatment groups on any of the days. Figure 3 shows the proportions of resistant bacterial counts across days and treatment groups, and the resulting *P* values of the Wilcoxon test comparing the proportions of each resistance indicator between days.

Microbial richness and diversity

Sequencing of the 16S rRNA gene amplicon library generated a total of 4 970 688 reads, with an average of 28 243 reads per sample (range 1878–56 947; median 28 168).
Table S2 provides the abundance matrix (reads) at the genus level. Both antibiotic treatments resulted in decreased α-diversity of the faecal microbiota during treatment, as shown by a small but significant drop in the Shannon ($P = 1.1 \times 10^{-5}$ for univariate analysis) and Chao1 ($P = 1.3 \times 10^{-3}$ for univariate analysis) indices (Figure 4). The composition of the bacterial community also changed during treatment, as shown by the principal coordinates analysis of unweighted (Figure 5) and weighted (not shown) UniFrac distances, corresponding to a reduction of the phylum Firmicutes and an increase of Proteobacteria (Table S3 and Figure S1). These changes of α- and β-diversity indices were reversed after the termination of

treatment. Treatment with amoxicillin or amoxicillin/clavulanic acid had a similar impact on the α- and β-diversity of the microbiota.

Effect of amoxicillin (AMX) and amoxicillin/clavulanic acid (AMC) treatments on the α-diversity of the intestinal microbiota. Mean±SD values of the Shannon index (a) and Chao1 index (b) in the AMX and AMC groups before, during and after antibiotic treatment. ANOVAs on variables *Day* (Before, During and After) showed a statistical difference for sampling times ($P = 1 \times 10^{-5}$ and 1×10^{-3} for the Shannon and Chao1 indices, respectively, univariate analysis). The indicated *P* values correspond to *post-hoc* Tukey tests performed on the variable *Day*.

The spectra of activity of the two drugs were analysed comparing the abundance of taxa at the family and genus levels between treatment groups on each day and between pairs of days within each treatment group. The family- and genus-based analysis showed a similar microbiota composition of both treatment groups before the start of treatment, yet different dynamics of specific taxa followed. For example, at the genus level, *Clostridium* and *Turicibacter* decreased in both groups during treatment, while other genera (*Dialister*, *Oscillospira*, *Roseburia* and an unclassified genus within Lachnospiraceae) decreased only in the amoxicillin/clavulanic acid group. Another remarkable finding was the increase of *Escherichia* in the amoxicillin/clavulanic acid group during treatment and decrease in the amoxicillin group after treatment. On average, the proportion of *Escherichia* increased during treatment with either amoxicillin ($P = 0.09$) or amoxicillin/clavulanic acid ($P = 9.4 \times 10^{-4}$) from approximately 4%–5% to approximately 20%–21% of the total reads per sample. However, whereas this proportion dropped drastically (down to 2.5%) after treatment with amoxicillin, it remained high (23%) after treatment with amoxicillin/clavulanic acid.

The dynamics observed at the family level reflected the results of the genus-based analysis in Enterobacteriaceae, Turicibacteriaceae and Succinivibrionaceae, whereas other genera belonging to Clostridiaceae, Peptostreptococcaceae, Bifidobacteriaceae, Ruminococcaceae, Helicobacteraceae and Veillonellaceae did not translate into significant changes at the family level. Dynamics of certain families, i.e. Streptococcaceae and Lachnospiraceae, reflected the genus results only when referring to the genera *Streptococcus*, *Dorea* and *Blautia*. Differential abundance analysis between days and treatment groups is shown in Table 3.

Discussion

It is generally accepted that antimicrobial drugs negatively influence the gut microbiome, but the impact of some drugs is more deleterious than that of others depending on their spectrum of activity. This has been shown by numerous studies assessing the ecological impact of antimicrobial drugs belonging to different drug classes.[8,10,14–16] This is the first study assessing the impact of two closely related formulations (amoxicillin and amoxicillin/clavulanic acid), and it provides interesting indications about the effect of clavulanic acid on the faecal microbiota.

Clavulanic acid broadens the spectrum of amoxicillin by including bacterial taxa that can be resistant to amoxicillin due to β-lactamase production. These taxa include *Bacteroides* spp. and other bacteria that are normal commensals of the gut and carry chromosomal or plasmid-mediated β-lactamases.[19] Moreover, although clavulanic acid displays limited antibacterial activity, there is evidence that it also increases the activity of β-lactams by mechanisms other than the inhibition of β-lactamases. Due to these properties of clavulanic acid, amoxicillin/clavulanic acid is generally regarded as a drug with a broader spectrum and higher selective potential than amoxicillin. Indeed, our study indicates that oral administration of clavulanic acid in combination with amoxicillin reduces the abundance of the genera *Dialister*, *Oscillospira*, *Roseburia* and one unclassified Lachnospiraceae taxon compared with amoxicillin treatment alone. Direct comparison between the amoxicillin and amoxicillin/clavulanic acid groups revealed higher amounts of *Dialister* and *Roseburia* during treatment, and *Dialister* and *Lactococcus* after treatment in the amoxicillin group (Table 3). These differences suggest that inclusion of clavulanic acid affects certain fractions of the commensal gut microbiota that are not affected by amoxicillin alone. Some members of *Lactococcus*, *Roseburia*, Lachnospiraceae and the less known *Oscillospira* have been reported as short-chain fatty acid (SCFA) producers, biomarkers of health or organisms with probiotic potential.[20–24] Thus, it appears that some organisms that are regarded as beneficial to gut health could be affected by the inclusion of clavulanic acid in amoxicillin formulations. The degree of the changes observed at the genus level and the importance of a genus within the structure of its particular family reflect the dynamics observed in the family-based analysis. Families such as Lachnospiraceae and Streptococcaceae are composed of taxa associated with both health and disease, and therefore

analysis at lower taxonomic levels (genus, or species when possible) may allow a better understanding of the health implications of antimicrobial treatment.

The negative consequences of antimicrobial exposure include selection of opportunistic pathogens that reside as commensals in the gut microbiome. The most notable example is *E. coli*, which is one of the most common bacterial pathogens in both humans and dogs. Based on culture, total *E. coli* counts increased slightly during treatment with both drugs (Figure 2). This increase of *E. coli*, together with the observed increase of Proteobacteria in our study is in line with previous metagenomics studies reporting an increase of *E. coli*, Enterobacteriaceae and/or Proteobacteria following treatment with amoxicillin or amoxicillin/clavulanic acid in humans,[13,25] dogs,[7] piglets,[8,9] Wistar rats[10] and farmed mink.[12] Our sequencing data, however, showed that the genus *Escherichia* increased significantly following treatment with amoxicillin/clavulanic acid only, suggesting a possible additional negative effect attributable to clavulanic acid. High counts of intestinal *E. coli* are associated with pathological conditions such as type 2 diabetes, colorectal cancer and allergies in humans,[26–28] and intestinal bowel disease in humans and dogs.[29,30] Moreover, dysbiosis characterized by lower bacterial diversity and increased Enterobacteriaceae has been associated with increased susceptibility to specific pathogens, improper cognitive or immune development and poor response to drugs, including antibiotics, as seen in murine infection models and human patients undergoing therapy.[28] High concentrations of *E. coli* in faeces may also be a risk factor for urinary tract infections (UTIs), which are normally caused by uropathogenic *E. coli* strains resident in the faecal microbiota of the patient. In that regard, previous studies suggest that exposure to antimicrobials is a risk factor for UTI,[31] including UTI caused by resistant strains.[32] The *E. coli* enrichment observed by 16S rRNA gene sequencing following treatment with amoxicillin/clavulanic acid might be explained by the limited effects of clavulanic acid on this bacterial species. It has been shown that inhibition of amoxicillin/clavulanic acid-susceptible or intermediate *E. coli* (as defined by *in vitro* testing using a 2:1 ratio of amoxicillin and clavulanic acid) requires drug concentrations that are difficult to achieve by oral treatment in the presence of high bacterial concentrations ($\geq 10^7$ cfu/mL)[33] such as those occurring in faeces. However, this result was not corroborated by phenotypic counts of total *E. coli*, which seemed to be higher in the amoxicillin/clavulanic acid-treated group but the increase was not statistically significant (models including variables *Treatment* or *Day* returned the highest AIC values and showed no significant differences between treatments when run; $P = 0.332$). Phenotypic *E. coli* counts could be

inaccurate due to inclusion of lactose-positive colonies belonging to other Enterobacteriaceae, as indicated by the detection of *Klebsiella* or *Buttiauxella* among the single colonies that were selected for MALDI-TOF identification.

As for the selection of β-lactam resistance, inclusion of clavulanic acid in the treatment did not significantly influence the counts of any of the resistance indicators. Oral treatment with amoxicillin and amoxicillin/clavulanic acid had similar effects on selection of ampicillin-resistant *E. coli* and enterococci, namely an increase in the number of dogs carrying ampicillin-resistant enterococci (Table 1) and in the proportion of ampicillin-resistant *E. coli* and ampicillin-resistant enterococci during treatment (Figure 3). While the proportion of ampicillin-resistant *E. coli* returned to the initial values after cessation of therapy, the proportion of ampicillin-resistant enterococci remained high. These different dynamics suggest a longer term impact of treatment on resistance in enterococci compared with *E. coli*. The low number of samples harbouring cefotaxime-resistant *E. coli* in the two groups (19 samples in total) did not allow us to assess the effect of treatment on this important indicator. It should be noted that the methodology selected to analyse proportions was different from that used to analyse bacterial counts due to the lack of linearity of the data and poor behaviour of the models fitting the proportions as a binomial distribution (as number of successful/failed cases).

Both treatments reduced the richness and diversity of the faecal microbiota during treatment but α- and β-diversity indices returned to baseline levels after cessation of treatment. This finding is corroborated by numerous previous studies on the effect of amoxicillin or amoxicillin/clavulanic acid.[7,14,25] The short-lived effect found in our study is in accordance with the results of a previous study of amoxicillin exposure in healthy dogs, where the diversity index returned to pre-exposure levels within 2 weeks.[7] However, it is only partly in agreement with two previous studies on human volunteers treated with amoxicillin or amoxicillin/clavulanic acid (PO 500 mg twice daily for 3/7 days and 875/125 mg twice daily for 7 days, respectively).[14,25] These studies reported changes in microbial diversity associated with the antimicrobial treatment that persisted for longer periods of time (>6 months),[14] or that recovered only partially 2 weeks after the termination of treatment. This divergence between studies could be due to variations in amoxicillin dosage and treatment duration as well as methodological or host-related differences.

In a recent study, pigs were used as a model to evaluate differences between the effects of amoxicillin and ertapenem on microbiome composition and selection of antimicrobial resistance genes.[8] The study evidenced the usefulness of animal models to compare the impact of antimicrobial agents on development of dysbiosis and selection of antimicrobial resistance. This information is useful for developing recommendations on prudent antimicrobial use, since there is an increasing demand for therapeutic guidelines that consider the negative consequences of antimicrobial treatment in addition to clinical efficacy. Dogs were chosen in this study, as amoxicillin and amoxicillin/clavulanic acid are widely used drugs for managing bacterial infections in these companion animals. Moreover, their gut microbiome is closer to the human microbiome than the microbiome of either pigs or mice,[34] suggesting that dogs are better models for studying antimicrobial-mediated dysbiosis of the human gut microbiota.

The time and conditions used to store faecal samples in this study (up to 26 h at room temperature) are not expected to have a significant impact on the microbiome based on previous studies on the feline faecal microbiota.[35] There is evidence that variations observed between individuals are larger than the changes introduced by storage conditions such as temperature, duration and preservation media.[36–39] Moreover, various studies argue that temperature or storage duration may not have a radical influence on operational taxonomic unit (OTU) composition,[37–40] and that the observed changes may not be large enough to mask the effect of other conditions under study such as treatment or disease.[38] As for other studies investigating antimicrobial effects on gut microbiota, the relatively small sample size (21 dogs/group) is an obvious limitation of the study and it cannot be excluded that additional differences between the two treatment groups could have been detected in a larger population.

In conclusion, our study shows that oral treatment with amoxicillin and amoxicillin/clavulanic acid similarly affects microbial richness and increases carriage of ampicillin-resistant enterococci and ampicillin-resistant *E. coli* in dog faeces. The impact attributable to clavulanic acid appears to be limited to a reduction of some bacterial taxa that are known to have beneficial effects on gut health, and may include a possible increase of the opportunistic pathogen *E. coli*.

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Table 1. Characteristics of the 42 dogs included in the study. Dogs were treated with either amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC).

	AMX-treated group	AMC-treated group
N dogs	21	21
N dogs with all three samples	16	17
Female:male	13:8	12:9
Age (m)	Mean= 83m (6y 11m)	Mean= 68m (5y 8m)
	Min.= 6m	Min.= 4m
	Max.= 180m (15y)	Max.= 150m (12y 6m)
Weight (Kg)	Mean= 24.2 Kg	Mean= 23.6 Kg
	Min.= 3.5 Kg	Min.= 2.1 Kg
	Max.= 46 Kg	Max.= 52.5 Kg
Treatment duration	5 days (n=7)	5 days (n=9)
	7 days (n=12)	7 days (n=9)
	10 days (n=1)	10 days (n=1)
	13 days (n=1)	11 days (n=1)
		14 days (n=1)
Day of collection of second sample (during treatment)	Day 3 (n=4)	Day 3 (n=2)
	Day 4 (n=12)	Day 4 (n=13)
	Day 5 (n=5)	Day 5 (n=5)
		Day 6 (n=1)
Day of collection of third sample (during treatment)	Day 10 (n=2) Day 14 (n=2)	Day 9 (n=1) Day 13 (n=6)
	Day 11 (n=2) Day 15 (n=1)	Day 10 (n=1) Day 14 (n=1)
	Day 12 (n=4) Day 16 (n=1)	Day 11 (n=4) Day 17 (n=1)
	Day 13 (n=3) Day 17 (n=1)	Day 12 (n=2) Day 18 (n=1)
Days between cessation of third sample	5 days (n=5) 10 days (n=1)	4 days (n=1) 7 days (n=2)
	6 days (n=6) 12 days (n=1)	5 days (n=2) 8 days (n=1)
	7 days (n=3)	6 days (n=11)
System involved in diagnosis	dermatology (n=14)	dermatology (n=11)
	urogenital (n=5)	postoperative (n=3)
	dental (n=1)	trauma (n=2)
	ophthalmology (n=1)	urogenital (n=1)
		dental (n=1)
		gastrointestinal (n=1)
		respiratory (n=1)

Table 2. Summary of the Poisson regression analysis of bacterial counts (cfu) in dogs treated with amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC) taking into account repeated measures. For each bacterial population, the best model was selected based on AIC analysis. The list of variables tested included Day (before/during/after), Treatment (AMX/AMC) and Treatment duration (expressed in days). Bold indicates statistical significance ($P \leq 0.05$).

Analysed bacterial population (independent variable)		Variables included in the model best fitting the data (dependent variables)	Significant outcome
	random effect	fixed effects	
Total *E. coli*	(1|Dog:Day)	*Treatment duration*	none (*Treatment duration P*=0.138)
AMP-resistant *E. coli*	(1|Dog:Day)	none	none
CTX-resistant *E. coli*	(1|Dog:Day)	none	none
Total enterococci	(1|Dog:Day)	*Treatment*	*Treatment* (*P*=0.0164)
AMP-resistant enterococci	(1|Dog:Day)	none	none

Table 3. Significantly different abundant genera between treatment days (from before to during treatment, from during to after treatment and from before to after treatment) within each group of dogs treated with amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC), and significantly different genera between treatment groups (AMX and AMC) on each treatment day (before, during and after treatment)

Genus	**Log fold change**	***P* value**	**Adjusted *P* value**
Differentially abundant genera comparing before and during AMX treatment			
Clostridium[1a]	–6.92	6.47×10^{-5}	1.65×10^{-3}
Bifidobacterium	8.12	5.42×10^{-3}	4.60×10^{-2}
Clostridium[2a]	–1.69	2.37×10^{-3}	4.03×10^{-2}
Clostridium[3a]	–3.59	5.10×10^{-3}	4.60×10^{-2}
Streptococcus[b]	–4.92	5.33×10^{-3}	4.60×10^{-2}
Turicibacter[b]	–12.10	5.34×10^{-16}	2.73×10^{-14}
Differentially abundant genera comparing during and after AMX treatment			
Escherichia[b]	–3.69	1.19×10^{-4}	4.37×10^{-3}
Lactococcus[b]	22.00	9.06×10^{-14}	4.98×10^{-12}
Turicibacter[b]	28.20	4.35×10^{-45}	4.78×10^{-43}
Differentially abundant genera comparing before and after AMX treatment			
Anaerobiospirillum[b]	–27.8	4.56×10^{-21}	4.70×10^{-19}
Lactococcus	22.6	1.89×10^{-14}	9.73×10^{-13}
Differentially abundant genera comparing before and during AMC treatment			
Clostridium	–7.06	3.20×10^{-5}	2.49×10^{-4}
Dialister	–24.00	2.20×10^{-16}	4.62×10^{-15}
Escherichia[b]	3.72	1.57×10^{-4}	9.44×10^{-4}
Oscillospira	–6.01	3.55×10^{-5}	2.49×10^{-4}
Roseburia	–7.00	2.14×10^{-6}	2.25×10^{-5}
Turicibacter[b]	–27.90	8.84×10^{-71}	3.71×10^{-69}
unclassified *Clostridiales*	–6.32	1.25×10^{-6}	1.75×10^{-5}
unclassified *Lachnospiraceae*	–2.64	3.64×10^{-03}	1.91×10^{-2}
Differentially abundant genera comparing during and after AMC treatment			
*Dorea*b	3.81	3.00×10^{-4}	8.51×10^{-3}
Sarcina	24.20	2.54×10^{-17}	1.08×10^{-15}
Turicibacter[b]	25.50	5.71×10^{-42}	4.85×10^{-40}
Differentially abundant genera comparing before and after AMC treatment			
Blautia[b]	2.26	0.000630	0.0274
Helicobacter	–4.77	0.000607	0.0274
Differentially abundant genera between treatment groups AMX and AMC before treatment			
None			
Differentially abundant genera between treatment groups AMX and AMC during treatment			
Dialister	–24.20	1.04×10^{-16}	9.76×10^{-15}
Roseburia	–6.88	5.63×10^{-6}	2.65×10^{-4}
Differentially abundant genera between treatment groups AMX and AMC after cessation of treatment			
Dialister	–23.60	6.96×10^{-16}	3.69×10^{-14}
Escherichia[b]	3.08	1.23×10^{-3}	3.27×10^{-2}
Lactococcus	–23.30	1.69×10^{-15}	5.97×10^{-14}
Sarcina	23.50	6.46×10^{-16}	3.69×10^{-14}

a. *Clostridium* is classified as three families according to the Greengenes v13_8 database: Peptostreptococcaceae (*Clostridium*[1]), Clostridicaceae (*Clostridium*[2]) and Lachnospiraceae (*Clostridium*[3]).
b. The significant differences in this genus were also observed at their corresponding family level.

Figure 1. Percentage of dogs carrying resistance indicators ampicillin (AMP)-resistant *E. coli*, cefotaxime (CTX)-resistant *E. coli* and AMP-resistant enterococci before, during and 1 week after cessation of treatment with amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC). AMP-resistant enterococci increased significantly during treatment in both treatment groups, analysed together ($P = 4.35 \times 10^{-5}$) and separately (AMX, $P = 5.3 \times 10^{-3}$; AMC, 2.9×10^{-3}).

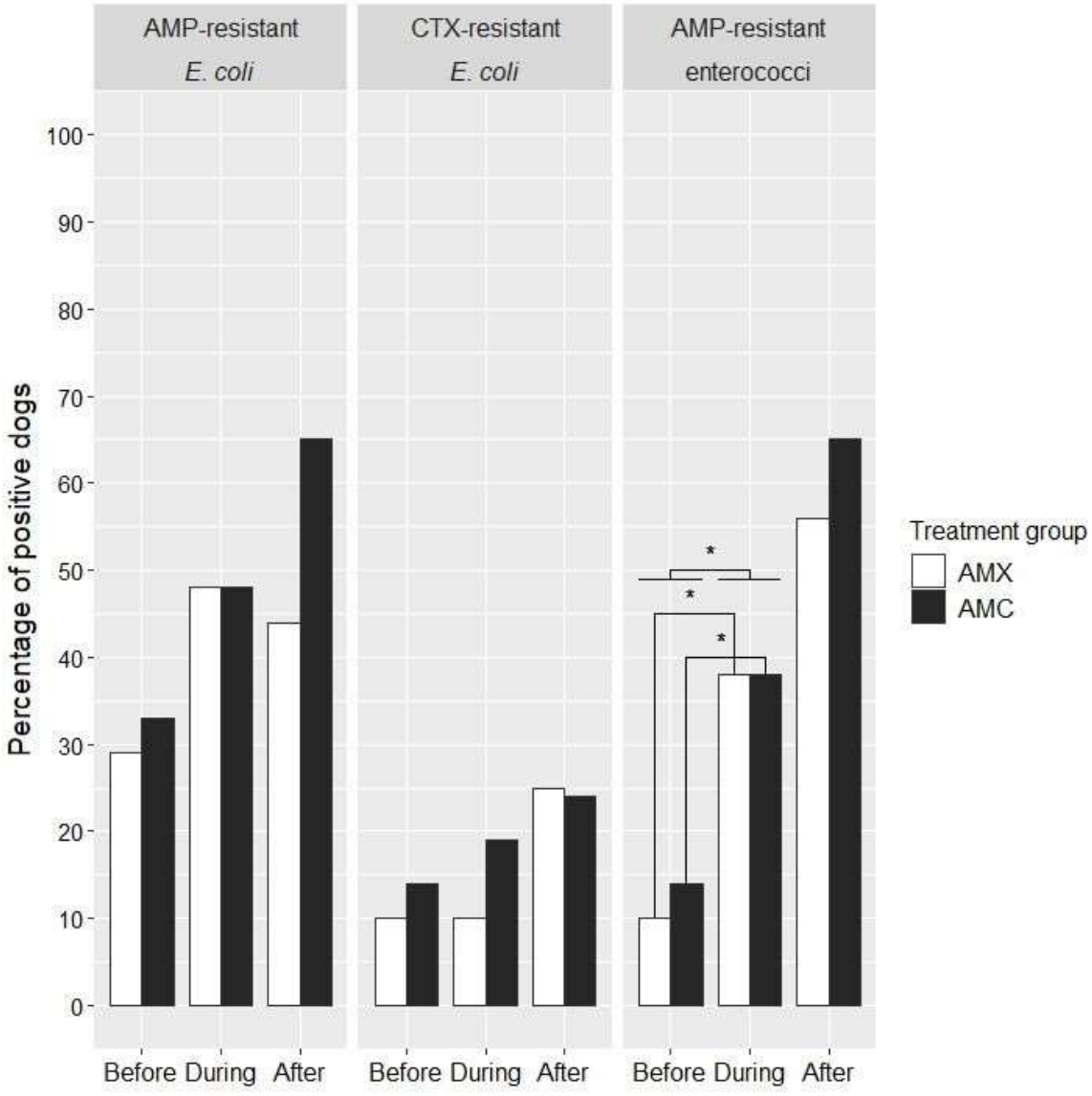

Figure 2. Counts of total and resistant bacterial indicators ampicillin (AMP)-resistant *E. coli*, cefotaxime (CTX)-resistant *E. coli* and AMP-resistant enterococci in faecal samples from dogs before, during and after antibiotic treatment with amoxicillin (AMX, n = 21) or amoxicillin/clavulanic acid (AMC, n = 21).

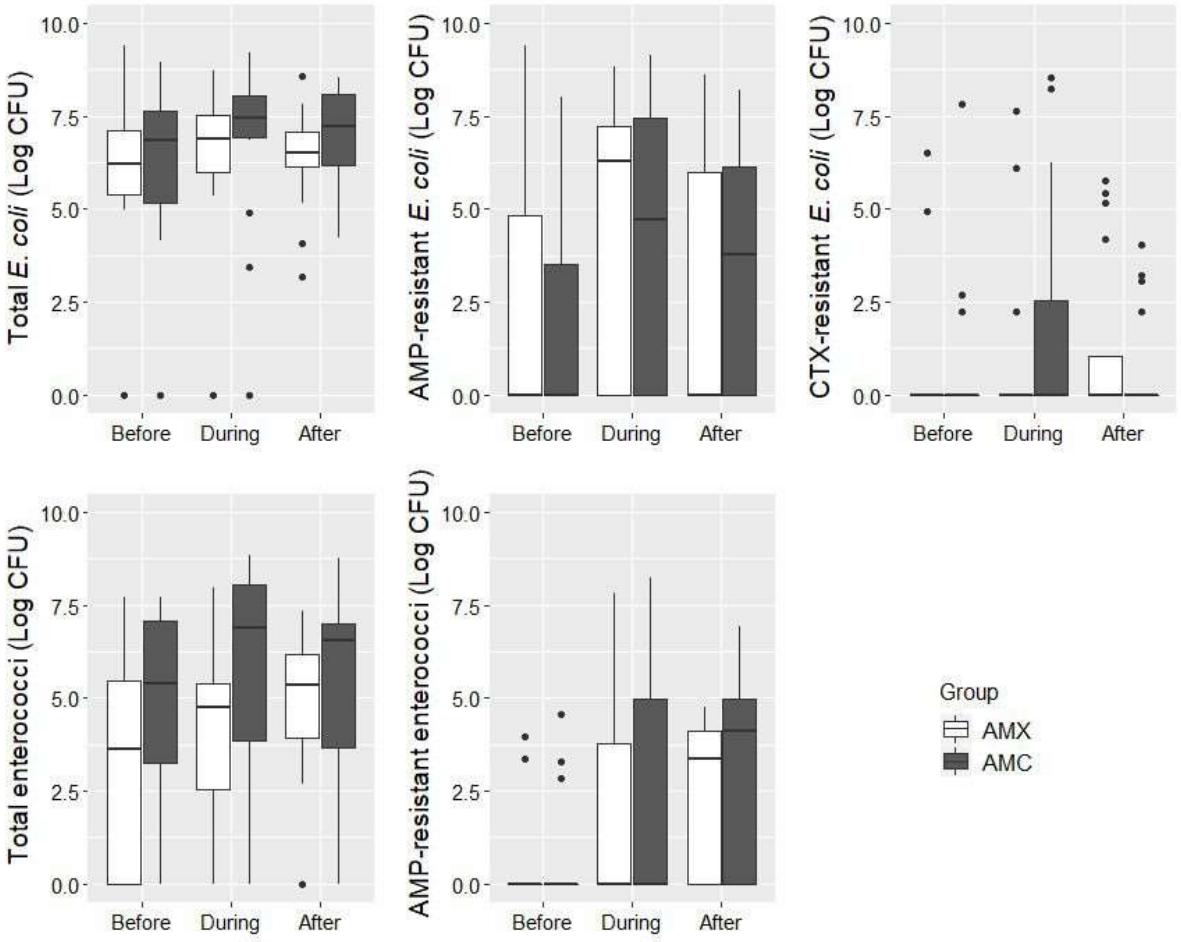

Figure 3. Proportion of antimicrobial-resistant bacterial indicators (resistant population/total population) ampicillin (AMP)-resistant *E. coli*, cefotaxime (CTX)-resistant E. coli and AMP-resistant enterococci in faeces from dogs before, during and after antibiotic treatment with amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC). Plots include P values resulting from comparing the proportions between treatment days (Before, During, After) by Wilcoxon test. Analysis comparing the two antimicrobial treatments did not reveal any significant differences.

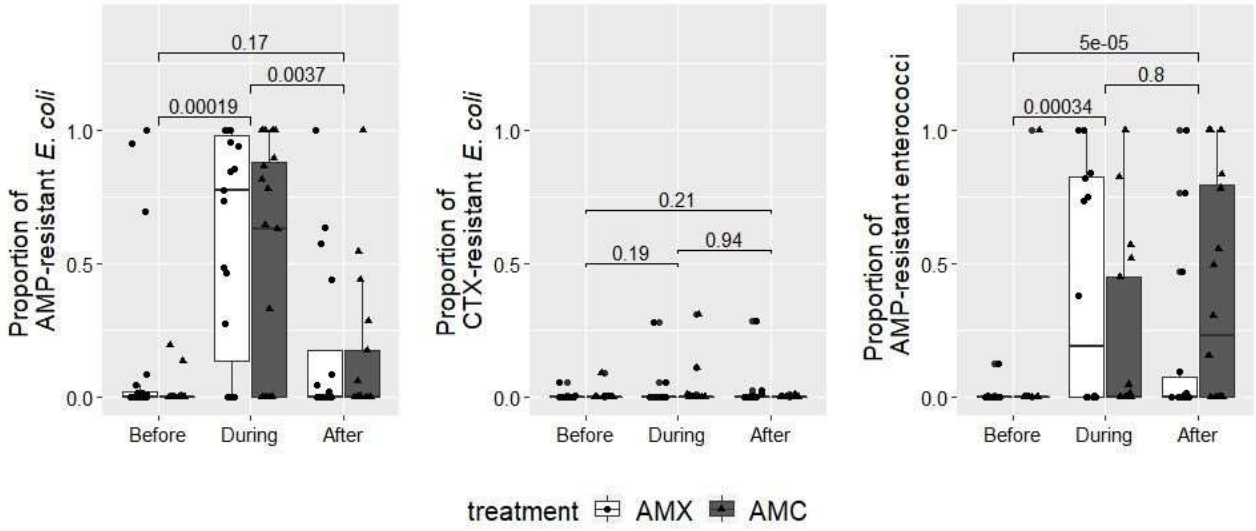

Figure 4. Effect of amoxicillin (AMX) and amoxicillin/clavulanic acid (AMC) treatments on the α-diversity of the intestinal microbiota. Mean±SD values of the Shannon index (a) and Chao1 index (b) in the AMX and AMC groups before, during and after antibiotic treatment. ANOVAs on variables *Day* (Before, During and After) showed a statistical difference for sampling times ($P = 1 \times 10^{-5}$ and 1×10^{-3} for the Shannon and Chao1 indices, respectively, univariate analysis). The indicated *P* values correspond to *post-hoc* Tukey tests performed on the variable *Day*.

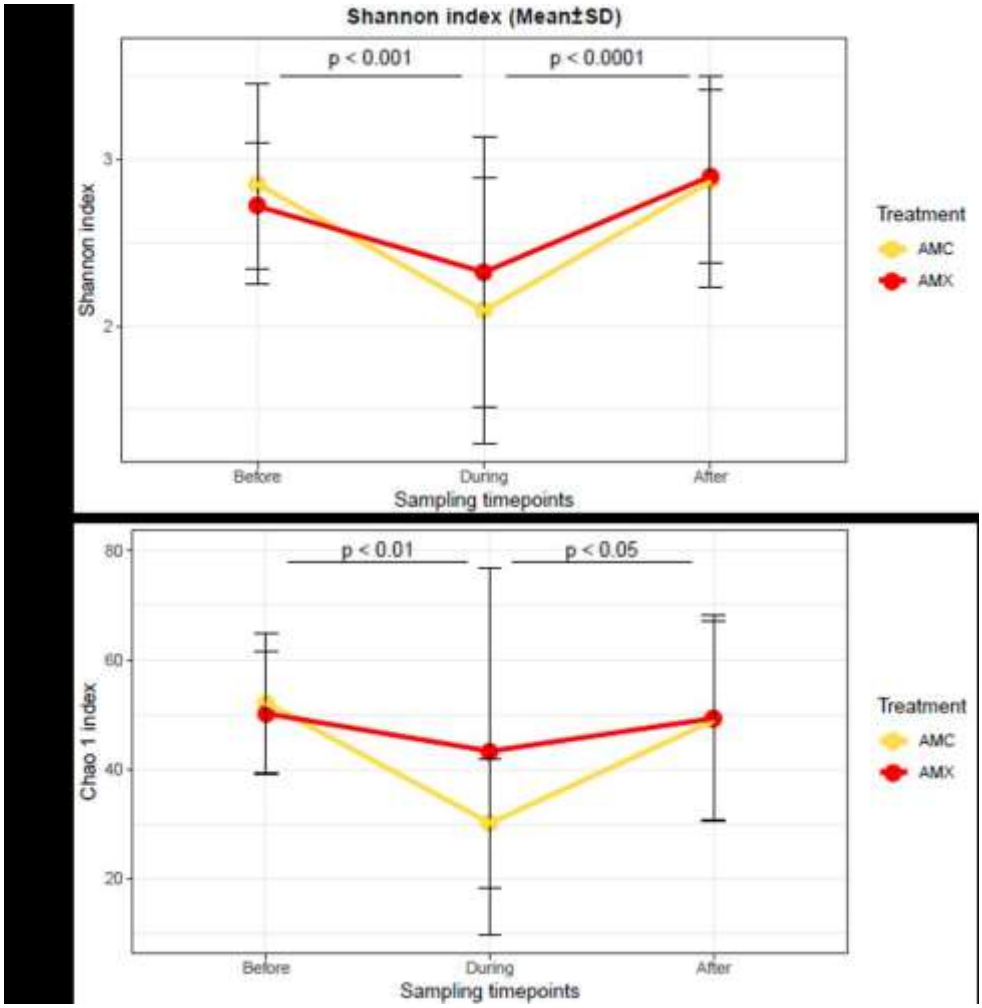

Figure 5. Principal coordinates analysis (unweighted UniFrac distances) of the faecal microbiota composition of dogs before (green), during (blue) and after (red) undergoing treatment with amoxicillin (AMX) or amoxicillin/clavulanic acid (AMC).