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CULTIVABLE ORAL MICROBIOTA IN PUPPIES

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ABSTRACT

The oral microbiota has been shown to be different in children born by caesarean section and delivered vaginally. The aim of this study was to investigate the oral microbial diversity in healthy puppies and to determine whether the birth mode affects the composition of the oral microbiota. A total of 19 puppies from 4 dams were included in the study. The puppies were divided into two groups depending on the birth mode, vaginal delivery (vaginal born VB) or caesarean delivery (caesarean section CS). On the seventh day after birth, swabs of the oral cavity were taken. All samples were analysed by bacteriological cultivation under aerobic and anaerobic conditions. Bacterial colonies were identified by Sanger sequencing of 16S rRNA. A total of 64 bacterial strains belonging to 10 genera were obtained from the oral swabs. The genera *Staphylococcus* (30.23 % VB and 47.62 % CS) and *Enterococcus* (25.58 % VB and 33.33 % CS) were the most abundant in both groups. The genera *Escherichia* (18.60 %) and *Enterobacter* (16.28 %) were largely present in puppies delivered vaginally, they were not found in puppies born by caesarean section. The other detected genera were present at lower proportions (< 5 %) and varied between the groups. The oral microbiota of the puppies in the litter was similar, but differed between litters and between groups. Based on these results, we can assume that the birth mode affects the oral microbiota of puppies.

Key words: microbial cultivation; oral bacteria; puppies

INTRODUCTION

It has long been believed that under normal conditions, intrauterine foetal development occurs in an aseptic environment and the colonization begins at birth. However, recent studies have shown that amniotic fluid is colonized by oral microorganisms in up to 70 % of pregnant women [15]. In addition, a similar composition of the microbial community was detected in samples of the maternal oral cavity, amniotic fluid, placenta, and neonatal oral cavity

[20]. To date, it has not been investigated whether canine amniotic fluid contains bacteria. However, genera *Staphylococcus, Streptococcus, Actinomyces, Arthrobacter, Cutibacterium, Bacillus, Kocuria, Fingoldia, Pasteurella, Moraxella, Escherichia* and *Pantoea* have been isolated from the placenta of puppies [22].

The mode of birth has an impact on the oral bacterial community of infants. Vaginally delivered infants exhibit a greater bacterial diversity compared to infants delivered by caesarean section [4, 11]. After birth, the newborn is exposed to a wide range of microbes from a variety of sources, including the environment in which it lives and maternal bacteria. The maternal microbiome is considered a key factor in the initial colonization of the neonatal microbiome [2]. Early colonization of the mucosa of mammalian host plays a pivotal role in the maturation of the host's immune system [23]. The composition of the oral microbiota in the first days of life appears to be a very important factor in achieving and maintaining good health in the following years [20].

There have been several studies which focused on the oral microbiology of babies [2, 20], kittens [17] or foals [6]. However, there has been no study focused on the oral microbiology of puppies that we know of which have used conventional culture or molecular methods.

The aim of this study was to investigate the microbial diversity of the oral cavity in healthy puppies and examine whether there are variations in the oral microbiota between puppies delivered vaginally and by caesarean section.

MATERIAL AND METHODS

Study population and swab collection

Nineteen puppies from 4 litters were enrolled in this study: Rottweiler (n = 7), Border Terrier (n = 5), Maltese (n = 4) and Czechoslovak Wolfdog (n = 2). The Rottweilers and the Malteses were born vaginally. The Border Terrier and the Czechoslovak Wolfdog were born by caesarean section. Oral swab samples were taken on the 7th day after birth at the Clinic of Small Animals, University of Veterinary Medicine and Pharmacy in Košice. Samples were collected for 15—20 seconds while rotating the swab (Amies Agar Gel Transport Swabs) throughout.

Ethical statement

All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (Protocol number 3323/16-221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by the Ethics Commission of the University of Veterinary Medicine and Pharmacy (Košice, Slovakia). The animals were handled in a humane manner in accordance with the guidelines established by the relevant commission. All applicable international, national and institutional guidelines for the care and use of animals were followed.

Microbiological analysis of samples

The oral samples were inoculated onto Brain Heart Infusion agar (BHI; HiMedia, Mumbai, India), deMan-Rogosa-Sharpe agar (MRS; Carl Roth GmbH, Karlsruhe, Germany), M-Enterococcus agar (ME; Becton, Dickinson and Co., Le Pont de Claix, France), Mitis Salivarius agar (MSA; Sigma Aldrich, Steinheim, Germany); supplemented with 1% potassium tellurite solution (Sigma Aldrich, Steinheim, Germany), and blood agar. The blood agar (BA) was prepared as Trypticase soy agar (Carl Roth GmbH, Karlsruhe, Germany), and supplemented with 5% of sterile defibrinated ram's blood. BHI plates were cultured under aerobic conditions, ME, MSA and BA plates were cultured under aerobic and anaerobic conditions (BBL GasPak™ Plus, Becton, Dickinson and Co., Maryland, USA), and MRS plates were cultured under anaerobic conditions. The plates were incubated at 37 °C for 48 hours at aerobic and 72 hours at anaerobic cultivation. Colonies with different morphological characteristics such as growth form, shape, size and colour were selected and subsequently sub-cultured to obtain pure bacterial cultures. The purity of the culture was determined by staining according to Gram.

16S rRNA sequencing of isolates

Bacterial DNA was extracted from the individual isolates using a DNAzol® Direct (Molecular Research Centre Inc., Cincinnati, USA) according to the manufacturer's recommendations. The PCR was carried out in a thermal cycler (TProfesional Basic, Biometra GmbH, Göttingen, Germany) using the universal primers, 27F and 1492R, and OneTaq 2X Master Mix with Standard Buffer (New England Biolabs, Foster City, USA). The PCR mixtures were subjected to an initial denaturation phase of 5 min

at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 3 min and a final extension step at 72 °C for 10 min. The amplified PCR products were separated on 2 % agarose gel and sent for Sanger sequencing using primer 1492R (Microsynth, Wien, Austria). All sequencing results were analyzed using Geneious 8.0.5 program (Biomatters, Auckland, New Zealand) and compared to the NCBI GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify the isolates.

RESULTS

Across all samples, a total of 64 bacterial strains were obtained from the oral swab samples. The bacteria belonged to 3 phyla, namely: *Actinobacteria, Firmicutes* and *Proteobacteria*. In the first group of puppies born vaginally (VB), *Firmicutes* accounted for 55.81 % and *Proteobacteria* for 44.19 %. In the second group of puppies born by caesarean section (CS), *Firmicutes* accounted for 90.48 %, and both *Actinobacteria* and *Proteobacteria* for 4.76 %. In general, bacterial taxa belonged to 10 genera. Seven genera were detected in the VB group and six genera in the CS group. Genera *Staphylococcus* (30.23 % VB and 47.62 % CS) and *Enterococcus* (25.58 % VB and 33.33 % CS) were the most abundant in both groups. The representation of individual genera in both groups is shown in Fig. 1.

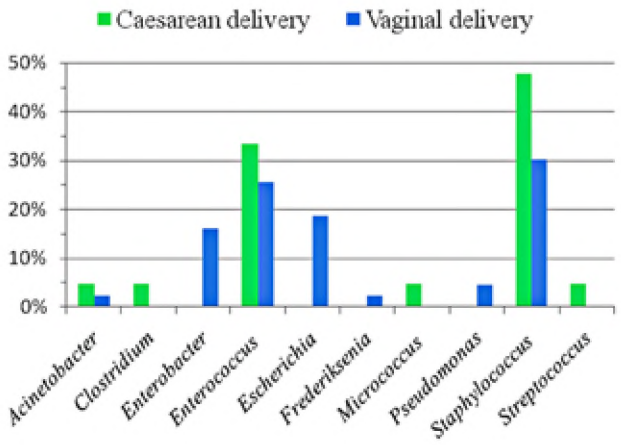

Fig. 1. The representation of bacterial genera in the oral cavity of puppies

Table 1. Prevalence of oral bacterial species in puppies in relation to delivery mode

Species (Phyllum)	Caesarean delivery (n = 7)	Vaginal delivery (n = 10)
Acinetobacter pittii **(Proteobacteria)**	1	1
Clostridium perfringens **(Firmicutes)**	1	0
Enterobacter ludwigii **(Proteobacteria)**	0	7
Enterococcus avium **(Firmicutes)**	0	2
Enterococcus canintestini **(Firmicutes)**	2	1
Enterococcus faecalis **(Firmicutes)**	5	8
Escherichia coli **(Proteobacteria)**	0	8
Frederiksenia canicola **(Proteobacteria)**	0	1
Micrococcus luteus **(Actinobacteria)**	1	0
Pseudomonas aeruginosa **(Proteobacteria)**	0	2
Staphylococcus epidermidis **(Firmicutes)**	1	3
Staphylococcus hominis **(Firmicutes)**	1	0
Staphylococcus haemolyticus **(Firmicutes)**	5	0
Staphylococcus pseudintermedius **(Firmicutes)**	3	10
Streptococcus fryi **(Firmicutes)**	1	0

The most common bacterial species in the CS group were *Enterococcus faecalis* and *Staphylococcus haemolyticus*, while in the VB group *Enterococcus faecalis, Escherichia coli* and *Staphylococcus pseudintermedius* were the most common. *Acinetobacter pittii, Enterococcus canintestini, Enterococcus faecalis, Staphylococcus epidermidis* and *Staphylococcus pseudintermedius* were present in both groups. All detected oral bacterial species in relation to the mode of delivery are shown in Table 1. The number of species isolated from individual puppies varied. On average, three species were isolated from puppies in the CS group. The number of isolated species was higher in puppies in the VB group, on average 4.3 species.

DISCUSSION

To our knowledge, this is the first study to focus on bacteria in the oral cavity of puppies. Previous studies of the canine oral microbiota have concentrated on the sampling of adult dogs. In this study, a total of 3 bacterial phyla, Actinobacteria, Firmicutes and Proteobacteria, which are commonly detected in the oral cavity of adult dogs [3, 13], were detected by culture-dependent methods. The phylum Actinobacteria was represented only by a species Micrococcus luteus isolated from one CS-born puppy, while the Firmicutes had a high proportion in all samples. Bacterial species belonging to the phylum Firmicutes accounted for more than 90 % of the microorganisms in the CS group.

The most frequently isolated bacteria were members of the genus *Staphylococcus*, specifically species: *S. epidermidis, S. hominis, S. haemolyticus* and *S. pseudintermedius*. In the study by Suepaul et al. [18], *S. epidermidis* and *S. haemolyticus* were isolated from canine and human mouths, while *S. hominis* were isolated only from humans and *S. pseudintermedius* was not detected. On the other hand, in the study by Sahin-Tóth et al. [14], *S. pseudintermedius* was commonly isolated from dogs from the mouth, nose and skin. *S. pseudintermedius* has been shown to be transmitted from the dam to puppies at birth and may persist in the offspring for a long time. However, intrauterine transmission is also plausible because this bacterium has also been isolated from samples of placenta and meconium of puppies born by caesarean section [22].

The second most numerous genus in this study was the genus *Enterococcus*. Enterococci are natural inhabitants of the mammalian gastrointestinal tract. Generally, enterococci are considered low pathogenic bacteria with the potential to improve health as a probiotic. *Enterococcus canintestini* and *Enterococcus faecalis* were isolated from faecal samples and vaginal mucosa of dogs [9, 10, 22]. Enterococci are considered in humans to be a transient component of the oral microbiota [8]. In the study by Isaiah et al. [5], enterococci were significantly present in the oral samples from patrol and narcotics detection dogs.

Large proportions of cultivable microbiota from the oral cavity of puppies in the VB group were represented by *Enterobacter* species and *Escherichia coli*. On the other hand, *Enterobacter* species and *Escherichia coli* were not detected in the CS group. *Enterobacter* species are part of the microbiota of the gastrointestinal tract of mammals, while other *Enterobacter* species may be present on skin, in water, certain foods, soil, and wastewater. Several *Enterobacter* species are responsible for causing many nosocomial infections [12]. *Enterobacter ludwigii*, isolated in this study, is a fermentative Gram-negative environmental species and an accidental human pathogen that belongs to the *Enterobacter cloacae* complex [16]. *Escherichia coli* along with *Lactobacillus, Pseudomonas, Staphylococcus* and *Streptococcus* are highly prevalent in the first several months of an infant's life, before tooth eruption [21]. Unclassified *Escherichia-Shigella* sp. was the most abundant saliva taxon of adult dogs in the study by Rupparell et al. [13].

Compared to adult dogs, genera such as *Actinomyces, Corynebacterium, Neisseria* or *Pasteurella*, commonly isolated from the oral cavity of adult dogs [3], were not isolated from the oral cavity of puppies. However, bacteria commonly isolated from the skin [19] or vaginal mucosa [22] of adult dogs were isolated. Potentially pathological species like *Clostridium perfringens* and *S. haemolyticus* were detected in the oral cavity of puppies in the CS group. *Acinetobacter pittii*, isolated from both groups, is an important nosocomial pathogen in humans and animals [1, 7].

CONCLUSIONS

The findings of this study provide new information about the oral microbiota in healthy puppies. The oral microbiota of puppies differs significantly from the oral microbiota of adult dogs. Oral microbiota also differs between puppies born vaginally and born by caesarean sec-

tion. The mode of birth seems to affect the bacterial colonization of the oral cavity of puppies in the first days of life. Further studies focusing on the oral microbiota of puppies, its development and the factors influencing it are necessary to identify physiological and pathological bacterial composition of oral microbiota in dogs.

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