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DOI: 10.1002/vms3.1144

ORIGINAL ARTICLE

WILEY

Normal bacterial flora of the oral cavity in healthy pet rabbits (*Oryctolagus cuniculus*)

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[Correction added on 9 May 2023, after first online publication: Affiliations 3 and 4 were added in this version.]

Abstract

Background: Rabbits often suffer from dental disease, including dental abscesses and periodontal/apical infections. With odontogenic infection and abscessation, a bacterial aetiology can be proven by bacterial culture and identification. Although studies exist on the bacterial flora of dental abscesses, the information available to date on the bacterial flora of the oral cavity in healthy rabbits is limited.

Objectives: This study aims to evaluate the cultivable bacterial flora in the oral cavity of healthy, young, pet rabbits and to compare this flora with the pathologic flora of odontogenic abscesses described in the literature.

Methods: Samples were collected from the oral cavity of 33 healthy, young pet rabbits undergoing routine procedures. Oral cavity culture specimens were collected by rolling a sterile flocked paediatric swab in the mouth. Identification was first attempted by morphological assessment, Gram staining and mass spectrometry (MALDI-TOF). Colonies that could not be identified by mass spectrometry were identified by amplification and molecular sequencing of a part of the 16s rRNA gene.

Results: Bacteria were recovered from 100% of oral swabs; 220 isolates of 35 different genera of bacteria were cultured. The most frequently isolated bacteria were *Streptococcus* sp. (19.8%), *Rothia* sp. (17.9%), *Enterobacter* sp. (7%), *Staphylococcus* sp. (6.6%) and *Actinomyces* sp. (5.7%). Four phyla are represented: *Proteobacteria* (38.3%), *Firmicutes* (30.5%), *Actinobacteria* (26.9%) and *Bacteroidota* (4.3%).

Conclusions: A wide range of commensal bacteria are present in the mouths of rabbits. Bacterial cultures taken from cases of dental abscesses often reveal bacteria. *Streptococcus* sp., *Staphylococcus* sp. and *Actinomyces* sp. are frequently found in cultures from dental abscesses, in contrast to *Rothia* and *Enterobacter* species. Our findings enhance the knowledge of rabbit microbial communities throughout oral cavity.

KEYWORDS
ARNr 16s, bacteria, dental abscesses, mass spectrometry, microbiome, oral cavity, rabbit

1 | INTRODUCTION

In humans, the oral cavity is a complex habitat of exceptional richness (Deo & Deshmukh, 2019). Many microorganisms colonise its different niches, from the hard surfaces of the teeth to the soft tissues of the oral mucosa (Deo & Deshmukh, 2019). The normal microbiome of the oral cavity consists of protozoa, archaea, viruses, fungi and many bacteria (Deo & Deshmukh, 2019). Each bacterium colonises a preferential environment due to specific adhesins present on their surface, allowing them to bind to complementary receptors on a given oral surface (Gibbons et al., 1976; Gibbons, 1989; Jaas et al., 2005).

With more than 700 species of bacteria identified, the oral cavity represents the second most important and diverse microbiota in humans after the gastrointestinal tract (Jaas et al., 2005; Yamashita & Toru Takeshita, 2017). The starting point of digestion is the oral microbiome which also has a role in maintaining oral and systemic health (Deo & Deshmukh, 2019). This microbiome is the most studied to date (McLean, 2014), probably because of the ease of sample collection, but the information available to date on the bacterial flora of the oral cavity in healthy rabbits is limited.

Rabbits commonly suffer from dental disease, including dental abscesses and periodontal/apical infections (Benato, 2017; Harcourt-Brown, 1995; Mullan & Main, 2006). The role of the oral microbiome in dental disease manifestation is unknown. In cases of odontogenic infection and abscess, a bacterial aetiology can be proven by bacterial culture and identification. Other methods of bacterial diagnosis are possible such as cytology, histology and PCR.

This study aims to evaluate the cultivable bacterial flora in the oral cavity of healthy pet rabbits and to compare this flora with the pathologic flora of odontogenic abscesses described in the literature.

2 | MATERIALS AND METHODS

2.1 | Study animals

This study includes privately owned pet rabbits admitted to the primary investigator's veterinary clinic either for a health check or routine vaccinations, between December 2021 and April 2022. An informed consent was obtained from the owner before inclusion of animals in the study. Elements such as age, sex, breed, living environment and diet were recorded. To reduce the variable microbiome part, only small-breed, single-housed, indoor rabbits ranging from 2 months to 3 years of age were included in the study. All rabbits were considered healthy, based on the history provided by the owners and the results of a complete general examination including a thorough examination of the oral cavity with a video otoscope in the conscious animal. Animals with any history of dental disease were excluded from the study. Animals with a recent history of illness (e.g. dermatologic, ophthalmic or systemic disease) or the administration of antimicrobial agents less than 2 months before sampling were excluded from the study. Animals were not fed for at least 1 h prior to sampling.

2.2 | Sampling procedure

To facilitate sampling, a towel was wrapped around the rabbit for restraint. In all animals, the lips were first cleaned with sterile materials (saline solution, swabs and gloves). Oral cavity culture samples were collected by rolling a sterile, flocked paediatric swab (COPAN eSwab, Mast group, Amiens, France) in the mouth for 5 s, while avoiding contact with the vibrissae. The swab was brought into contact with all areas of the oral cavity (tooth surfaces, dorsal and lateral tongue surfaces, buccal epithelium and hard palate). The COPAN eSwab incorporates a modified Liquid Amies transporting medium. After sampling, the oral cavity was thoroughly examined with a video otoscope. If food debris, caecotrophs, stools, oral lesions or other anomalies were present in the oral cavity, the rabbits were excluded from the study. The samples were stored for 24 h at the clinic and refrigerated at 4°C, before being transferred to the laboratory by courier. Transportation time was 24 h.

2.3 | Laboratory methods

The analysis was carried out at diagnostic laboratories for identification by morphological assessment, Gram staining, MALDI-TOF mass spectrometry (Bruker France SAS, Champs sur Marne, France) and by amplification and molecular sequencing of a part of the 16s rRNA gene (Microsynth France SAS, Vaulx-en-Velin, France).

A volume of 200-µl swab transport liquid was taken and stored from each swab after vortex homogenisation. Then samples were spread on a slide for Gram staining and plated onto Schaedler agar, Columbia agar, MacConkey agar and Columbia ANC agar culture media using the three-phase streak-plating technique. The petri dishes were then incubated at 37°C, and those for anaerobic culture were placed in an anaerobic jar, at 37°C.

They were examined for growth at 24 h; each colony identified was picked and spread on a culture medium for incubation (Columbia for aerobic strains and Schaedler for anaerobic strains) and re-examined every 24 h for strain isolation confirmation. Identification was first attempted by mass spectrometry (MALDI-TOF), and strains were systematically stored at −20°C for possible further analysis.

Colonies that could not be identified by morphological assessment, Gram staining and mass spectrometry were identified by amplification and molecular sequencing of a part of the 16s rRNA gene.

3 | RESULTS

3.1 | Animals

Altogether 33 rabbits met the inclusion criteria (17 females and 16 males), and after examination, none were excluded. The median age of the animals was 9 months (range: 2 months to 2.5 years). The breeds represented were Netherland Dwarf ($n = 20$), Holland Lop ($n = 11$) and

Lionhead rabbits ($n = 2$). All rabbits were fed an appropriate diet: ad libitum hay in addition a limited amount of good quality commercial extruded pellets as well as varied greens and fruits. Rabbit bedding consisted of hemp (43%), wood shavings (30%), corn cobs (15%) or wood pellets (12%).

3.2 | Bacterial isolation

Thirty-three buccal swabs were collected during the study period. Bacteriological examination of buccal samples demonstrated a positive result on culture in 33 of 33 (100%) swabs; 220 isolates of 35 different genera of bacteria were cultured (Table 1).

Morphological assessment, Gram staining and MALDI-TOF analysis were sufficient to determine bacterial identification in 150 cases (of 220). Seventy bacterial strains required molecular sequencing for identification. Molecular sequencing enabled identification of 62 bacteria strains, whereas 8 bacterial strains remained unidentified.

The most frequently isolated bacteria were *Streptococcus* sp. (42 of 212 isolates; 19.8%), *Rothia* sp. (38/212; 17.9%), *Enterobacter* sp. (15/212; 7%), *Staphylococcus* sp. (14/212; 6.6%) and *Actinomyces* sp. (12/212; 5.7%) representing 57% of total isolated bacteria. Four phyla were represented: *Proteobacteria* (38.3%), *Firmicutes* (30.5%), *Actinobacteria* (26.9%) and *Bacteroidetes* (4.3%). The same predominant flora (*Streptococcus* sp., *Rothia* sp., *Enterobacter* sp., *Staphylococcus* sp. and *Actinomyces* sp.) were found in young rabbits (≤6 months), adult rabbits (>6 months and <3 years) as well as in male rabbits. In female rabbits, *Neisseria* sp. (5.9%) replaced *Actinomyces* sp. (3%) in the predominant flora. Seventeen rabbits had two or more bacteria of the same genus (mostly of the *Streptococcus* genus). A mean of 6.4 bacterial species were isolated per rabbit. One rabbit mouth led to the isolation of 3 bacterial species, 1 mouth to 4, 5 mouths to 5, 10 mouths to 6, 8 mouths to 7 and 8 mouths to 8.

TABLE 1 Oral bacterial isolates.

Phylum	Genus	Species	No (%)
Firmicutes	*Streptococcus*		42 (19.8)
		Streptococcus ferus	7 (3.3)
		Streptococcus sanguinis	4 (1.9)
		Streptococcus gordonii	4 (1.9)
		Streptococcus suis	1 (0.5)
		Streptococcus minor	1 (0.5)
		Streptococcus mitis	1 (0.5)
		Streptococcus pneumoniae	1 (0.5)
		Streptococcus ratti	1 (0.5)
Actinobacteria	*Rothia*		38 (17.9)
		Rothia nasimurium	32 (15)
		Rothia endophytica	3 (1.4)
		Rothia mucilaginosa	1 (0.5)
		Rothia dentocariosa	1 (0.5)
Proteobacteria	*Enterobacter*		15 (7)
		Enterobacter cloacae	6 (2.8)
		Enterobacter cowanii	4 (1.9)
		Enterobacter xiangfangensis	4 (1.9)
		Enterobacter asburiae	1 (0.5)
Firmicutes	*Staphylococcus*		14 (6.6)
		Staphylococcus xylosus	4 (1.9)
		Staphylococcus saprophyticus	3 (1.4)
		Staphylococcus aureus	1 (0.5)
		Staphylococcus chromogenes	1 (0.5)
		Staphylococcus pettenkoferi	1 (0.5)
		Staphylococcus epidermidis	1 (0.5)
		Staphylococcus warneri	1 (0.5)
Actinobacteria	*Actinomyces*		12 (5.7)
		Actinomyces gashouyii	6 (2.8)
		Actinomyces denticolens	5 (2.4)
Proteobacteria	*Neisseria*		10 (4.7)
Proteobacteria	*Pelistega*		8 (3.8)
Proteobacteria	*Moraxella*		7 (3.3)
Proteobacteria	*Cronobacter*		7 (3.3)
Actinobacteria	*Corynebacterium*		6 (2.8)
Proteobacteria	*Escherichia*		6 (2.8)
Bacteroidota	*Bergeyella*		5 (2.3)
Proteobacteria	*Klebsiella*		5 (2.3)
Proteobacteria	*Pantoea*		4 (1.9)

(Continues)

4 | DISCUSSION

In the present study, the most frequently isolated organisms from the oral cavity in healthy pet rabbits were *Streptococcus* sp., *Rothia* sp., *Enterobacter* sp., *Staphylococcus* sp. and *Actinomyces* sp.

The *Streptococcus* genus includes commensal, pathogenic and opportunistic aerobic Gram-positive organisms in humans and animals (Haenni et al., 2018). Their natural niches are the body cavities (nasopharynx and respiratory, gastrointestinal and genitourinary tracts) and the skin (Nguyen et al., 2015). The *Rothia* genus is Gram-positive aerobic bacteria, commensals of the oral cavity and respiratory tract (Elkattawy et al., 2021). They can appear as opportunistic pathogens causing various infections in immunocompromised and immunocompetent persons (Fatahi-Bafghi, 2021). The *Enterobacter* genus includes commensal Gram-negative anaerobic organisms of the microflora of the mammalian gastrointestinal tract, and they are also described in the environment and reported as opportunistic pathogens in plants, animals and humans (Davin-Regli et al., 2019).

TABLE 1 (Continued)

Phylum	Genus	Species	No (%)
Proteobacteria	*Actinobacillus*		4 (1.9)
Proteobacteria	*Acinetobacter*		3 (1.4)
Firmicutes	*Aerococcus*		2 (0.9)
Firmicutes	*Enterococcus*		2 (0.9)
Proteobacteria	*Kosakonia*		2 (0.9)
Firmicutes	*Lactobacillus*		2 (0.9)
Firmicutes	*Macrococcus*		2 (0.9)
Proteobacteria	*Ochrobactrum*		2 (0.9)
Proteobacteria	*Rahnella*		2 (0.9)
Proteobacteria	*Achromobacter*		1 (0.5)
Actinobacteria	*Arthrobacter*		1 (0.5)
Bacteroidota	*Bacteroides*		1 (0.5)
Proteobacteria	*Bordetella*		1 (0.5)
Proteobacteria	*Caviibacterium*		1 (0.5)
Bacteroidota	*Cloacibacterium*		1 (0.5)
Bacteroidota	*Empedobacter*		1 (0.5)
Firmicutes	*Gemella*		1 (0.5)
Proteobacteria	*Leclercia*		1 (0.5)
Proteobacteria	*Pseudomonas*		1 (0.5)
Proteobacteria	*Serratia*		1 (0.5)
Bacteroidota	*Weeksella*		1 (0.5)
	Total 35		212

[Correction added on 9 May 2023, after first online publication: Table 1 was corrected in this version.]

The *Staphylococcus* genus includes commensal Gram-positive aerobic organisms whose natural niches extend from the skin to the mucous membranes in humans and other mammals (Paharik & Horswill, 2016). They are also reported as opportunistic pathogens in immunocompromised individuals (Paharik & Horswill, 2016). The *Actinomyces* genus is Gram-positive, primarily facultative anaerobic bacteria in the normal flora of the upper respiratory, female genital and gastrointestinal tracts. *Actinomyces* species are generally considered to have a low virulence potential (Hsiao et al., 2021; Sakko et al., 2016).

After exhaustive bibliographic research, we found only one recent study (Hu et al., 2021) focusing on oral bacteria, but as mouth bacteria were studied in a group, including skin and lungs, straight comparison is not possible.

Dental abscesses are a major reason for consultation in rabbit medicine, and bacteriology can have a determining role in the therapeutic choices to increase the chances of recovery and limit resistance development (Benato, 2017; Harcourt-Brown, 1995; Harcourt-Brown & Chitty, 2013; Mullan & Main, 2006).

As in humans (Robertson & Smith, 2009) and some pets such as dogs and cats (Dow et al., 1986; Kroemers et al., 2014), odontogenic abscesses in rabbits usually originate from a mixture of aerobic and anaerobic bacteria with more than three isolates most often detected on culture (Tyrrell et al., 2002; Taylor et al., 2010; Gardhouse et al., 2017).

Three main studies have been published on bacterial isolates from odontogenic abscesses in the rabbit (Tyrrell et al., 2002, Taylor et al., 2010; Gardhouse et al., 2017). *Streptococcus* sp., *Staphylococcus* sp. and *Actinomyces* sp. are bacteria frequently found in cultures from dental abscesses (Tyrrell et al., 2002; Taylor et al., 2010; Gardhouse et al., 2017). These three bacteria are commensal but can have an opportunistic pathogenic activity (Haenni et al., 2018; Paharik & Horswill, 2016; Hsiao et al., 2021; Sakko et al., 2016). *Rothia* and *Enterobacter* species, which have been found in large numbers in the present study, are not commonly found in cultures from dental abscesses.

A study on the standardisation of sampling techniques would be of interest to improve the quality of the bacterial isolates from odontogenic abscesses because an inadequate sampling technique can lead to negative culture or to the isolation of contaminating normal oral flora but also of contaminating faecal flora due to the practice of caecotrophy in rabbits (Tyrrell, 2002; Gardhouse et al., 2017; Kholes, 2014). This explains the presence of normal intestinal bacteria of the rabbit gut microbiota such as *Bacteroides* sp. or *Enterobacter* sp. in the current study (Combes et al., 2017, Cotozzolo et al., 2020, Fu et al., 2018, Monteils et al., 2008; Velasco-Galilea et al., 2018; Zeng et al., 2015).

In this study, we deliberately chose young, indoor, single-housed rabbits in order to achieve a relatively homogeneous study population. Indeed, in humans, the oral microbiome is composed of a core microbiome, common to all individuals and a variable microbiome exclusive to the individual and dependent on his/her physiology and environment (Deo & Deshmukh, 2019; Zarco et al., 2012). In our study, we tried to reduce this variation as much as possible by choosing only small-breed, single-housed, indoor rabbits. Rabbits living together and having a friendly relationship, including allogrooming and licking (Crowell-Davis, 2010; Donnelly & Vella, 2021; Saunders, 2014; Varga, 2014), could have changes in their oral microbiome. This behaviour can be reproduced with a companion of another pet species which is why only rabbits living alone, with no other pets in the home were accepted in our study. Furthermore, rabbits practice caecotrophy with the ingestion of soft faeces rich in essential amino acids, vitamins and minerals (Crowell-Davis, 2010; Donnelly & Vella, 2021; Saunders, 2014; Varga, 2014) but can also practice coprophagy by the ingestion of hard faeces which are poor in nutrients but rich in raw fibre (Ebino et al., 1993). Choosing only single-housed rabbits for our study is therefore essential to avoid disturbing the oral microbiome with the intestinal microbiome of another rabbit or other animal species.

Each oral niche has its own bacterial signature in humans; the profile of bacterial species differs according to the surface from which the sample was taken (Jaas et al., 2005; Mager et al., 2003). In our study, the swab was brought into contact with all areas of the oral cavity to define the predominant bacterial flora of the whole healthy oral cavity.

In rabbits, acquired malocclusions affecting the cheek teeth do not usually occur before the age of 3 years (Böhmer, 2015). This increase in dental problems with age may influence the bacterial oral flora. Therefore, in our study, we only included young rabbits ranging from 2 months to 3 years of age. Furthermore, as humans age and teeth are

lost, the flora changes and becomes similar to that of a child before tooth eruption (Deo & Deshmukh, 2019; Patil et al., 2013) which is why our study only included young rabbits. Similar studies should be conducted in older rabbits to highlight a possible evolution of the oral bacterial flora with age and in rabbits living in groups or outdoors to highlight the probable influence of the environment and diet on the bacterial flora. Additionally, a study on the oral bacterial flora of rabbits with dental disease will highlight the disease impact on the oral flora, whereas a study on rabbits with dental pathology and under antibiotic treatment could reveal the effect of antibiotic therapy on the oral flora. Results of such studies would allow us to determine whether the bacteria involved in these abscesses are part of the physiological oral flora that has become contaminants or pathogens. The ultimate aim is to find out whether dental disease induces a dysbiosis of the oral flora. If this is the case, an analysis of this bacterial flora could be a means of early detection of a dental bacterial infection. It would be based on a non-invasive sampling technique, but this would need to be standardised.

Other limitations of our present study were that we had a relatively small number of cases (33 rabbits), and we only identified the cultivable flora. There may be many other non-cultivable bacteria present in the mouth of a healthy rabbit and bacteria which may die before they can be cultured (unsuitable substrate, temperature, error in manipulation etc.). Fungi also play an important role in the microbiome of many mammalian species (Krumbeck et al., 2021) but were not addressed in this study. A study of the fungal flora of the oral cavity in healthy rabbits would be of interest to improve the interpretation of fungal cultures from dental abscesses even if they are rarely looked for.

AUTHOR CONTRIBUTIONS

Conceptualisation; data curation; investigation; methodology; writing – original draft: Lucas Flenghi. *Data curation; writing – original draft*: Maeva Mazouffre. *Data curation; methodology; writing – review and editing*: Aurélie Le Loc'h. *Conceptualisation; data curation; methodology; project administration; writing—review and editing*: Guillaume Le Loc'h. *Conceptualisation; methodology; supervision; writing—review and editing*: Christophe Bulliot.

ACKNOWLEDGEMENTS

The authors would like to thank Céline Levrier and Françoise Rey for their kind proofreading of this research and Alexandre Flenghi for designing the graphical abstract figure.

FUNDING INFORMATION

Self-funding.

ETHICS STATEMENT

Favourable opinion of the SSA Ethics Committee No 115 concerning the use of animals for scientific purposes. File registered under number SSA_2022_006.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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TRANSPARENT PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.1144.

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How to cite this article: Flenghi, L., Mazouffre, M., Le Loc'h, A., Le Loc'h, G., & Bulliot, C. (2023). Normal bacterial flora of the oral cavity in healthy pet rabbits (*Oryctolagus cuniculus*). *Veterinary Medicine and Science*, *9*, 1621–1626. https://doi.org/10.1002/vms3.1144