Effect of spiramycin and metronidazole on canine dental biofilm bacteria

Jana Kačírová[1], Miriam Sondorová[1], Natália Šurín Hudáková[1], Tomáš Lipták[2], Aladár Maďari[2], Kristína Mravcová[3], Lívia Kolesár Fecskeová[4], Rastislav Mucha[5], Marián Maďar[1]

[1]University of Veterinary Medicine and Pharmacy in Košice, Department of Microbiology and Immunology, Košice, Slovakia
[2]University of Veterinary Medicine and Pharmacy in Košice, University Veterinary Hospital, Košice, Slovakia
[3]Czech Academy of Sciences, v.v.i, Institute of Vertebrate Biology, Brno, Czech Republic
[4]P.J. Šafárik University in Košice, Faculty of Medicine, Associated Tissue Bank, Košice, Slovakia
[5]Biomedical Research Center of the Slovak Academy of Sciences, Institute of Neurobiology, Košice, Slovakia

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Abstract

Periodontal diseases are the most common condition in companion animal practice. The administration of antibiotics is associated with the therapy of these diseases. The aim of the present study was to verify the effect of antibiotics on canine dental biofilm bacteria due to increasing antibiotic resistance. Dental biofilm samples were taken from six dogs before and after administration of antibiotics, specifically, the combination of spiramycin and metronidazole. The samples were cultured on solid media under aerobic and anaerobic conditions. Sequencing analysis of the 16S rRNA gene was used to identify bacterial isolates. In addition, total bacterial DNA was extracted from samples from one dog and the V3−V4 region of the 16S rRNA gene was sequenced using the Illumina MiSeq platform. Using cultivation, 55 isolates belonging to 4 phyla were isolated before antibiotics administration, and 36 isolates belonging to 3 phyla were isolated after antibiotics administration. A significant decrease was noted in the genera *Porphyromonas*, *Neisseria* and *Frederiksenia*, whereas there was a significant increase in the genus *Streptococcus*. Of the total microbiota, there were 69 operational taxonomic units (OTUs) belonging to 11 phyla before antibiotics administration, and 51 OTUs belonging to 8 phyla after antibiotics administration. A significant decrease was recorded in the genus *Porphyromonas*, while a significant increase in the genus *Capnocytophaga*. The significant effect of spiramycin and metronidazole on the genus *Porphyromonas* at the time of their administration was confirmed by both cultivation and amplicon sequencing.

Oral bacteria, periodontal diseases, antibiotics, dog

Periodontal diseases are a group of multi-factorial inflammatory diseases with multiple clinical forms that occur in humans, dogs, cats, and other mammalian species (Rodrigues et al. 2021). Among the aetiological factors involved in the initiation and development of periodontal diseases, dental biofilm bacteria, especially Gram-negative anaerobic bacteria, play an important role (Di Bello et al. 2014). These bacteria cause abnormal host immune responses, followed by destruction of periodontal tissues such as cementum, periodontal ligaments and alveolar bone, which can lead to tooth loss (Nomura et al. 2020). Periodontal diseases based on the severity of clinical and radiographic lesions are classified into four stages: stage I – gingivitis, stage II – early periodontitis, stage III – moderate periodontitis and stage IV – advanced periodontitis (Carvalho et al. 2015).

The therapy of periodontal diseases in dogs ranges from the removal of dental biofilm and calculus using hand and ultrasonic instruments to the extraction of affected teeth and mucogingival surgery (Albuquerque et al. 2012). In severe and progressive cases, mechanical periodontal therapy is insufficient and additional antibiotic therapy to reduce periodontal pathogens is indicated (Giboin et al. 2012).

The aim of the present study was to identify bacteria present in the dental biofilm of dogs with periodontal diseases and to verify the effect of antibiotics, specifically spiramycin and metronidazole, on these bacteria.

Address for correspondence:
Jana Kačírová
University of Veterinary Medicine and Pharmacy in Kosice
Komenskeho 73, 041 81 Kosice, Slovakia,
Phone: +421915326232
E-mail: kacirova.jana@gmail.com
http://actavet.vfu.cz/

Materials and Methods

Animals and periodontal diseases therapy

In total, 6 dogs (3 females and 3 males) with periodontal diseases aged 3 to 14 years (mean age 8.5 years) and with a mean weight of 4.3 kg were included in the present study (Table 1). As part of periodontal disease therapy, they were orally administered STOMORGYL 2 (150 000 IU spiramycin and 25 mg metronidazole; MERIAL S.A.S., Lyon, France) at the recommended dose of 75 000 IU spiramycin and 12.5 mg metronidazole/kg bw/day for 7 days. On the fourth day, their dental biofilm and calculus were removed under general anaesthesia.

Table 1. General information about the studied animals.

Dog	Sex	Breed	Age (years)	Weight (kg)
1	♂	Jack Russell Terrier	14	6.2
2	♀	Yorkshire Terrier	6	3.8
3	♀	Maltese	8	3.1
4	♀	Maltese	3	3.6
5	♂	Prague Ratter	11	4.2
6	♂	Chihuahua	9	5.2

Prior to anaesthesia, preoperative clinical examinations, biochemical and haematological blood tests were performed. Based on these examinations, patients were classified into categories of the American Society of Anesthesiologists. Subsequently, an intravenous catheter was inserted into the vena cephalica antebrachii and premedication was first performed by intravenous (i.v.) administration of butorphanol (0.2 mg/kg; Butomidor 10 mg/ml, Richter Pharma, Wels, Austria). Approximately 2 min later, i.v. administration of medetomidine (0.005–0.01 mg/kg; Cepetor 1 mg/ml, CP-Pharma, Burgdorf, Germany) was performed. Induction by i.v. administration of propofol (Propofol 1% MCT/LCT Fressenius, Fresenius Kabi, Graz, Austria) was performed after another 2 min until the desirable effect was achieved (1–2 mg/kg). After the jaw tone disappeared, the endotracheal tube was inserted into the trachea and its cuff was inflated. The patient was also connected to a multi-parameter vital signs monitor (BeneView T8, Mindray, Shenzhen, China) using which ECG, haemoglobin oxygen saturation, end-tidal carbon dioxide, blood pressure, heart rate and rhythm, respiratory rate, and temperature were monitored. If the anaesthesia was shallow (jaw tone and swallowing reflex present) it was deepened or maintained by repeated administration of i.v. bolus of propofol at half the induction dose (0.5–1.0 mg/kg). During anaesthesia, a balanced electrolyte solution of Ringer-Lactate (Compound Sodium Lactate Ringer-Lactat, B.BRAUN, Melsungen, Germany) was administered i.v. at a rate of 5 ml/kg to support blood pressure and acid-base balance.

Dental prophylactic therapy of dogs according to the 2019 AAHA (the American Animal Hospital Association) Dental Care Guidelines for Dogs and Cats was performed. Under general anaesthesia, a complete dental examination, which includes periodontal probing of gingival sulcus depth of the entire tooth, was performed. Subsequently, the teeth were completely brushed using hand and ultrasonic instruments. After scaling, teeth were polished using a prophy cup and paste (KRUUSE, Langeskov, Denmark). Finally, the subgingival debris was rinsed off with saline or chlorhexidine.

Sample collection

Dental biofilm samples were collected twice; before antibiotic therapy and on day 4 of antibiotic therapy before teeth brushing. Using a sterile syringe needle, the dental biofilm was scraped off and transferred to an Eppendorf tube containing 300 µl of sterile phosphate-buffered saline. All biofilm samples were taken from dogs undergoing routine oral therapy at the Small Animal Clinic of the University Veterinary Hospital, University of Veterinary Medicine and Pharmacy in Košice. Informed consent was obtained from the owners of the dogs for the study. The study was approved by the Ethics Committee of the University of Veterinary Medicine and Pharmacy in Košice (EKVP/2022-02). The animals were handled in a humane manner in accordance with the guidelines established by the relevant committee. All applicable international, national and institutional guidelines for the care and use of animals were followed. None of the dogs involved in present study had taken antibiotics or oral antimicrobial agents (e.g. chlorhexidine) during the last 3 months prior to the start of the study.

Microbiological cultivation

Samples were vortexed at the maximum speed for 20 s and shaken at 1400 r.p.m. (Thermoshaker TS-100C, BioSan, Riga, Latvia) for 5 min for content homogenization. Homogenized samples were decimally serially diluted in phosphate-buffered saline and plated on Trypticase Soy Agar (TSA; Carl Roth GmbH and Co., Karlsruhe, Germany) supplemented with 5% sheep blood and Mitis Salivarius agar (Sigma Aldrich, Steinheim, Germany) supplemented with 1% potassium tellurite solution (Sigma Aldrich). The plates of both media were incubated under aerobic and anaerobic conditions at 37 °C. BBL GasPak Plus (Becton, Dickinson and Co., Maryland, USA) was used to achieve anaerobic conditions. The plates were examined after 2 days of aerobic and after 3 and 7 days of anaerobic cultivation, respectively. Solitary colonies with different morphological characteristics, such as shape, size, colour and growth form, were selected and subsequently sub-cultured on TSA with 5% sheep blood for 24 h under aerobic or 48 h under anaerobic conditions at 37 °C.

16S rRNA sequencing of isolates

Bacterial DNA was extracted from pure bacterial cultures using a DNAzol direct (Molecular Research Center Inc., Cincinnati, USA) according to the manufacturer's instructions. The PCR was processed using 1.0 µl of sample (DNA template) added to 30 µl of reaction mixture containing OneTaq 2X Master Mix with Standard Buffer (New England Biolabs, Foster City, USA), universal primers (27F and 1492R) and molecular grade water. The thermal cycling programme consisted of initial activation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min and an extension step at 72 °C for 3 min. Finally, the amplification was completed by the extension step at 72 °C for 10 min. The positive PCR products were visualized on 2% agarose gel under UV light, using GelRed (Biotium, Inc., Hayward, USA). If there was no amplification product in the samples, DNA was isolated from pure bacterial cultures using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Irvine, USA) and PCR was performed under the same conditions. All positive PCR products were sent for purification and sequencing to the Microsynth (Vienna, Austria). The obtained chromatograms of sequences were edited using Geneious 8.0.5 (Biomatters, Auckland, New Zealand). The final sequences were compared with other bacterial sequences of the 16S rRNA genes in the National Center for Biotechnology Information (NCBI) database using BLASTn analysis to identify isolates.

Sample DNA extraction and processing

Bacterial genomic DNA was extracted from both samples taken from dog no. 3, before and after antibiotics administration, according to the protocol by Vesty et al. (2017) with some modifications. Briefly, aliquots of homogenized samples were centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was discarded. The pellets were resuspended in 180 µl of lysis buffer (50 mM Tris-HCl, 10 mM EDTA and 1% sodium dodecyl sulphate) and 25 µl of proteinase K was added to the mixture. The tubes were incubated at 55 °C for 2 h, with shaking at 300 r.p.m. (Thermoshaker TS-100C), after which proteinase K was inactivated by heating at 95 °C for 5 min. The tubes were then centrifuged at 10,000 × *g* for 5 min at 23 °C and the supernatant was transferred to new Eppendorf tubes. Phenol and chloroform were equally (1:1) added to the supernatant and centrifuged at 10,000 × *g* for 5 min at 23 °C. The upper aqueous phase was transferred to a new Eppendorf tube. Subsequently, isopropanol (0.6 volume of supernatant) and 3 M sodium acetate solution (0.1 volume of supernatant) were added. The nucleic acids were precipitated overnight at 4 °C. The following day, DNA was pelleted at 10,000 × *g* for 10 min at 4 °C, washed with 100 µl of cold 75% ethanol and dried at 35 °C for 5–10 min. The pellets were resuspended in 30 µl of DNA Elution Buffer (Zymo Research). Extracted bacterial DNA was sent for sequencing of the V3−V4 region of the bacterial 16S rRNA gene on Illumina MiSeq (2 × 250 bp) to the Microsynth (Balgach, Switzerland).

Sequence data processing

Locus specific primer sequences (341F and 805R) were trimmed in Microsynth. Initial processing of the obtained sequences was carried out in SEED2 (Větrovský et al. 2018). Reads were joined using the fastq-join function with default settings, the sequences were filtered for mean sequence quality ≥ 30 and the correct length of the amplicon (approx. 420 bp). A chimera check was performed using UPARSE (built in SEED2). Further processing was carried out using the Silva NGS online platform (https://www.arb-silva.de/ngs/) with operational taxonomic unit clustering threshold set at 98% similarity.

Statistical analysis

Paired *t*-test in the GraphPad Prism 9.3.1 (GraphPad Software, San Diego, USA) statistical program was used to analyse the difference between samples before and after antibiotics administration. Data were reported as means and *P*-value less than 0.05 ($P < 0.05$) was considered significant.

Results

The composition of dental biofilms of 6 dogs before and after administration of antibiotics was examined by standard cultivation on solid media and in one of the dogs by 16S rRNA gene amplicon sequencing as well.

Cultivable bacterial community

In total, 55 isolates from samples before antibiotics administration and 36 isolates from samples after antibiotics administration were obtained by cultivation. Bacterial strains isolated from samples before antibiotics administration belonged to 4 phyla, namely Proteobacteria, Actinobacteriota, Bacteroidota and Firmicutes. Representatives of the phylum Bacteroidota were not isolated after antibiotics administration, only representatives of the phyla Actinobacteriota, Firmicutes and Proteobacteria were isolated (Plate II, Fig. 1).

Bacteria of the genera *Porphyromonas* and *Bergeyella* from the phylum Bacteroidota were detected before, but not after the administration of antibiotics. Bacteria of the genera *Neisseria*, *Corticibacter*, *Kingella*, *Lampropedia*, *Lysobacter* and *Moraxella* from the phylum Proteobacteria were also detected before, but not after the administration of antibiotics. On the other hand, genera *Actinobacillus* and *Escherichia* from the phylum Proteobacteria, *Gemella* from the phylum Firmicutes and *Tessaracoccus* from the phylum Actinobacteriota were detected only after antibiotics administration. All detected genera and their relative abundances are shown in Fig. 2 (Plate II). Relative abundance of genera *Porphyromonas* ($P < 0.0001$), *Neisseria* ($P < 0.01$) and *Frederiksenia* ($P < 0.05$) was significantly higher before antibiotics administration than after antibiotics administration, whereas relative abundance of genus *Streptococcus* ($P < 0.05$) was significantly higher after antibiotics administration (Table 2).

Table 2. Relative abundance of cultivable bacterial genera present in canine dental biofilms before and after the administration of antibiotics at the genus level.

Genus (phylum)	Before antibiotics (n = 55)	After antibiotics (n = 36)	*P* value
Actinomyces (Actinobacteriota)	9.20%	13.21%	0.3474
Corynebacterium (Actinobacteriota)	9.39%	19.76%	0.1206
Gleimia (Actinobacteriota)	7.30%	9.88%	0.6597
Schaalia (Actinobacteriota)	11.05%	11.55%	0.9132
Tessaracoccus (Actinobacteriota)	0.00%	5.00%	0.2031
Bergeyella (Bacteroidota)	1.85%	0.00%	0.3632
Porphyromonas (Bacteroidota)	11.05%	0.00%	< 0.0001
Gemella (Firmicutes)	0.00%	3.33%	0.3632
Staphylococcus (Firmicutes)	3.70%	5.71%	0.6213
Streptococcus (Firmicutes)	8.86%	18.21%	0.0308
Actinobacillus (Proteobacteria)	0.00%	1.67%	0.3632
Corticibacter (Proteobacteria)	1.67%	0.00%	0.3632
Escherichia (Proteobacteria)	0.00%	3.33%	0.3632
Frederiksenia (Proteobacteria)	8.97%	1.67%	0.0267
Kingella (Proteobacteria)	1.67%	0.00%	0.3632
Lampropedia (Proteobacteria)	1.52%	0.00%	0.3632
Lysobacter (Proteobacteria)	1.67%	0.00%	0.3632
Moraxella (Proteobacteria)	2.08%	0.00%	0.3632
Neisseria (Proteobacteria)	10.64%	0.00%	0.0098
Pasteurella (Proteobacteria)	9.39%	6.67%	0.6403

Total bacterial community

The 17 059 assembled sequences (9 367 before and 7 692 after administration of antibiotics) were divided in 143 operational taxonomic units (OTUs) at 98% clustering threshold. In total, 0.02% of sequences could not be assigned to any taxa. Only OTUs representing at least 0.1% of sequences in at least one sample were selected for further calculations, resulting in 69 OTUs before and 51 OTUs after the administration of antibiotics. The OTUs comprised 11 phyla before and 8 phyla after antibiotics administration. The phyla Actinobacteriota, Bacteroidota, Campylobacterota, Chloroflexi, Firmicutes, Fusobacteriota, Patescibacteria and Proteobacteria were detected before and also after antibiotics administration, whereas Desulfobacterota, Spirochaetota and Synergistota were detected only before administration of antibiotics (Plate III, Fig. 3).

The OTUs assigned to Bacteroidota predominated in the bacterial community in both samples, before and after antibiotics administration, representing more than 40% of the sequences. The genus *Porphyromonas*, commonly associated with periodontal diseases, decreased from 33.84% to 1.61% after antibiotics administration. Other genera of the phylum Bacteroidota, except *Capnocytophaga* and *Petrimonas*, also decreased after antibiotics. The genus *Capnocytophaga* increased from 0.31% to 39.64%. All OTUs assigned to phyla Campylobacterota, Chloroflexi and Fusobacteriota had a higher proportion before administration of antibiotics. The OTUs assigned to Firmicutes, except *Acholeplasma*, *Streptococcus* and uncultured Aerococcaceae, also had a higher proportion before administration of antibiotics. On the other hand, all OTUs assigned to phylum Actinobacteriota had a higher proportion after administration of antibiotics with *Actinomyces* and *Corynebacterium* as representative genera. Overall, the relative abundance of the phyla Patescibacteria and Proteobacteria increased after antibiotics administration, but some OTUs belonging to these phyla decreased. The relative abundance of individual families contributing more than 1% of the total bacterial community in at least one sample is shown in Fig. 4 (Plate III).

Discussion

Periodontal antibiotic therapy is considered an effective adjunct to conventional mechanical debridement in the therapeutic management of periodontal diseases due to evidence for bacterial specificity in periodontitis (Kapoor et al. 2012). A combination of spiramycin and metronidazole was used in the present study. Spiramycin is a macrolide antibiotic and antiparasitic used to treat a variety of infections, including periodontitis (Calcagnile et al. 2018; Howard et al. 2021). To our knowledge, there are currently no studies available describing the effect of spiramycin on *Porphyromonas gulae*, a major periodontal pathogen in dogs. However, several studies have described the effect of spiramycin on the human periodontal pathogen *Porphyromonas gingivalis*. In the study Rams et al. (2011), only one of 15 isolated *P. gingivalis* strains was resistant to spiramycin under *in vitro* conditions. In the randomized placebo-controlled clinical trial (Chiappe et al. 2011), *P. gingivalis* was suppressed after 7 days of systemic administration of spiramycin.

Metronidazole is highly active against both Gram-negative and Gram-positive anaerobic bacteria (Löfmark et al. 2010). In Beagle dogs, topical application of metronidazole improved the clinical features of the experimentally induced periodontitis and eliminated some of the microorganisms associated with periodontitis (Klinge et al. 1992). In the study of Stephan et al. (2008), only one of 320 *Prevotella* strains and one of 310 *Porphyromonas* strains were fully resistant to metronidazole. In the study by Senhorinho et al. (2012), all isolated strains of *Porphyromonas macacae*, *Fusobacterium nucleatum* and *Fusobacterium canifelinum* were sensitive to metronidazole. However, 2.6% of *P. gulae* strains isolated from dogs with periodontitis and 13.3% of *P. gulae* strains isolated from dogs without periodontitis were resistant to metronidazole. In the study of Yoshida et al. (2021), high doses of metronidazole were required to inhibit the growth of *P. gulae*.

In the study by Rams et al. (2011), several strains of human periodontal pathogens were resistant to spiramycin and several strains were resistant to metronidazole. However, none of them was resistant to both antibiotics. In the present study, the combination of spiramycin and metronidazole was effective against *P. gulae*. At the time of antibiotics administration, *P. gulae* was not detected by cultivation in any sample. Also, amplicon sequencing showed a decrease in the genus *Porphyromonas* in the dental biofilm sample. Other periodontal pathogens such as *P. macacae*, *Fusobacterium* spp. or *Prevotella* spp. were not detected at all by cultivation in the samples. Amplicon sequencing demonstrated

the effect of antibiotics on members of the family Fusobacteriaceae. In addition, members of the family Prevotellaceae were not detected by amplicon sequencing in sample after antibiotics administration.

Amplicon sequencing showed a higher relative abundance of the genus *Capnocytophaga* after antibiotics administration. In the study by Poulet et al. (2005), *Capnocytophaga* spp. isolated from human oral cavity were resistant to metronidazole and also frequently to spiramycin. Based on the classification of microorganisms involved in human periodontal diseases according to Socransky et al. (1998), *Capnocytophaga* species belong to a green complex that is associated with periodontal health. Species *Capnocytophaga canimorsus* and *Capnocytophaga cynodegmi* constitute normal microbiota of the oral cavity of dogs and cats (Suzuki et al. 2010; Zajkowska et al. 2016). In the study of Santibáñez et al. (2021), the relative abundance of the genus *Capnocytophaga* was higher in dogs with healthy periodontium than in dogs with periodontitis.

After antibiotics administration, the genera *Streptococcus*, *Actinomyces* and *Corynebacterium* were higher than before antibiotics in both cultivation and amplicon sequencing. In humans, streptococci are the first microorganisms to colonize oral surfaces and are the dominant species in the oral cavity (Abranches et al. 2018). On the other hand, streptococci appear to represent a minor genus in dogs (Dewhirst et al. 2012). However, their relative abundance was higher in dogs with healthy periodontium than in dogs with periodontitis in the study by Santibáñez et al. (2021). It was assumed that *Actinomyces* species and *Corynebacterium* species may be involved in canine periodontitis due to their trypsin-like activity (Takada and Hirasawa 2000). It has been proven that they represent a large proportion of the cultivable microbiota of canine dental biofilm (Elliott et al. 2005). However, their increased occurrence in dogs with periodontitis has not been proven (Santibáñez et al. 2021). On the other hand, genera *Actinomyces* and *Corynebacterium*, along with *Capnocytophaga*, *Flavobacterium*, *Gemella*, *Abiotrophia*, *Streptococcus*, and *Frederiksenia* were shown to be present in all healthy dogs regardless of breed, sex, diet or other factors in the study of Bell et al. (2020).

In conclusion, the combination of spiramycin and metronidazole seems to be suitable as a complementary therapy for periodontal diseases to conventional mechanical debridement. The results of both cultivation and amplicon sequencing confirmed the effect of spiramycin and metronidazole on the genus *Porphyromonas*. During the administration of antibiotics, there was an increase in the representation of genera *Capnocytophaga*, *Streptococcus*, *Actinomyces* and *Corynebacterium* associated with oral health. Future studies analysing samples even after antibiotics discontinuation will be needed to verify these results and assess whether recolonization by periodontal pathogens occurs.

Conflict of interest

None declared.

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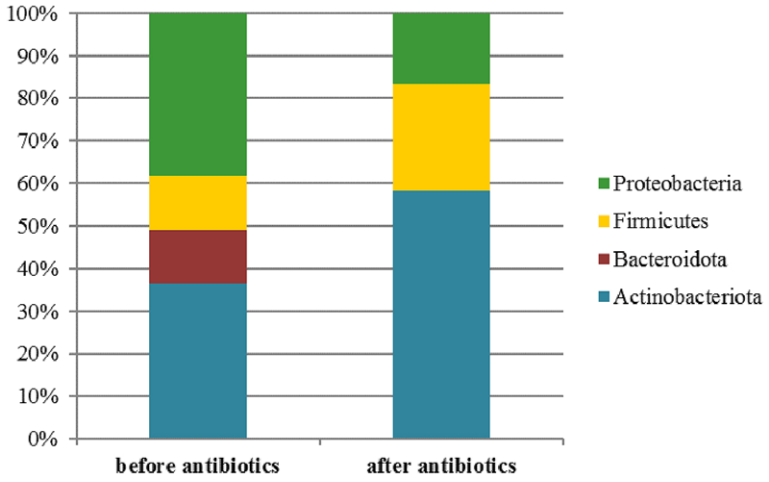

Fig. 1. Composition of the cultivable bacterial community of canine dental biofilms before and after the administration of antibiotics at the phylum level.

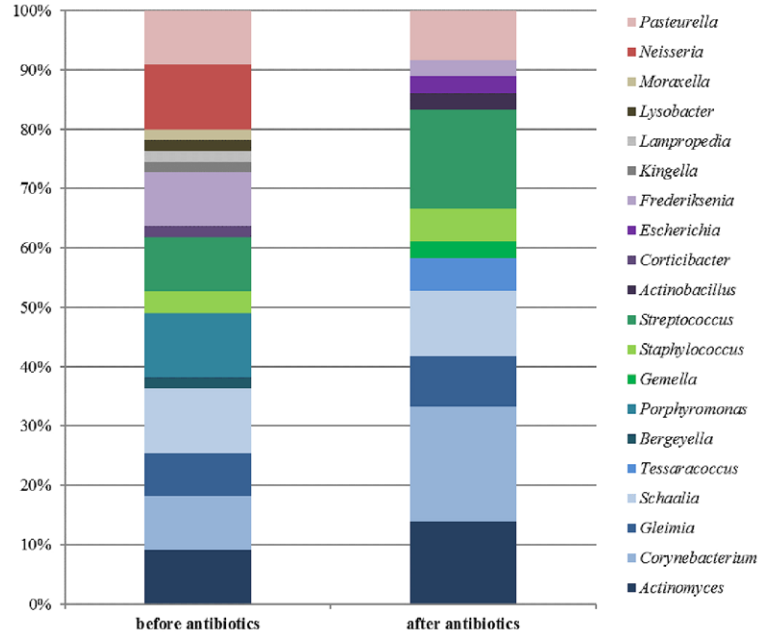

Fig. 2. Composition of the cultivable bacterial community of canine dental biofilms before and after the administration of antibiotics at the genus level.

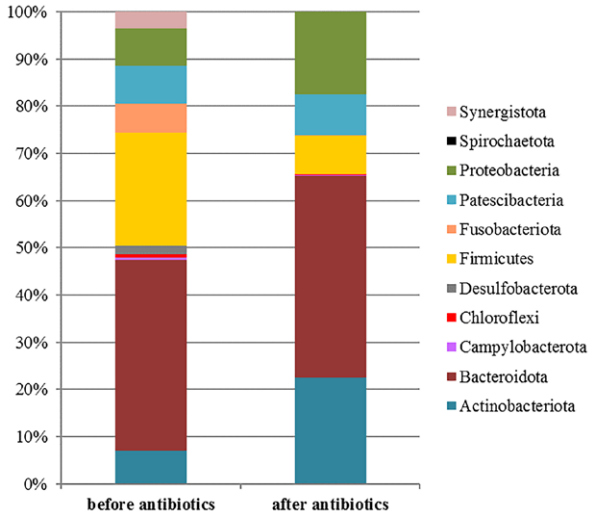

Fig. 3. Composition of the total bacterial community of dental biofilm of dog no. 3 before and after the administration of antibiotics at the phylum level by Illumina amplicon sequencing.

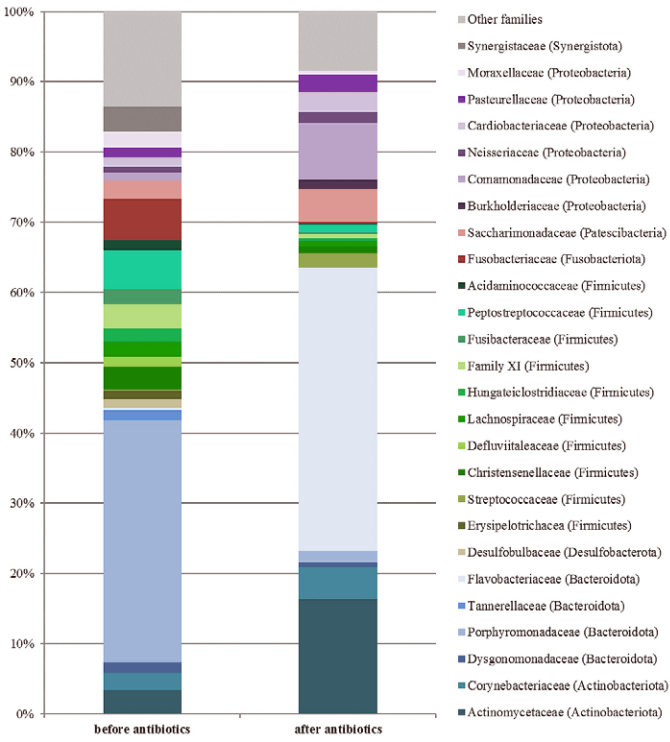

Fig. 4. Families with a relative abundance representing more than 1% of total sequence reads by Illumina amplicon sequencing present in at least one sample from dog no. 3.