Abbreviations

AMOVA	Analysis of molecular variance
B group	Brachycephalic breeds group
CR	Chronic idiopathic rhinitis
CRS	Chronic rhinosinusitis
CT	Computed tomography
DL group	Meso- to dolichocephalic medium to large breeds group
DNA	Deoxyribonucleic acid
FR	Fungal rhinosinusitis
HOMOVA	Homogeneity of molecular variance
LDA	Linear discrimination analysis
LEfSe	Linear discriminant analysis effect size
MALDI-TOF	Matric assisted laser desorption ionisation/time of flight
MALT	Mucosa-associated lymphoid tissue
NM	Nasal microbiota
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RA	Relative abundance
RDA	Redundancy analysis
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
T group	Terrier breeds group
Th	T helper

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Summary - Résumé

Summary

Fungal rhinosinusitis in dogs is most commonly associated with infection by *Aspergillus fumigatus*. The disease typically originates in the paranasal sinuses and may extend into the nasal cavities, where it induces tissue destruction, chronic inflammation, and mucosal damage. Diagnosis and treatment rely heavily on advanced imaging, particularly computed tomography, combined with rhinoscopic evaluation. Although the condition is suspected to result from impaired innate and/or adaptive immune responses against the fungus, definitive evidence supporting this hypothesis remains lacking (Peeters and Clercx 2007; Sharman and Mansfield 2012). In humans, the gut microbiota and microbial communities at extraintestinal sites play a central role in modulating immune function (Zheng et al., 2020), and the nasal microbiota in particular has been implicated in the pathogenesis of upper airway diseases such as chronic rhinosinusitis and allergic rhinitis (Tai et al., 2021). Despite these insights, knowledge of the nasal microbiota in dogs remains extremely limited. Further investigation of the nasal microbiota in health and disease may provide novel perspectives on the pathophysiology, diagnosis, and treatment of chronic nasal disorders in canine patients.

The aim of this thesis was to elucidate the composition and determinants of the canine nasal microbiota in health and disease, and to assess its potential involvement in the pathogenesis of fungal rhinosinusitis. By combining culture-based and DNA sequencing approaches, it sought to characterize healthy and dysbiotic nasal microbial profiles, evaluate the diagnostic relevance of conventional culture methods, and provide new insights for improving the diagnosis and treatment of chronic nasal diseases in dogs.

Study 1 focused on the bacterial communities present in dogs with nasal discharge, regardless of the underlying pathology, and compared results obtained by conventional agar-plate culture of nasal swabs with those obtained by 16S rRNA gene amplicon sequencing. In practice, nasal swab culture results are commonly performed and used as a guide for antimicrobial treatment (local or systemic) in dogs with nasal discharge. However, the usefulness of this complementary exam as well as the use of systemic antimicrobials in such cases are questionable. This study highlighted that standard culture does not reliably predict the bacterial profile detected by 16S rRNA gene amplicon sequencing and did not support the routine use of a standard nasal swab for the diagnostic workup in dogs with nasal diseases.

The primary objective of **study 2** was to characterize the core nasal microbiota in healthy dogs using DNA sequencing. Both breeds predisposed to fungal rhinosinusitis (medium to large mesocephalic and dolichocephalic breeds) and those considered less predisposed (such as brachycephalic breeds and terriers) were included. The study also assessed the potential influence of intrinsic and extrinsic factors on microbial composition. The findings revealed that facial and body conformation significantly influenced the composition of the nasal microbiota. Notably, brachycephalic dogs exhibited distinct microbial profiles, marked by higher species richness and increased alpha-diversity compared to the other breed groups. No consistent associations were identified between microbial composition and sex or environmental exposure. The effect of age remained inconclusive. This study also offers valuable insight into the baseline microbial composition of the healthy canine nasal cavity, where *Moraxella* was identified as the predominant genus in most samples. These findings provide essential reference data for assessing microbiota shifts in dogs with nasal disease.

Study 3 aimed to assess whether a specific dysbiosis of the nasal microbiota was associated with dogs with fungal rhinosinusitis, compared to the nasal microbiota of healthy breed-matched dogs and dogs with chronic idiopathic rhinitis. Results confirmed major alterations of the nasal microbiota in dogs affected with fungal rhinosinusitis and dogs with chronic idiopathic rhinitis, consisting mainly in a decrease of the genus *Moraxella*. Increase in unique bacterial families were also associated with fungal rhinosinusitis (*Staphylococcaceae*, *Porphyromonadaceae*, *Enterobacteriaceae* and *Neisseriaceae*) and chronic idiopathic rhinitis (*Pasteurellaceae* and *Lactobacillaceae*), showing that distinct patterns of dysbiosis can be observed with different chronic nasal diseases. Whether these dysbiotic patterns influence the course of the disease remains to be investigated.

This work underscores the limitations of conventional culture approaches in their ability to capture the complexity of the nasal microbial environment in dogs with nasal discharge. It further demonstrates that in healthy dogs, in which a consistent core microbial pattern was observed, intrinsic anatomical factors can shape the composition of the nasal microbiota. Finally, it provides a comprehensive characterization of dysbiosis patterns associated with different chronic nasal conditions, offering novel insights into their pathogenesis and potential diagnostic and therapeutic strategies.

Résumé

La rhinosinusite fongique implique principalement une infection par *Aspergillus fumigatus*. L'infection commence généralement dans les sinus paranasaux et peut s'étendre aux cavités nasales. Elle peut entraîner une destruction des tissus, provoquant une inflammation chronique et des dommages à la muqueuse. La tomodensitométrie des cavités nasales et la rhinoscopie sont des éléments importants du diagnostic et du traitement. On pense que la maladie est secondaire à une réponse immunitaire innée et/ou adaptative altérée contre le champignon, mais les tentatives pour confirmer cela ont cependant échoué (Peeters et Clercx 2007, Sharman et Mansfield 2012). Il a été démontré que le microbiote, dans les intestins ainsi que dans les organes extra-intestinaux, joue un rôle clé dans la coordination des fonctions du système immunitaire chez les humains (Zheng et al., 2020). Plus précisément, on suppose que le microbiote nasal participe à l'induction de maladies des voies respiratoires supérieures telles que la rhinosinusite chronique et la rhinite allergique (Tai et al., 2021). À ce jour, très peu de choses sont connues sur le microbiote nasal canin en santé et en maladie. L'étude du microbiote nasal pourrait ouvrir de nouvelles perspectives concernant la physiopathologie, le diagnostic et le traitement des maladies nasales chroniques chez les chiens.

L'objectif de cette thèse était d'élucider la composition et les déterminants du microbiote nasal canin, en conditions saines et pathologiques, et d'évaluer son implication potentielle dans la physiopathologie de la rhinosinusite fongique. En combinant des approches basées sur la culture et le séquençage, ce travail visait à caractériser les profils microbiens nasaux sains et dysbiotiques, à évaluer la pertinence diagnostique des méthodes de culture conventionnelles, et à apporter de nouvelles perspectives pour améliorer le diagnostic et le traitement des maladies nasales chroniques chez le chien.

L'**étude 1** s'est intéressée aux communautés bactériennes présentes chez des chiens présentant un écoulement nasal, quelle que soit la pathologie sous-jacente, en comparant les résultats obtenus par culture bactérienne standard sur écouvillon nasal à ceux issus du séquençage de l'amplicon d'un fragment du gène de l'ARN ribosomial 16S. En pratique, les résultats de la culture nasale sont fréquemment utilisés pour orienter le traitement antimicrobien (local ou systémique) chez ces chiens. Toutefois, la pertinence de cet examen complémentaire, ainsi que l'utilisation d'antibiotiques systémiques dans ce contexte, reste discutable. Les résultats ont montré que la culture standard ne prédit pas de manière fiable le profil bactérien détecté par le séquençage de

l'amplicon du gène codant l'ARN ribosomial 16S et n'ont pas soutenu l'utilisation de routine d'un écouvillon nasal standard pour le bilan diagnostique des chiens atteints de maladies nasales.

L'objectif de l'**étude 2** était de déterminer le microbiote nasal de base chez les chiens en bonne santé, en incluant des races prédisposées (chiens de taille moyenne à grande à tête mésocéphalique ou dolichocéphalique) et non prédisposées (chiens brachycéphales et races terriers) à la rhinosinusite fongique, tout en évaluant l'influence potentielle de facteurs intrinsèques et extrinsèques. Cette étude a révélé que la conformation faciale et corporelle influence significativement la composition du microbiote nasal. En particulier, les chiens brachycéphales présentaient un profil microbien distinct, caractérisé par une richesse spécifique et une diversité alpha accrues par rapport aux autres groupes de races. Aucune corrélation constante avec le sexe ou l'environnement n'a été observée, et l'effet de l'âge est resté incertain. Cette étude a également permis de clarifier la composition du microbiote nasal attendu chez un chien en bonne santé, dominé dans la majorité des cas par le genre *Moraxella* dans la plupart des échantillons. Ces résultats constituent des données de référence essentielles pour évaluer les modifications du microbiote chez les chiens atteints d'affections nasales.

L'**étude 3** visait à évaluer si une dysbiose spécifique du microbiote nasal était associée aux chiens atteints de rhinosinusite fongique, en comparaison avec le microbiote nasal de chiens en bonne santé appariés sur la race et de chiens atteints de rhinite idiopathique chronique. Les résultats ont confirmé des altérations majeures du microbiote nasal chez les chiens atteints de rhinite fongique et de rhinite idiopathique chronique, consistant principalement en une diminution du genre *Moraxella*. Une augmentation de familles bactériennes spécifiques a également été observée : *Staphylococcaceae*, *Porphyromonadaceae*, *Enterobacteriaceae* et *Neisseriaceae* dans la rhinite fongique ; *Pasteurellaceae* et *Lactobacillaceae* dans la rhinite idiopathique chronique. Ces observations suggèrent que des profils de dysbiose distincts sont associés à différentes maladies nasales chroniques. Il reste à déterminer si ces profils influencent l'évolution de la maladie.

En conclusion, ce travail met en évidence les limites de la culture bactérienne conventionnelle, pour appréhender la complexité de l'écosystème microbien nasal chez les chiens atteints de maladies nasales chroniques. Il démontre également qu'en conditions physiologiques, le microbiote nasal canin présente une structure relativement homogène entre individus, pouvant être influencée par des facteurs anatomiques intrinsèques. Enfin, il permet de caractériser des profils spécifiques de dysbiose associés à différentes affections nasales chroniques, offrant ainsi de

nouvelles perspectives pour mieux comprendre leur physiopathologie et envisager des stratégies diagnostiques et thérapeutiques ciblées.

General preamble

Chronic nasal disorders are a common cause of referral in veterinary practice and often pose significant diagnostic and therapeutic challenges. *Aspergillus fumigatus*, the predominant etiologic agent of fungal rhinosinusitis, is an ubiquitous saprophytic fungus, and its transition from environmental commensal to pathogenic organism suggests an underlying failure of host defense mechanisms (Peeters and clercx, 2007; Barrs and Dear, 2024). While local immune dysregulation is strongly suspected to contribute to the pathogenesis of the disease, the precise mechanisms involved remain incompletely understood (Peeters and Clercx, 2007; Billen and Peeters, 2017; Sharman and Mansfield, 2012). Despite significant advances in therapeutic protocols for canine fungal rhinosinusitis, no universally accepted gold standard has yet been established. More recently, shortened antifungal infusion protocols have also been evaluated (Vangrinsven et al., 2018). Nevertheless, treatment outcomes remain variable, recurrences can occur, and complete eradication is not always achieved (Schuller and Clercx, 2007; Billen et al., 2010; Sharman and Mansfield, 2012), highlighting the need for a deeper understanding of the disease's biological underpinnings.

In parallel, advances in microbiome research have highlighted the role of commensal microbial communities in maintaining immune homeostasis and preventing pathogen overgrowth (Zheng et al., 2020). In human medicine, growing evidence implicates alterations in the nasal microbiota, commonly referred to as dysbiosis, in the development and progression of several chronic inflammatory upper airway diseases, including chronic rhinosinusitis, allergic rhinitis and fungal rhinosinusitis (Tai et al., 2021; Dellière et al., 2021). These observations suggest that disruption of the resident microbial ecosystem may play a central role not only in disease initiation but also in its chronicity and resistance to conventional therapy (Tai et al., 2021).

Within the One Health framework, antimicrobial stewardship has become a major priority in veterinary medicine, as unnecessary antimicrobial use contributes to the emergence and dissemination of antimicrobial resistance (Weese et al., 2015). Dogs with nasal disease are often treated empirically or based on questionable nasal swab culture results, which may lead to misinterpretation and inappropriate antimicrobials prescriptions. Reducing unnecessary antimicrobial use in companion animals is essential to improve clinical practice and to preserve antibiotic efficacy for human health, underscoring the need for robust data to refine diagnostic and therapeutic strategies (Weese et al., 2015).

Our current understanding of the nasal microbiota in dogs remains very limited. In contrast to other microbial ecosystems, such as the gastrointestinal microbiota, the nasal cavity has received little investigative attention in the dog. Existing data stem primarily from a few exploratory studies, often conducted on small sample sizes (Tress et al., 2017; Ericsson et al., 2016). As a result, the composition of the normal nasal microbiota, its interindividual variability, and its potential role in respiratory health or disease remain largely unknown in dogs. Further research is needed to better characterize the canine nasal microbiota, its interactions with the host, and its dynamics in inflammatory or infectious context.

Introduction

1 The nasal cavities in healthy dogs

1.1 Anatomy

The respiratory tract is anatomically divided into two main sections: the upper and lower respiratory tracts. The upper respiratory tract includes the external nares, nasal vestibule, nasal cavities, paranasal sinuses, and nasopharynx. The morphology of the nasal cavities is largely determined by the anatomical conformation of the skull. Through selective breeding practices aimed at producing specific phenotypic traits, canine breeds have been progressively diversified and can now be classified into three major groups based on their cephalic index: brachycephalic, mesocephalic, and dolichocephalic (Figure 1).

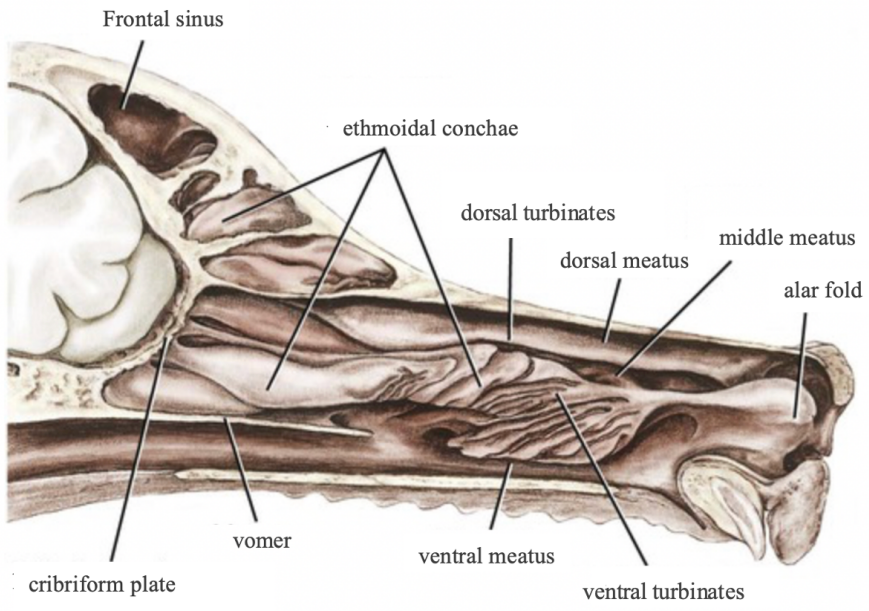
Figure 1: Skull conformation distinguishing the brachycephalic (e.g. pug), mesocephalic (e.g. Labrador) and dolichocephalic (e.g. greyhound) breeds (Source: NATOM Explorer dog and cat).



Dolichocephalic and mesocephalic dogs exhibit relatively similar nasal cavity anatomy. The nasal passage extends from the external nares rostrally to the choanae caudally, which provide access to the nasopharynx. The two nasal cavities are separated by the nasal septum. Immediately caudal to the nostrils and nasal vestibule, the nasal cavities are divided into three distinct passages by the nasal turbinates: the dorsal, middle, and ventral meatuses, each of which communicates with the common meatus. These meatuses are delimited by the dorsal and ventral turbinates (Figure 2). Progressing caudally, the ventral turbinate becomes subdivided and expands to occupy nearly the entire transverse section of the nasal cavity, leaving only the dorsal meatus clearly identifiable above it. The ventral meatus leads into the nasopharyngeal meatus, choanae, and ultimately the

nasopharynx. The dorsal meatus merges caudally with the common meatus, while the middle meatus provides access to the ethmoidal turbinates (comprising endo- and ectoturbinals), which are attached to the cribriform plate. Located caudo-dorsally to the ethmoidal turbinates is the frontal sinus, typically divided into three compartments: rostral, medial, and lateral. These air-filled cavities within the skull bones communicate with the nasal cavities. In contrast, the maxillary and sphenoidal sinuses are generally less developed. All three paranasal sinuses drain into the middle meatus.

Figure 2: Sagittal section showing the nasal turbinates. (Source: Miller's anatomy of the dog, 4th edition).



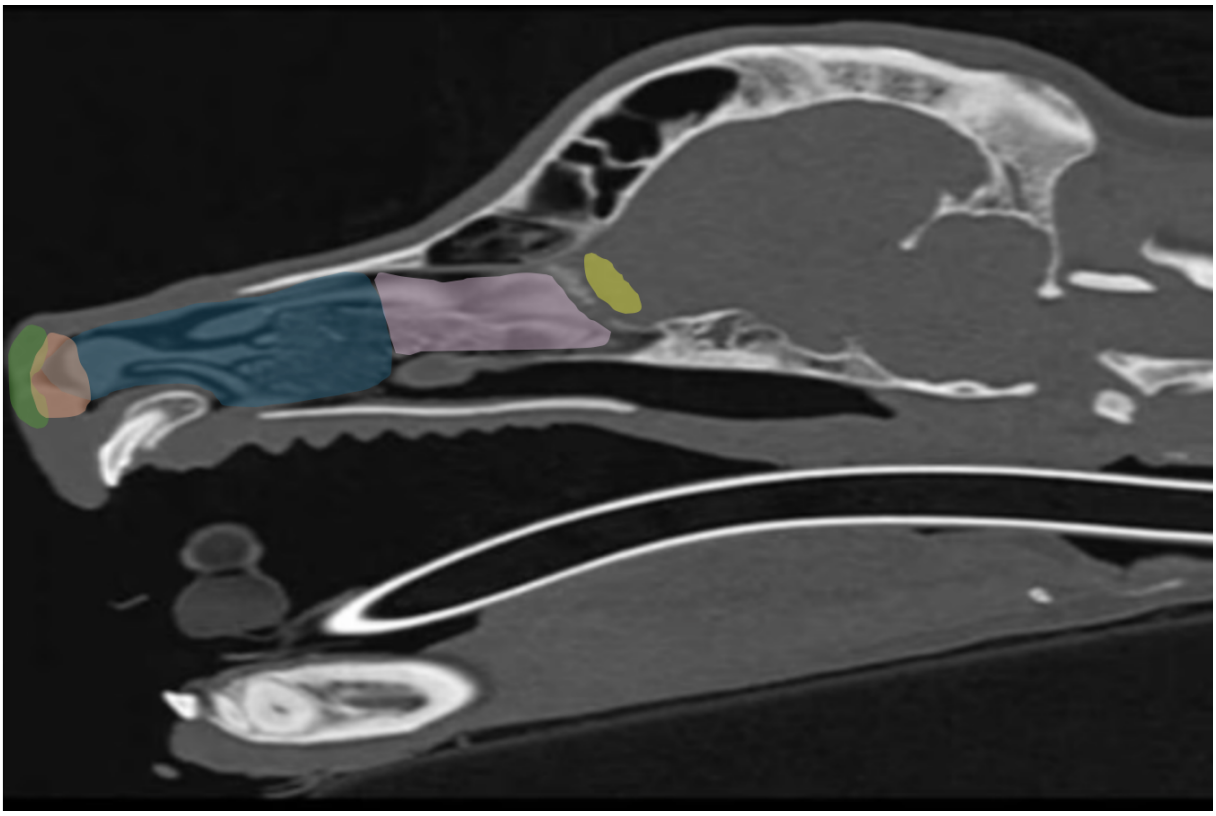
Notably, the overall conformation of the nasal structures is strongly influenced by cranial morphology, which has been significantly altered by artificial selection in modern dog breeds, particularly in brachycephalic types that exhibit extreme facial shortening and associated structural changes in the viscerocranium (Selba et al., 2020). In these breeds, selective breeding has led to a substantial reduction in nasal cavity size, the nostril entrance becomes stenotic, the nasal turbinates deform and grow abnormally, the frontal sinus diminishes in size or may even be absent, and excess soft tissues narrow the lumen of the nasopharynx. All of these changes contribute to the obstruction and increased airway resistance in the nasal cavities of brachycephalic dogs (Oechtering et al., 2016).

1.2. Physiology

The nasal cavities play a crucial role in various functions related to the inspired air. They enhance air quality by warming it via submucosal vascular plexuses, humidifying it through nasal gland secretions, and filtering and purifying it using the mucociliary clearance system and sneezing reflexes. The dorsal meatus channels airflow carrying odorant molecules through the intricate labyrinth formed by the turbinates, particularly the ventral turbinate. Beyond its role in airflow modulation, the ventral turbinate contributes significantly to thermoregulation in dogs. In brachycephalic dogs, the markedly reduced size and complexity of this structure are believed to contribute to impaired heat dissipation and are likely involved in the pathogenesis of heat and exercise intolerance commonly observed in these breeds (Oechtering et al., 2016).

The nasal cavities are lined with distinct epithelial types that differ in both morphology and function, including squamous, transitional, respiratory, and olfactory epithelia (Craven et al., 2007). The luminal surface of the nasal vestibule is covered by a squamous epithelium resembling that of the skin. This transitions into a zone of transitional epithelium, which in turn gives way to ciliated respiratory epithelium lining most of the non-olfactory nasal mucosa. Goblet cells and subepithelial glands within this region produce mucus, which is propelled by the coordinated beating of cilia, forming the mucociliary clearance system. This system serves as a primary defense mechanism by trapping inhaled particles and irritants, which are then transported toward the oropharynx to be swallowed and eliminated, aided by the mucus's bactericidal properties. In contrast, the ethmoidal turbinates are lined by olfactory epithelium, characterized by non-motile olfactory cilia embedded in a thin mucus layer. This specialized structure facilitates the capture of odorant molecules, which are subsequently transmitted to the olfactory bulb for processing (Figure 3).

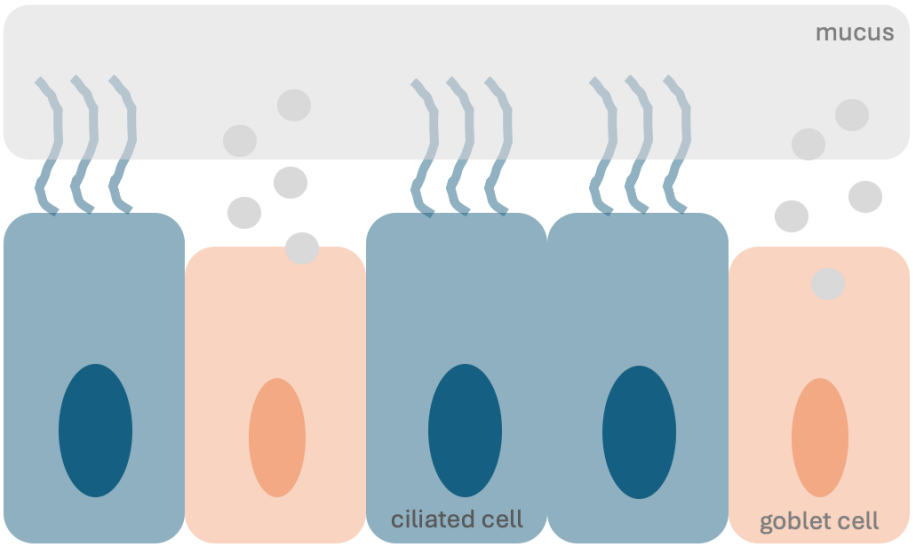
Figure 3: Computed tomography (sagittal reconstruction) highlighting the localisation of the different epithelia and oflactory bulb (Source: created by the author).



Green: squamous epithelium; orange: transitional epithelium; blue: respiratory epithelium; pink: transitional epithelium; yellow: olfactory bulb.

The nasal cavities also play an active role in immunological defense. In dogs, the nasal immune system constitutes a complex network of protective mechanisms that act synergistically to defend against inhaled pathogens and preserve mucosal integrity. This system integrates several layers of defense, including physical barriers, mucociliary clearance mechanisms (Figure 4), innate immune responses, mucosal immunity, and locally secreted immunoglobulins. Together, these components enable the nasal mucosa to detect, neutralize, and eliminate potentially harmful agents while maintaining immune homeostasis.

Figure 4: Mucociliary apparatus of the nasal cavity (Source: created by the author).



Goblet cells produce the mucus which entraps inhaled particles. These particles are discharged by the coordinate movements of the cilia.

The upper respiratory tract is protected by both innate and adaptive immune mechanisms. While potentially harmful airborne microorganisms are recognized and eliminated, commensal microbial communities are generally tolerated due to immune hyporesponsiveness at the mucosal surface (Kumpitsch et al., 2019). The innate immune system provides the first line of defense through the action of neutrophils, macrophages, and natural killer cells, which identify and destroy pathogens via phagocytosis and the release of antimicrobial molecules. The innate immune system also involves the respiratory epithelium which acts both as a physical and immunological barrier through mucociliary clearance, tight junction-mediated epithelial integrity, and the production of antimicrobial peptides, cytokines and chemokines that regulate local immune responses. The nasal cavities also support local mucosal immune responses. Although the canine nasal mucosa lacks organized mucosal lymphoid structures, it harbors diffuse lymphoid populations (Day, 2009). This mucosa-associated lymphoid tissue includes specialized immune cells such as lymphocytes, plasma cells, and antigen-presenting cells, which collectively orchestrate antigen recognition and immune activation. Immunoglobulins (particularly IgA, but also IgG and IgM) play a central role in mucosal immunity. Within the nasal mucosa, IgA-secreting plasma cells are the predominant subset and contribute to pathogen neutralization and inhibition of microbial adherence to the epithelium. The nasopharyngeal tonsil, located in the caudal nasopharynx just distal to the openings of the auditory tubes, functions as a primary antigen sampling site, where IgA-producing plasma cells also predominate (Billen et al., 2006).

2 Diseases of the nasal cavities in dogs

2.1 In general

Diseases affecting the nasal cavities are commonly encountered in canine clinical practice. Depending on the underlying etiology, a variety of clinical signs may be observed, including sneezing, nasal discharge of varying characteristics (serous, mucoid, purulent, or hemorrhagic), reverse sneezing, ulceration or depigmentation of the nares, facial asymmetry, deformity, nasal pain, and stertorous breathing.

When confronted with such clinical signs, several key factors can assist in narrowing down the differential diagnoses. These include the patient's age, breed predisposition, duration and progression of clinical signs, laterality of nasal discharge (unilateral vs bilateral), and the presence or absence of systemic manifestations. Among the most frequently diagnosed nasal conditions in dogs are nasal neoplasia, fungal rhinosinusitis, chronic idiopathic rhinitis, nasal foreign bodies, and periodontal disease (Tasker et al., 1999; Windsor et al., 2004; Meler et al., 2008; Lobetti, 2009; Plickert et al., 2014; Rösch et al., 2019). Less frequently, congenital anomalies and parasitic rhinitis caused by *Pneumonyssoides caninum* may be identified (Windsor et al., 2004; Meler et al., 2008; Rösch et al., 2019).

Certain systemic conditions, including coagulopathies, severe hypertension, hyperviscosity syndromes, and gastrointestinal disorders associated with vomiting or regurgitation, may present with secondary nasal signs (Cohn, 2020). In cases of nasal signs associated with systemic illness, or of isolated epistaxis in the absence of other nasal abnormalities, extranasal diseases should be considered as differential diagnosis (Cohn, 2020).

Among the primary causes of chronic nasal discharge in dogs, the most frequently diagnosed conditions include nasal neoplasia, fungal rhinosinusitis, and chronic idiopathic rhinitis (Tasker et al., 1999; Meler et al., 2008; Lobetti, 2009). These chronic conditions are more commonly observed in medium to large breed dogs with mesocephalic or dolichocephalic cranial conformations (Lefebvre et al., 2005; Meler et al., 2008).

2.2 Fungal rhinosinusitis

2.2.1 Etiopathogenesis

Fungal rhinosinusitis (FR) in dogs is caused by the inhalation of spores from the ubiquitous environmental mold *Aspergillus fumigatus*. Although *Aspergillus fumigatus* is the predominant etiological agent, other fungal species, including *Aspergillus flavus*, *Aspergillus niger, Aspergillus nidulans*, and *Penicillium* spp., have also been implicated in the disease, albeit much less frequently. Inhaled conidia that evade mucociliary clearance mechanisms may germinate under favorable local conditions, particularly warm, humid environments, leading to the formation of fungal hyphal plaques that adhere to the sinonasal mucosa (Barrs and Dear, 2024). Although exposure to *Aspergillus* spores is universal among dogs and humans, only a minority of individuals develop clinical disease.

In contrast to human medicine, where *Aspergillus* spp. primarily causes lower respiratory tract infections in immunocompromised hosts, canine FR occurs predominantly in immunocompetent individuals (Table 1). The condition in dogs is typically non-invasive, with fungal hyphae remaining confined to the nasal cavity and frontal sinuses, without breaching the respiratory mucosa (Peeters et al., 2005).

To transition from the saprophytic to the pathogenic form, *Aspergillus fumigatus* must adapt to the host environment by deploying multiple pathogenicity and virulence traits. These include intrinsic conidial characteristics that facilitate host entry, notably their small size (2–3 µm), which allows them to bypass mucociliary clearance, the RodA hydrophobin-coated rodlet layer, which masks pathogen-associated molecular patterns from host immune recognition, and DHN-melanin, which protects conidia against reactive oxygen species and phagocyte-mediated killing (van de Veerdonk et al., 2017; Latgé and Chamilos, 2019). Secreted metabolites and toxins further promote fungal persistence by impairing host defense mechanisms. Gliotoxin, together with other metabolites such as fumagillin and helvolic acid, reduces mucociliary function, facilitating contact of fungal elements with the respiratory epithelium (Tomee and Kauffmann, 2000; Gu et al., 2021) Gliotoxin also inhibits macrophage adherence and phagocytosis, while other fungal metabolites impair neutrophil function, complement-mediated opsonization, intracellular killing, and T-cell

proliferation, thereby promoting immune evasion (Tomee and Kauffman, 2000; van de Veerdonk et al., 2017; Gu et al., 2021). In addition, fungal adherence to extracellular matrix proteins, including laminin, fibronectin, collagen, fibrinogen, and complement component C3, facilitates colonization and may enhance tissue invasion (Tomee and Kauffman, 2000). Finally, galactosaminogalactan contributes to biofilm formation, epithelial persistence, and protection from host immune responses (van de Veerdonk et al., 2017; Gu et al., 2021; Earle et al., 2023).

The current state of knowledge regarding *Aspergillus fumigatus* virulence factors does however not explain why this pathogen causes severe rhinosinusitis in some dogs but no symptoms in others. As a result, host-related factors may also be involved. In canine sino-nasal aspergillosis, overt systemic immunodeficiency or generalized defects of innate and adaptive immune mechanisms are not typically identified, although concurrent disease, facial trauma, nasal foreign bodies, and dental disease have occasionally been implicated. A dysfunction in toll-like receptor (a class of proteins playing a role in the innate immune response) expression or signaling has been proposed as a primary immunological defect contributing to the pathogenesis of canine FR (Mercier et al., 2012; Mercier et al., 2014; Billen and Peeters, 2017). The exact T-helper (Th) cell responses required for effective immune defense against *Aspergillus fumigatus* remain unclear. It is hypothesized that a local imbalance between pro-inflammatory signals (mediated by Th1 cells) and anti-inflammatory responses (involving Th17 and possibly regulatory T cells) may contribute to disease persistence and the associated inflammatory cascade (Vanherberghen et al., 2012, Vanherberghen et al., 2013; Billen and Peeters, 2017). Additionally, the extensive infiltration of inflammatory cells into the affected mucosa, combined with the production of proteolytic enzymes and toxic secondary metabolites by *Aspergillus fumigatus*, is thought to play a central role in the progressive tissue damage and bone destruction observed in affected dogs (Peeters et al., 2005; Frisvad et al., 2016).

2.2.2 Diagnosis

The typical clinical profile raising suspicion for FR is a young to middle-aged, large-breed dog with a mesocephalic or dolichocephalic cranial conformation. Affected dogs commonly present with unilateral or bilateral epistaxis and mucopurulent nasal discharge. Systemic signs, such as lethargy and reduced appetite, may be present but are often nonspecific. On physical examination, findings such as facial pain, depigmentation and/or ulceration and/or hyperkeratosis of the nasal planum,

and normal to increased nasal airflow may further support clinical suspicion (Figure 5). For definitive diagnosis, rhinoscopy and sinusoscopy remain the most effective diagnostic modalities, allowing direct visualization of fungal plaques and facilitating localized therapeutic interventions (Figure 6). However, in certain cases, the fungal colonization is confined to the frontal sinuses and/or the caudal nasal cavity, regions that may not be accessible during standard rhinoscopic procedures. Computed tomography (CT) serves as a valuable complementary tool, particularly when rhinoscopic access is limited. Characteristic CT findings suggestive of sinonasal aspergillosis include moderate to severe turbinate destruction with varying degrees of abnormal soft tissue within the nasal passages; non-specific mucosal thickening adjacent to the bones of the frontal sinus, maxillary recess, and nasal cavity; and evidence of reactive osseous changes, such as dorsolateral thickening and localized lysis of the frontal bone crest (Figures 7 and 8) (Saunders et al., 2002). In cases of chronic clinical signs, CT is recommended to assess the integrity of the cribriform plate. Identification of cribriform plate lysis warrants caution with intranasal topical therapy, due to the theoretical risk of drug leakage into the cranial vault and subsequent neurological complications (Mathews et al., 1996; Peeters and Clercx, 2007). However, more recent studies have reported that topical antifungal treatments were well tolerated even in dogs with marked cribriform plate destruction, without resulting in adverse neurological outcomes (Belda et al., 2018; Ballber et al., 2018). Additional diagnostic modalities that can aid in reaching a diagnosis include cytology (De Lorenzi et al., 2006), fungal culture (Pomrantz et al., 2007), molecular diagnostics such as PCR targeting *Aspergillus fumigatus* and other *Aspergillus* spp. (Biénès et al., 2022), and serological testing (Billen et al., 2009). Cytological examination of nasal swabs from affected dogs typically reveals dark blue, septate hyphae with parallel walls and dichotomous branching at approximately 45°, characteristic of *Aspergillus* spp. (Figure 9). These fungal elements are often observed in association with neutrophilic inflammation and phagocytosed bacteria, consistent with concurrent secondary bacterial infection (De Lorenzi et al., 2006).

Figure 5: External appearrance of the nostrils in two dogs with fungal rhinosinusitis (Source: Image courtesy of the Internal Medicine Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



(A) Marked ulceration of the ventrolateral margin of the right nostril and mild hyperkeratosis of the dorsal nasal planum. (B) Mucopurulent, mildly hemorrhagic nasal discharge, dorsal nasal planum hyperkeratosis, and ventral depigmentation of the nasal planum of the left nostril.

Figure 6: Fungal plaques in the frontal sinus (upper panel) and nasal cavity (lower panel) of dogs with fungal rhinosinusitis (Source: Images courtesy of the Endoscopy Unit image database, Small Animal Internal Medicine, University of Liège, Belgium).

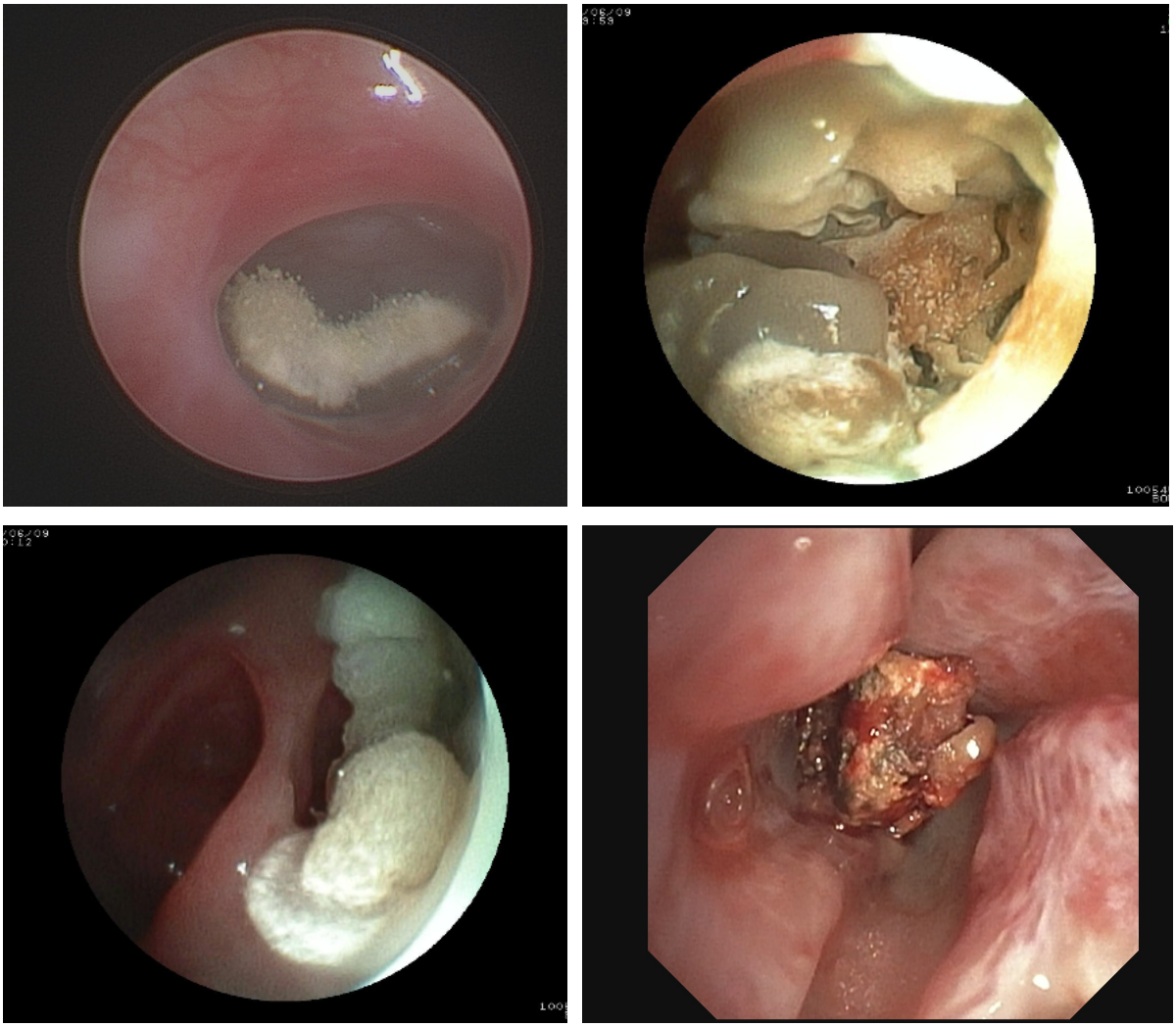
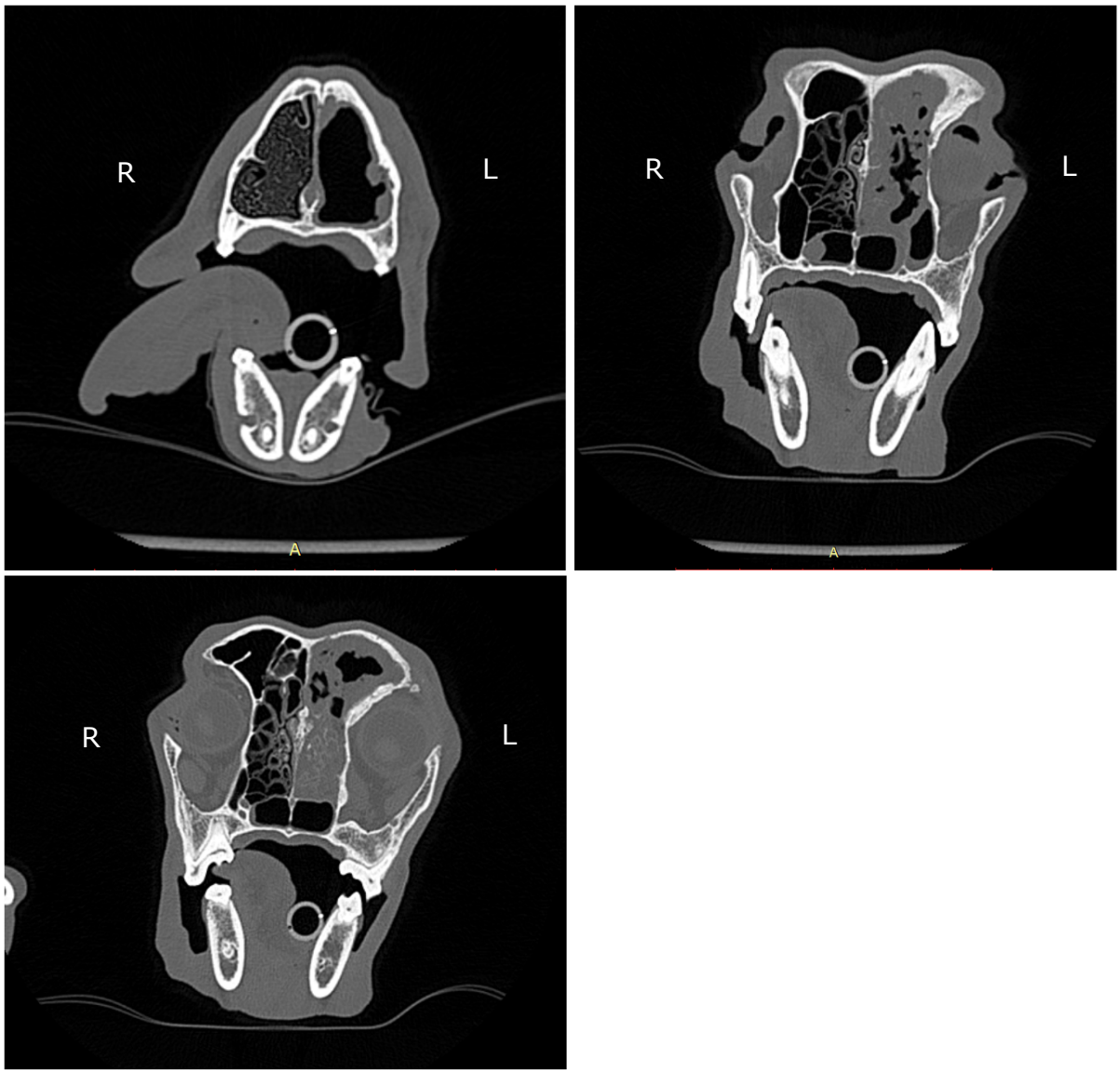
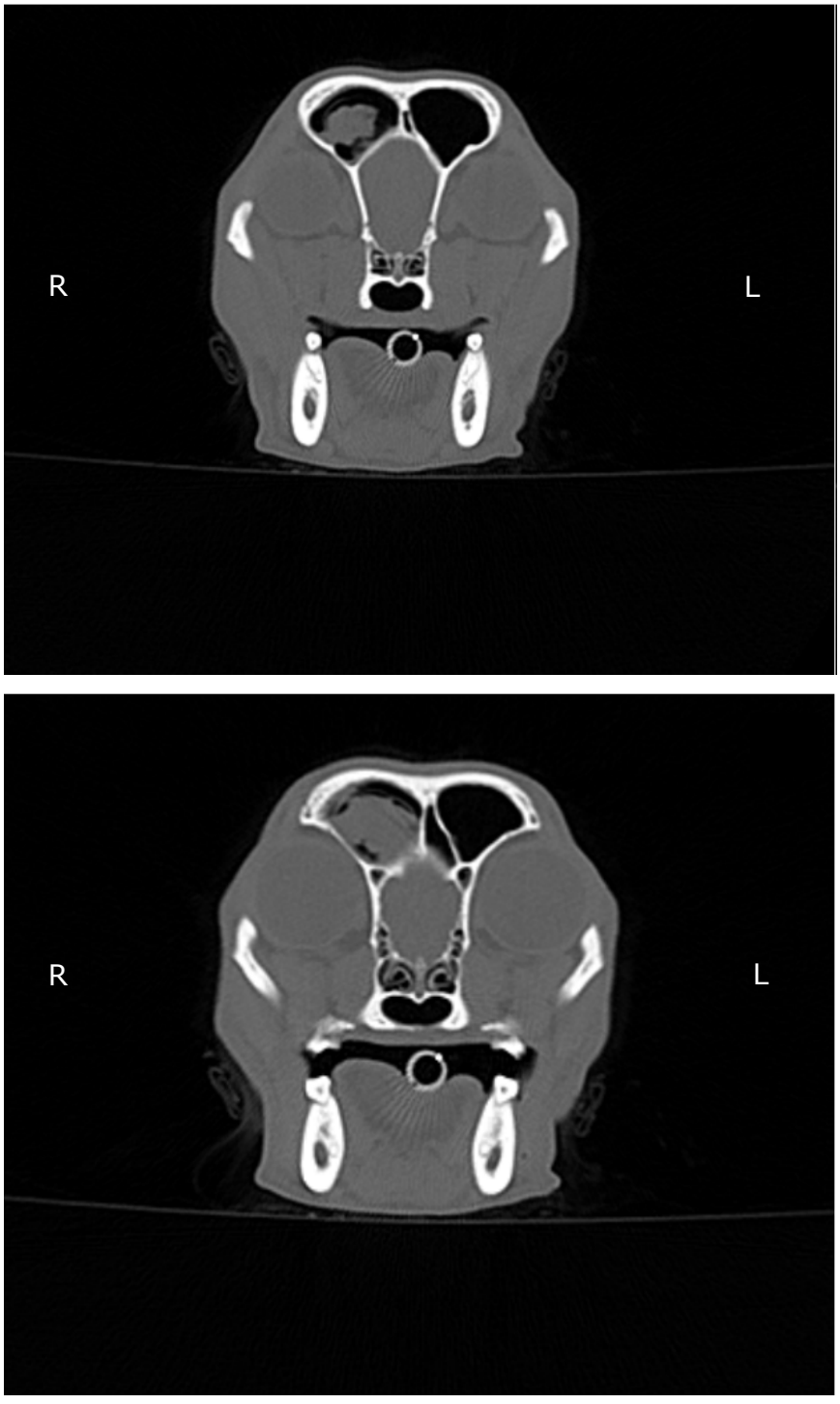


Figure 7: Computed tomography (coronal reconstructions) of a dog with fungal rhinosinusitis (Source: Images courtesy of the Medical Imaging Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



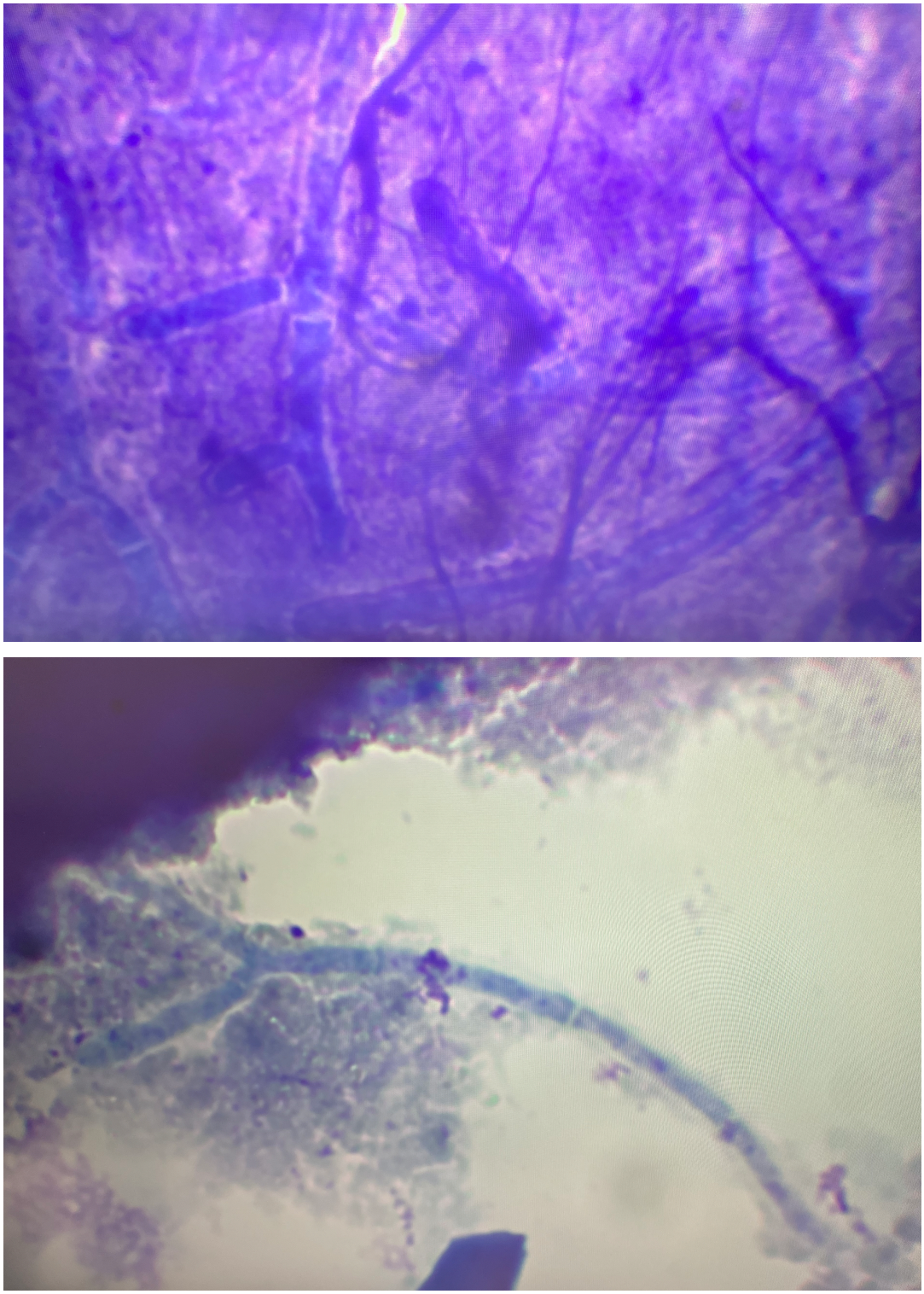
There is marked turbinate lysis on the left side associated with erosion of the nasal septum and maxillary bone. There is subtotal opacification of the left frontal and ethmoid sinuses with slightly heterogeneous soft tissue density. A mild reactive periosteal new bone formation and sclerosis are visible around the affected areas.

Figure 8: Computed tomography (coronal reconstructions) of a dog with fungal rhinosinusitis (Source: Images courtesy of the Medical Imaging Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



The right frontal sinus appears partially filled with soft tissue density with a convex contour, compatible with fungal material. The contralateral frontal sinus remains aerated, highlighting the unilateral distribution.

Figure 9: Cytology of a nasal swab showing septate hyphae compatible with *Aspergillus* spp. or *Penicillium* spp. in a dog with fungal rhinosinusitis (Diff-quick stain, 100x magnification), (Source: Images courtesy of the Internal Medicine Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



Dense aggregates of septate fungal hyphae, exhibiting regular septation and parallel walls, observed on Diff-Quik cytology.

2.2.3 Treatment

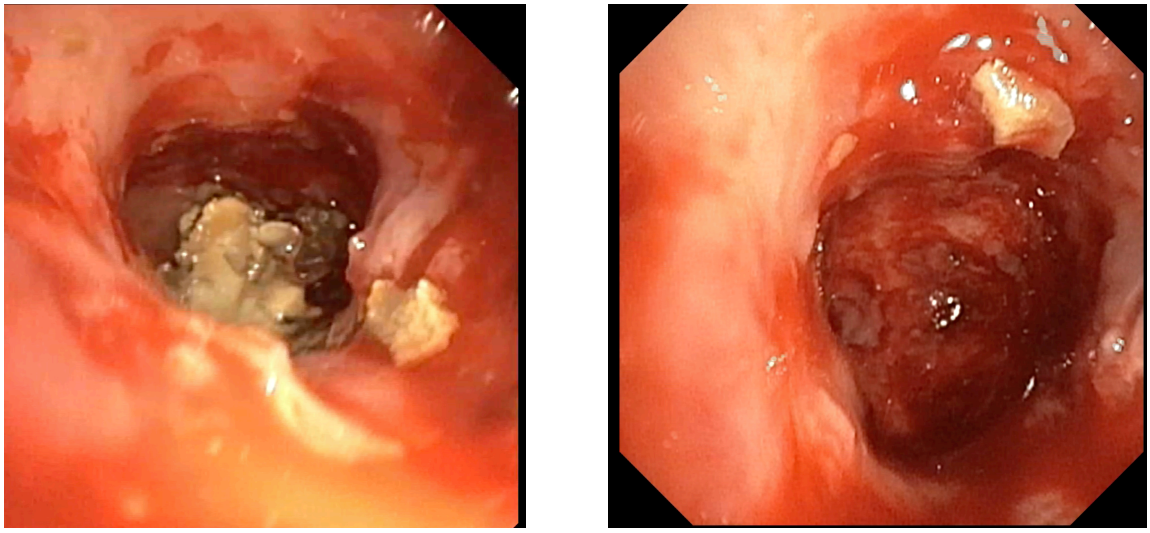
The treatment of FR remains challenging, and no universally accepted gold standard protocol has been established to date. Systemic antifungal therapy using azole derivatives generally demonstrates poor to moderate efficacy, requires prolonged administration, and is often associated with adverse effects (Peeters and Clercx, 2007). Nonetheless, more recent reports suggest improved clinical outcomes with combination therapy using posaconazole and terbinafine (Stewart and Bianco, 2017).

Topical administration of antifungal agents, such as enilconazole or clotrimazole, via catheters placed surgically, blindly, or endoscopically, has yielded higher success rates (Peeters and Clercx, 2007; Billen and Peeters, 2017). Minimally invasive protocols typically involve thorough endoscopic debridement (figure 10) of fungal plaques followed by a 1-hour infusion of 1% clotrimazole or 1–2% enilconazole through blindly placed catheters into each nasal cavity (Mathews et al., 1998; Zonderland et al., 2002; Saunders et al., 2003; Schuller and Clercx, 2007; Greci et al., 2008; Pomrantz and Johnson, 2010; Sharman et al., 2010) or endoscopically directed catheters (Figure 11) targeting the affected frontal sinuses and/or nasal cavities (Mathews et al., 1996; McCullough et al., 1998; Saunders et al., 2003; Schuller and Clercx, 2007). Comparison of treatment outcomes remains difficult due to a lack of standardized definitions of therapeutic success (Billen and Peeters, 2017). However, studies incorporating endoscopic follow-up have reported positive outcomes in 46.6% to 85.7% of cases after a single treatment, and in 89.5% to 100% of cases after repeated treatments (Zonderland et al., 2002; Saunders et al., 2003; Schuller and Clercx, 2007; Greci et al., 2008; Billen et al., 2010). A major limitation of these minimally invasive approaches is the duration of the procedure, mainly due to the required soaking period. Furthermore, complete cure and prevention of recurrence cannot be guaranteed. These limitations highlight the need for a better understanding of the predisposing factors and local conditions that facilitate fungal persistence, including the role of the nasal microbiota, biofilm formation, mucosal pH, and host immune responses that may fail to control or eradicate the infection effectively.

Prior to initiating the present thesis, we started exploring the therapeutic management of FR in dogs. This interest led to a clinical study aimed at comparing two minimally invasive intranasal enilconazole infusion protocols for the treatment of canine sinonasal aspergillosis. Specifically, the objectives of the study were to determine whether a simplified and time-reduced infusion protocol of 15 minutes could yield clinical outcomes non-inferior to those of a standard 1-hour protocol, and to evaluate the prognostic relevance of achieving complete endoscopic removal of fungal plaques.

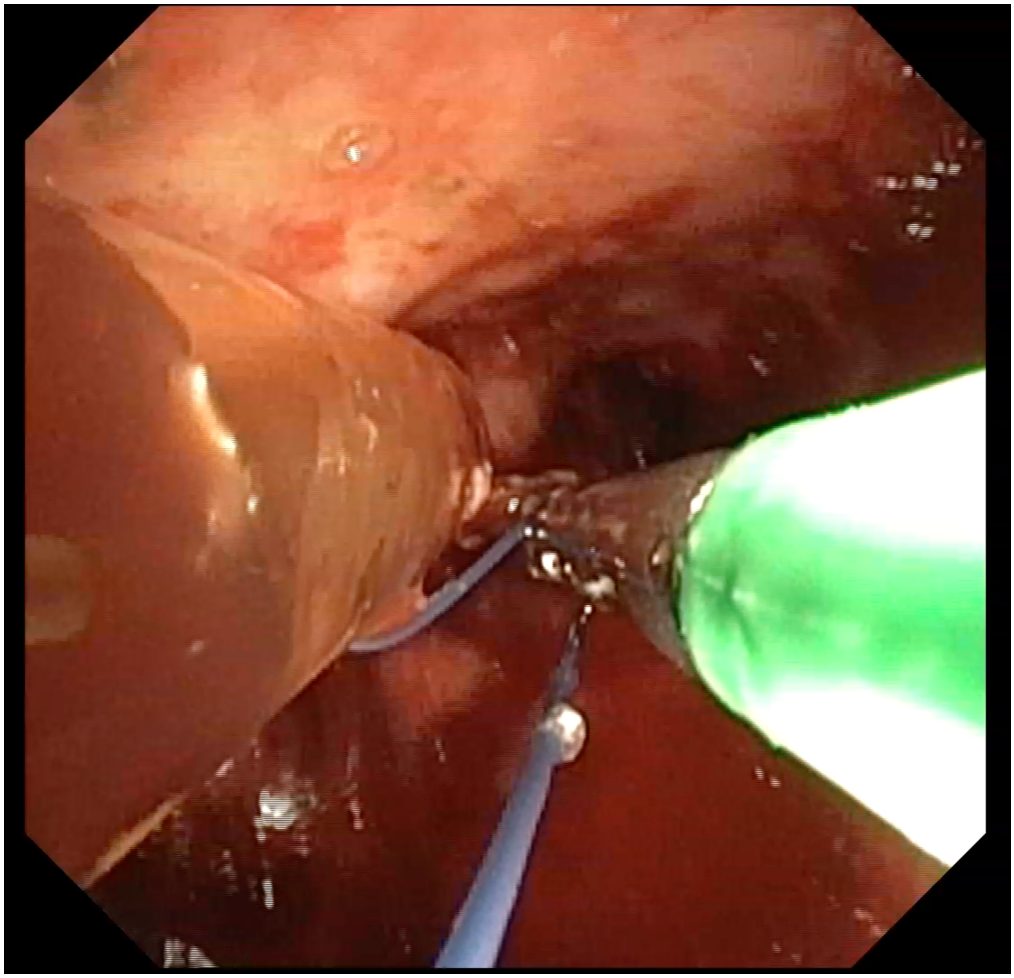
This work was motivated by a practical clinical need to optimize therapeutic approaches by improving procedural efficiency and reducing anaesthesia times, while maintaining high standards of efficacy and safety in the management of canine FR. Following thorough endoscopic debridement, dogs treated with the simplified protocol were positioned in dorsal recumbency with the head elevated at approximately 90°, and bilateral nasal infusion catheters were placed endoscopically to allow manual instillation of 2% enilconazole solution until reflux was observed at the nostrils (Figure 12). The simplified 15-minutes protocol significantly reduced treatment duration (median 92 minutes vs. 201 minutes) without compromising efficacy. First treatment success rates were 58% and 62.5% for the standard 1-hour protocol and for the simplified 15-minutes protocol respectively. Complete endoscopic debridement was also associated with better outcomes. These findings support the simplified protocol as an efficient and effective alternative for managing canine sinonasal aspergillosis (Vangrinsven et al., 2018).

Figure 10: Endoscopic appearance of the frontal sinus before and after debridement of fungal plaques (Source: Images courtesy of the Endoscopy Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



Left panel: Frontal sinus filled with abundant adherent fungal plaques characteristic of fungal rhinosinusitis. Right panel: Same frontal sinus following endoscopic debridement, demonstrating removal of the fungal plaques and exposure of the underlying mucosa.

Figure 11: Endoscopic placement of a catheter into the affected frontal sinus for topical antifungal treatment (Source: Images courtesy of the Endoscopy Unit image database, Caladrius Veterinary Specialist Centre, Belgium).



Endoscopic view showing grasping of a pre-positioned suture attached to the tip of the catheter using biopsy forceps passed through the endoscope working channel. The catheter is then guided through the frontal sinus ostium into the affected frontal sinus, allowing subsequent instillation of topical antifungal cream.

Figure 12: Topical antifungal treatment following endoscopic debridement (Source: Image courtesy of the Endoscopy Unit image database, Small Animal Internal Medicine, University of Liège, Belgium).



The dog is intubated and positioned in dorsal recumbency with the head maintained at approximately 90° and bilateral infusion catheters placed within the nasal cavities. Manual infusion of 2% enilconazole solution is performed through the indwelling nasal catheters. Leakage of the solution into the nasopharynx is prevented by packing the nasopharynx with gauze sponges combined with manual compression of the soft palate.

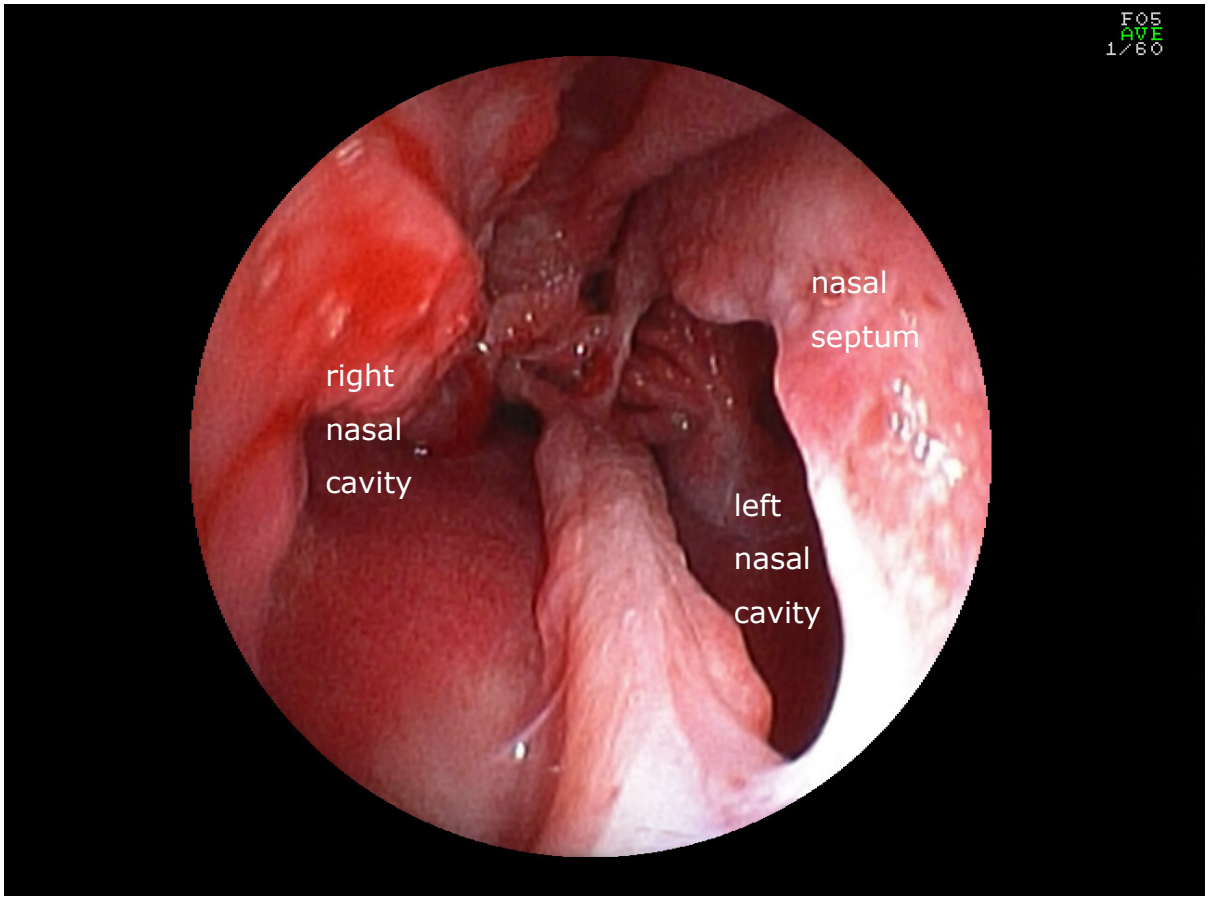
2.2.4 Long-term outcome

Recurrence of FR is considered uncommon but remains a recognized complication. When recurrence occurs, it typically develops between 2 and 36 months following an initially successful treatment (Schuller and Clercx, 2007; Greci et al., 2008; Pomrantz and Johnson, 2010). Isolated reports have described delayed recurrences, including a case in which clinical signs reappeared 4 years post-treatment (Schochet and Lappin, 2007).

Reduced treatment success rates have been attributed to incomplete debridement of fungal plaques prior to the administration of topical antifungal agents (Claeys et al., 2006; Billen et al., 2010; Sharman et al., 2010; Sharman et al., 2012; Sharman and Mansfield, 2012). In some cases, despite apparent mycological cure, mild to moderate nasal clinical signs may persist. In one study, 22% of treated dogs exhibited ongoing clinical signs; approximately half of these cases responded to topical antibiotic therapy, suggesting a secondary bacterial rhinitis (Schuller and Clercx, 2007). One dog in the same study responded favorably to anti-inflammatory treatment with methylprednisolone, raising suspicion of post-infectious lymphoplasmacytic rhinitis.

Extensive and often irreversible damage to the nasal turbinates caused by the fungal infection (Figure 13) is believed to predispose affected dogs to the development of chronic inflammatory rhinitis and/or recurrent bacterial infections (Schuller and Clercx, 2007; Greci et al., 2008; Sharman et al., 2010).

Figure 13: Control rhinoscopy 3 weeks after debridement and enilconazole infusion protocol in a dog with bilateral fungal rhinosinusitis (Source: Image courtesy of the Endoscopy Unit image database, Small Animal Internal Medicine, University of Liège, Belgium).



Note the important lytic lesions, especially in the nasal septum, that are consequences of the fungal infection.

2.2.5 Aspergillosis in humans and comparison with canine sinonasal aspergillosis

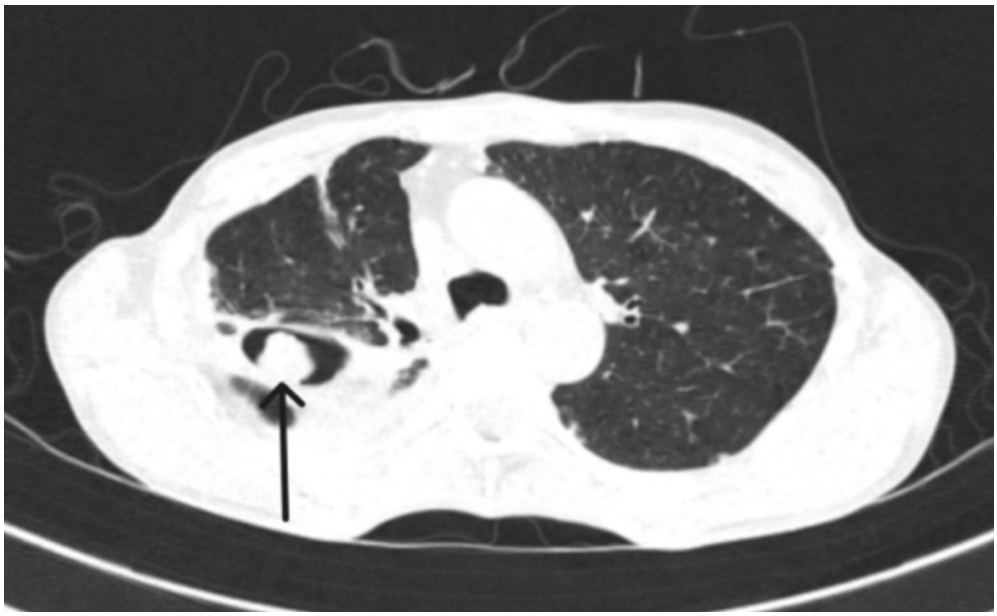
In humans, fungal infections of the respiratory tract caused by *Aspergillus* spp. most commonly affect the lower airways and are broadly classified into invasive and non-invasive forms (Cadena et al., 2021). Among the non-invasive manifestations are allergic bronchopulmonary aspergillosis and chronic pulmonary aspergillosis. The latter encompasses various conditions, including pulmonary aspergilloma and chronic cavitary pulmonary aspergillosis.

Pulmonary aspergilloma is characterized by the presence of a mobile fungal mass, commonly referred to as a fungal ball, composed of *Aspergillus* spp. hyphae, which develops within a pre-existing pulmonary cavity without invasion of the surrounding lung parenchyma. The fungal mass may be freely mobile within the cavity or partially anchored to the cavity wall by inflammatory

or granulomatous tissue (Latgé and Chamilos, 2019) (Figure 14). Pulmonary aspergilloma most commonly occurs in immunocompetent individuals and is often associated with minimal or absent clinical signs, as well as radiological stability over a period of at least three months (Chakraborty et al., 2024).

Invasive aspergillosis, by contrast, includes invasive pulmonary aspergillosis, tracheobronchial aspergillosis, and extrapulmonary dissemination. These forms predominantly affect immunocompromised patients and are associated with significant morbidity and mortality (Latgé and Chamilos, 2019).

Figure 14: Computed tomography (axial reconstructions) of a pulmonary aspergilloma (Source: Chakraborty et al., 2024).



Thick-walled cavitating lesion in the right lung. A nodular soft tissue lesion is noted inside the cavity compatible with and aspergilloma.

Paranasal aspergillosis has also been reported in humans, predominantly affecting the maxillary and sphenoidal sinuses (Pagella et al., 2007; Yang et al., 2024). This condition is categorized into invasive and non-invasive forms. Invasive presentations include acute fulminant fungal sinusitis, granulomatous invasive fungal sinusitis, and chronic invasive fungal sinusitis. These entities typically occur in immunocompromised individuals and may be potentially life-threatening (Pagella et al., 2007).

Non-invasive forms comprise allergic fungal sinusitis and paranasal sinus fungus ball (Chandrasekharan et al., 2012). A paranasal sinus fungus ball is characterized as an extramucosal,

intrasinusal fungal growth, most often presenting unilaterally in immunocompetent patients (Figure 15). The most implicated organisms are *Aspergillus fumigatus* and *Aspergillus flavus* (Pagella et al., 2007). Table 1 provides a comparative overview of the key features distinguishing canine and human fungal rhinosinusitis.

Given the similarities in localization and fungal species involved, canine sinonasal aspergillosis shares several features with human paranasal sinus fungus ball. However, notable differences remain. In humans, paranasal fungus balls are frequently asymptomatic and are often discovered incidentally on imaging (Akhondi et al., 2023). By contrast, in dogs, subclinical or mildly symptomatic presentations are uncommon, and extensive local bone destruction is commonly observed.

The mechanisms underlying this divergence in disease severity and tissue damage are not fully elucidated. Potential contributing factors include the nature and persistence of the inflammatory response elicited by the fungus, genetic predisposition in the host, and/or deficiencies in innate immune responses. Further investigation into these variables may enhance our understanding of the pathogenesis of sinonasal aspergillosis across species.

Figure 15: Computed tomography (axial reconstructions) of a paranasal fungus ball in a human patient (Source: Pagella et al., 2007).



Opacification of the left maxillary sinus with soft tissue density and central hyperdensity. Sclerosis and mild expansion of the maxillary walls. Findings compatible with fungal sinusitis and fungus ball.

Table 1: Comparison of canine fungal rhinosinusitis and human paranasal fungal ball.

	Canine	**Human**
Risk factors	Anatomical conformation of the skull, suspected local immunological deficiencies (Peeters and Clercx 2007). Nasal foreign body, dental disease and facial trauma (Peeters and Clercx 2007; Day, 2009).	Dental origine such as dental implant (Jang et al., 2022), sinus surgery.
Forms	Non-invasive.	Non-invasive.
Localisation	Nasal cavity(cavities) and/or frontal sinus(es).	Paranasal sinuses (maxillary > sphenoidal) (Deutsch et al., 2019).
Histology	Intense destructive inflammation dominated by lymphoplasmacytic infiltration with admixed macrophages and fewer neutrophils. No evidence of invasion beneath the level of the mucosal epithelium. Superficial ulceration and necrosis with deeper foci of necrosis, haemorrhages and granulation. Bony destruction in deeper mucosal biopsies (Peeters et al., 2005).	Dense mass of fungal hyphae adjacent to respiratory mucosa without no tissue invasion (Daudia and Jones, 2008). An entangled mass of fungal organisms in fibrinous, necrotic exudate (Montone, 2016).
Computed tomography	Turbinate destruction, variable amount of abnormal soft tissue in the nasal passages, non-specific thickening of the mucosa, thickened reactive bone (Saunders et al., 2002).	Paranasal sinus fungal ball: Opacification of the involved sinus, frequent flocculent calcifications. Sclerosis or bone destruction can occur (Daudia and Jones, 2008).
Causative agent	*A.fumigatus* >> *A. flavus*, *A. niger*, *A. nidulans* (Day, 2009) *Penicillium* spp.	*Aspergillus fumigatus*, *Penicillium* spp., *Aspergillus flavus*, *Crysosporium* (Pagella, 2007)
Treatment	Debridement of fungal plaques and endoscopic or surgical balneation (Sharman and Mansfield, 2012).	Surgical removal of the fungus ball.
Prognosis	Generally good with appropriate treatment; recurrence is possible.	Good prognosis, in some cases invasive fungal sinusitis may develop (Ferguson, 2000).

2.3 Other common chronic nasal diseases

2.3.1 Chronic idiopathic rhinitis

Chronic idiopathic rhinitis (CR), also referred to as chronic lymphoplasmacytic rhinitis, is characterized by a diffuse infiltration of the nasal mucosa with lymphocytes and plasma cells. The diagnosis of this condition remains challenging, primarily due to the absence of specific clinical or imaging findings, aside from the histopathological pattern. Moreover, similar histologic changes can

occur in the context of other nasal diseases, such as fungal or neoplastic conditions, limiting the specificity of histopathology alone (Cohn, 2014).

The diagnosis of CR is therefore based on the exclusion of other potential causes of chronic nasal inflammation and the identification of lymphoplasmacytic infiltration on biopsy. Although terms such as immune-mediated chronic rhinitis (Burgener et al., 1987; Tasker et al., 1999) and allergic rhinitis (Van Pelt and McKiernan, 1985; McCarthy et al., 1990) have been used interchangeably, the exact pathogenesis of CR remains poorly defined.

It is possible that CR does not represent a single disease entity, but rather a heterogeneous group of disorders sharing a similar histological response pattern. Alternatively, CR may represent a sequela of a previous disease whose underlying cause has already resolved. Although an allergic mechanism has been proposed in some cases, a consistent Th2-type inflammatory profile has not been demonstrated, and the response to therapy with glucocorticoids, alone or combined with antihistamines, remains highly variable among affected individuals. These uncertainties highlight the need for further research into the immunopathogenesis and classification of this pathology.

Given that some dogs diagnosed with CR exhibit clinical improvement with antibiotic therapy, the potential involvement of specific infectious agents has been explored. In a study by Windsor et al. (2006), PCR analyses were performed on nasal biopsies obtained from dogs with CR, FR, nasal neoplasia, and from healthy controls. The aim was to detect the presence of *Bartonella*, *Mycoplasma*, and *Chlamydophila* species, and to compare their distribution across these groups. The results did not support a significant role for these bacteria in the etiopathogenesis of CR. Interestingly, the fungal DNA load was found to be higher in dogs with either CR or FR, compared to healthy dogs and those with nasal tumors. This raises the possibility that fungal organisms may contribute to the chronic inflammatory process, although their exact role remains unclear.

Investigations into the cytokine and chemokine gene expression profiles in the nasal mucosa revealed distinct patterns between dogs with CR and those with FR, indicating that these are two separate disease entities, each driven by unique immunopathological mechanisms (Peeters et al., 2005).

In humans, chronic rhinosinusitis is a prevalent and heterogeneous inflammatory condition characterized by persistent inflammation of the nasal cavity and paranasal sinuses for more than 3 months. It affects a substantial proportion of the adult population and is associated with significant morbidity and reduced quality of life (Hastan et al., 2011; Fokkens et al., 2020). Although it is commonly classified according to the presence or absence of nasal polyps, these categories

ecompass a broad spectrum of underlying disorders (Fokkens et al., 2020). Among the different forms, chronic rhinosinusitis without nasal polyps represents the most frequent phenotype.

Chronic nasal inflammatory diseases in humans and dogs share several broad pathophysiological features, including persistent mucosal inflammation, epithelial barrier alterations, and disruption of mucociliary clearance mechanisms. Chronic idiopathic rhinitis in dogs is not typically associated with the development of nasal polyps, and a consistent fungal etiology has not been demonstrated. Although canine chronic rhinitis does not correspond directly to any single phenotype of human CRS, its clinical and pathological characteristics appear more closely aligned with the non-polypoid spectrum of human chronic rhinosinusitis.

2.3.2 Nasal neoplasia

A strong presumptive diagnosis of nasal neoplasia should be considered in older dogs presenting with a progressive and intermittent unilateral nasal discharge, particularly when the discharge is hemorrhagic and accompanied by reduced nasal airflow on the same side. Dogs with a larger surface area of nasal mucous membrane (such as dolichocephalic breeds) or dogs living in urban environments with increased exposure to pollutants may have a higher risk of developing nasal neoplasia (Reif and Cohen, 1971; Hayes et al., 1982; Bukowski and Wartenberg, 1998). Computed tomography is a key diagnostic tool for chronic nasal disease in dogs (Saunders et al., 2003). It allows for accurate differentiation between neoplastic and non-neoplastic conditions, and provides detailed information on lesion localization, extent, and bone involvement (Lefebvre et al., 2005; Kuehn, 2006). The combined use of CT imaging, rhinoscopy, and histopathological examination of biopsy samples substantially improves diagnostic sensitivity and specificity, enabling a more definitive diagnosis and informed therapeutic planning (Lefebvre et al., 2005).

In dogs carcinomas account for two-thirds of nasal neoplasia, with adenocarcinoma being the most common tumor type (Malinowski, 2006). The remaining one-third is predominantly composed of sarcomas. Histopathological evaluation of nasal tissue is essential for confirming the specific tumor type. Both nasal carcinomas and sarcomas exhibit local invasiveness.

3 The respiratory tract microbiota

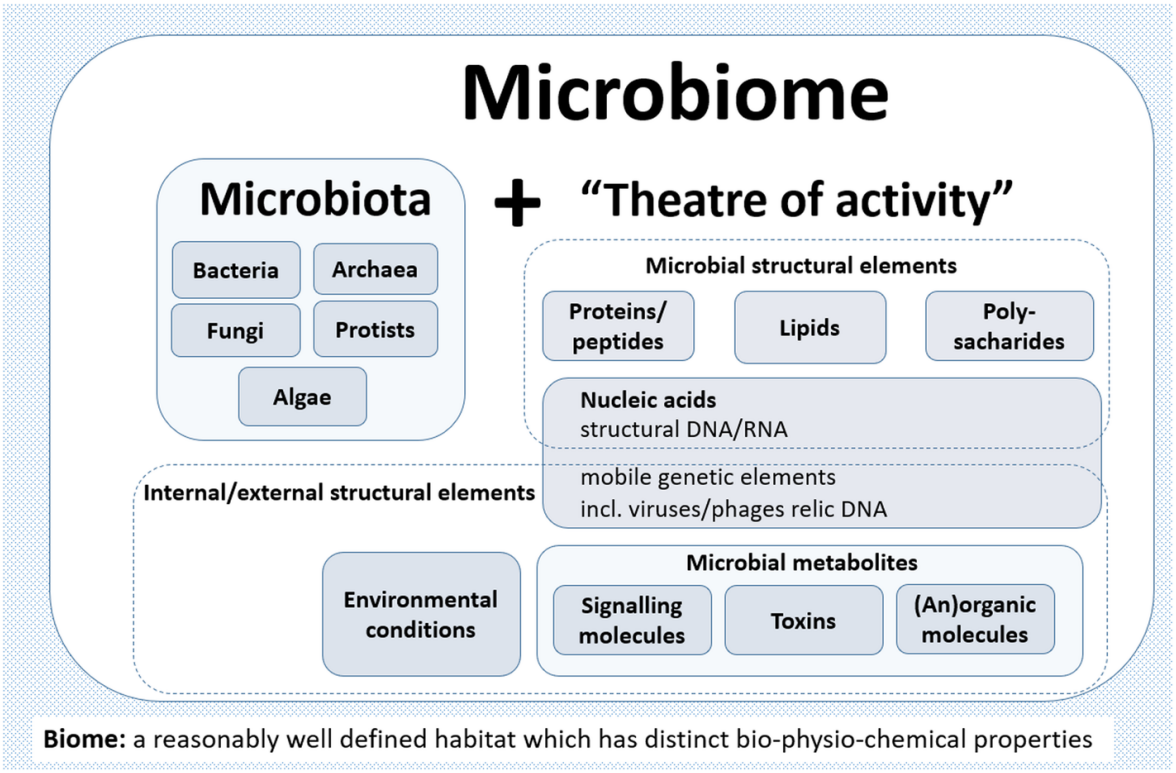
3.1 The general concepts of microbiota analysis

A comprehensive understanding of DNA sequencing methodologies is essential when interpreting and comparing studies on microbiota composition. It is not uncommon for studies investigating similar anatomical sites to report conflicting results. Factors such as the choice of 16S rRNA gene hypervariable regions targeted, differences in laboratory protocols, bioinformatics pipelines, sampling techniques, and the precise anatomical location of sample collection all contribute to inter-study variability and may significantly impact the observed microbial profiles (Ramakrishnan et al., 2013; Biswas et al., 2015).

3.1.1 Definition of microbiome, microbiota and related terms

The term microbiome refers to the collective community of microorganisms and their genetic material that inhabit a defined environment with specific physicochemical characteristics (Berg et al., 2020, Figure 16). It encompasses not only the microbiota, which includes microorganisms from various kingdoms such as bacteria, fungi, protists, archaea, and algae, but also their “theatre of activity”, which comprises microbial structures, metabolites, mobile genetic elements (e.g., transposons, phages, viruses), and relic DNA embedded within the environmental matrix of the habitat (Berg et al., 2020). Consequently, the concept of microbiome is broader and more integrative than that of microbiota. In the present work, the term “microbiota” is used specifically to describe the bacterial component of the microbial community; other microbial kingdoms (e.g., fungi, viruses) are not considered. The term core microbiota refers to the subset of microbial taxa that are consistently associated with a given host or environment and are thought to play important roles in maintaining homeostasis.

Figure 16: Schematic representation for the definition of the microbiome and microbiota (Source: Berg et al., 2020).



In bacterial taxonomy, hierarchical classification follows the descending order: phylum, class, order, family, genus, and species. In microbial ecology and sequencing-based studies, the term operational taxonomic unit (OTU) is commonly employed to define clusters of closely related sequences. OTUs serve as a practical proxy for microbial species and are typically defined using a threshold of 97% sequence similarity, corresponding to a clustering distance of 0.03. It is important to note that OTUs do not necessarily correspond to formal taxonomic ranks or biological species but rather represent an operational framework for analyzing and comparing microbial diversity based on sequence data.

3.1.2 Alpha- and beta-diversity

Alpha- and beta-diversity are essential statistical metrics used to evaluate the structure and complexity of microbial communities.

Alpha-diversity refers to the diversity within a single sample, considering both species richness (the number of distinct taxonomic units) and evenness (the relative distribution of these taxa). It yields a single numerical value that reflects the internal complexity of a microbial ecosystem, with higher values typically indicating a more diverse and balanced microbial community. In this

work, alpha-diversity was assessed using the Chao1 index, which estimates species richness by accounting for low-abundance taxa, and the Simpson index, which incorporates both richness and evenness while emphasizing dominant species. It is important to distinguish richness, which refers to the total number of species present, from abundance, which corresponds to the raw sequence read counts for each taxon. These raw counts are generally converted to relative abundances by expressing them as percentages, allowing for comparison across samples regardless of sequencing depth.

Beta-diversity examines the differences in microbial community composition between samples or groups, capturing the degree of dissimilarity based on both species' presence/absence and their relative abundances. This metric provides insights into the factors shaping microbial ecosystems and allows researchers to identify patterns, associations, or disruptions linked to specific clinical or environmental conditions. Multiple indices can be used to assess beta-diversity, each emphasizing different aspects of community heterogeneity. These variations often stem from whether the index accounts for species abundance or merely presence/absence, and how it interprets shared absences and rare taxa. Among these, the Bray-Curtis dissimilarity index is one of the most used. It considers both the overall size of the microbial communities (total abundance per sample) and the specific composition (relative abundance of individual taxa). In this work, the Bray-Curtis index was chosen to quantify compositional dissimilarity between groups, with a particular focus on differences in common taxa.

In the context of nasal microbiota analysis, alpha-diversity offers valuable insights into the richness and evenness of microbial species within an individual's nasal cavity. This measure reflects the internal complexity and potential resilience of the nasal microbiome, contributing to the assessment of its stability and overall health. In contrast, beta-diversity captures inter-individual differences in microbial community composition. By comparing these profiles across individuals or groups, beta-diversity helps uncover patterns influenced by factors such as disease status, age, environmental exposure, or host genetics.

3.1.3 AMOVA, HOMOVA, constrained ordination, LEfSe and Shotgun metagenomic sequencing

The Analysis of Molecular Variance (AMOVA) is a statistical method used to evaluate whether microbial community compositions differ significantly between predefined groups. In the context of

nasal microbiota studies, AMOVA quantifies how much of the observed variance can be attributed to differences between groups rather than within them. This approach allows for the identification of significant microbial shifts and supports the detection of distinct community structures associated with factors such as disease status or environmental conditions.

Hierarchical Analysis of Molecular Variance (HOMOVA) extends the principles of AMOVA by incorporating hierarchical structures into the analysis of microbial community variation. It provides insights into how different levels of grouping influence nasal microbiota composition and helps identify significant microbial community differences. In nasal microbiota research, HOMOVA provides a more nuanced understanding of how different levels of grouping, such as clinical categories, geographic origin, or environmental exposures, contribute to the overall variance in microbial profiles. When used alongside AMOVA, HOMOVA offers complementary insights, helping to identify both global and hierarchical patterns of microbial community differentiation.

In the context of nasal microbiota analysis, researchers frequently aim to determine how specific factors, such as disease status, age, environmental influences, or host-related characteristics, shape the composition of microbial communities. Constrained ordination, also referred to as constrained multivariate analysis, offers a statistical framework to explore these associations by incorporating external explanatory variables directly into the ordination process. Redundancy Analysis (RDA) is a widely used method for this purpose. It integrates multiple linear regression with ordination techniques, enabling the decomposition of variance in microbial composition while accounting for predefined explanatory variables. By doing so, RDA quantifies the proportion of variation in the microbial community that can be directly attributed to the selected factors, thus providing insight into the ecological or clinical drivers of microbiota structure.

Linear Discriminant Analysis Effect Size (LEfSe) is a statistical approach designed to identify microbial taxa whose relative abundance significantly differs between predefined groups. It combines non-parametric statistical testing with linear discriminant analysis (LDA), allowing not only the detection of taxa with statistically significant differences but also the estimation of their effect size. This dual strategy ensures that the identified microbial features are both statistically robust and biologically meaningful. In the context of nasal microbiota analysis, LEfSe facilitates the identification of taxa that are most strongly associated with specific conditions or groups, thereby highlighting potential microbial biomarkers relevant to health or disease.

Shotgun metagenomic sequencing represents an alternative and comprehensive approach for investigating uncultured microbial communities, as it allows the simultaneous assessment of microbial composition and functional potential. In this method, DNA is extracted from all cells present within a sample. Unlike amplicon-based sequencing, which targets a specific genomic region such as the 16S rRNA gene, shotgun sequencing involves random fragmentation of the entire DNA pool into small pieces, which are then individually sequenced. These reads map to diverse regions across the genomes of all organisms present, including non-microbial species. Some sequences align to taxonomically informative loci, such as 16S rRNA genes, while others correspond to coding regions that shed light on the functional capabilities of the microbial community. Although shotgun metagenomics was not applied in the present work, it constitutes a powerful tool for future investigations aiming to integrate taxonomic and functional insights.

3.2 What do we know about the nasal microbiota in healthy dogs?

To date, limited information is available regarding the respiratory tract microbiota in healthy dogs. Compared to the extensive research conducted on the gastrointestinal microbiota, studies focusing on the nasal microbiota remain scarce.

3.2.1 Current knowledge concerning the nasal microbiota using cultural methods

In the mid-20th century, several studies aimed to characterize the cultivable nasal microbial community of healthy dogs, though the findings varied considerably depending on the methodologies employed (Smith 1961; Clapper and Meade 1963; Abramson et al., 1976; Bailie et al., 1978). The most frequently isolated bacteria included members of the *Streptococcus/Enterococcus* genus (e.g., *Streptococcus faecalis*, *Streptococcus mitis*), species of the genus *Staphylococcus* (e.g., *Staphylococcus epidermidis*, *Staphylococcus aureus*), as well as *Escherichia coli*, *Bacillus* spp., *Corynebacterium* spp., *Neisseria* spp., and *Proteus* spp. (Clapper and Meade 1963; Abramson et al., 1976; Bailie et al., 1978). Quantitative and qualitative differences in culture results were observed between the anterior and posterior regions of the nasal cavities, with a higher number of bacterial species typically isolated from the anterior nasal passages (Abramson et al., 1976).

3.2.2 Current knowledge concerning the nasal microbiota using DNA sequencing methods

The upper respiratory tract is continuously exposed to inhaled air from the external environment, which carries aeroallergens, bacterial cells, and fungal spores. In addition to these biological particles, the respiratory mucosa is subjected to various physical and chemical atmospheric factors such as humidity, oxygen, and nutrients. A highly diverse and predominantly aerobic bacterial community colonizes the upper airways (Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017). Notably, many of the bacterial taxa identified are difficult to culture and were only recently described in association with the nasal cavities through the application of DNA sequencing techniques (Tress et al., 2017). Regional differences in microbial composition have been observed, with a certain degree of anatomical continuity in microbial populations progressing from the nasal cavities to the oropharynx and ultimately to the lungs (Ericsson et al., 2016).

Across studies, Proteobacteria has consistently been reported as the dominant phylum in the nasal passages, followed by Firmicutes, Bacteroidetes, Actinobacteria, and Tenericutes, although the order of abundance varies depending on the study (Tress et al., 2017: Figure 17; Rodrigues Hoffmann et al., 2014: Figure 18; Ericsson et al., 2016 : Figure 19; Isaiah et al., 2017: Figure 20). Within the Proteobacteria phylum, the family *Moraxellaceae*, and more specifically the genus *Moraxella*, represent the most prevalent taxa in the canine nasal microbiota (Rodrigues Hoffmann et al., 2014; Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017).

Figure 17: Phylum and family-level composition of the bacterial microbiota of the nose of 23 client-owned healthy dogs (Source: Tress et al., 2017).

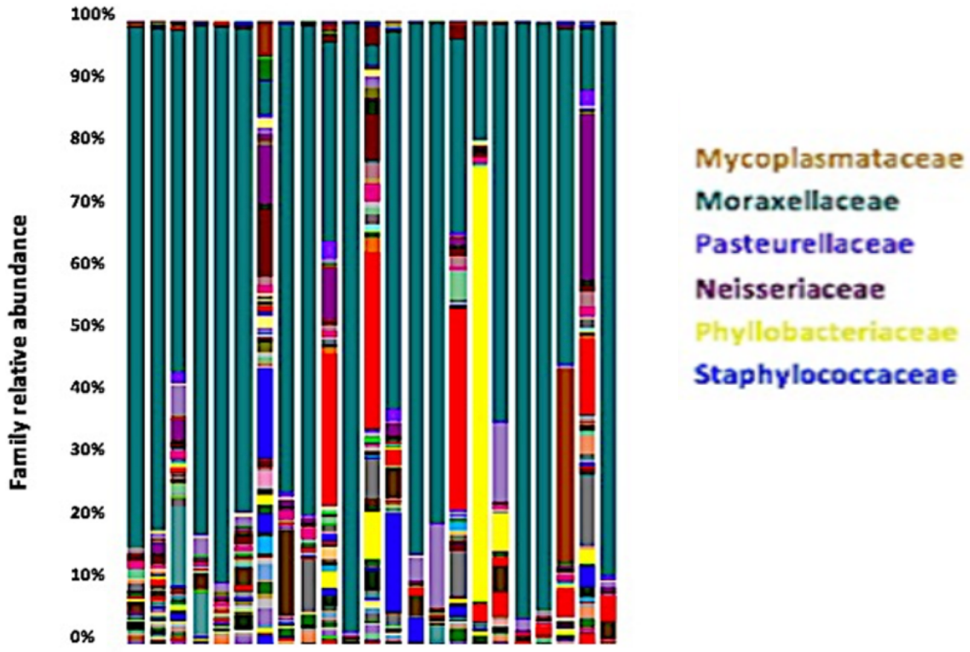


Figure 18: Average bacterial families in the nostril of 12 healthy client-owned dogs (Source: Rodrigues Hoffmann et al., 2014).

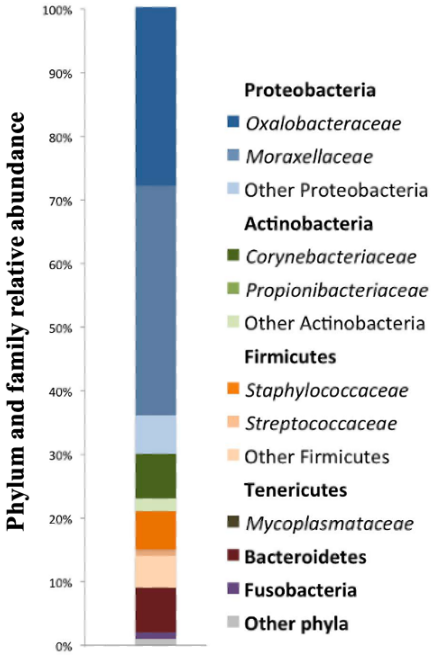


Figure 19: Relative abundance taxa at phylum- and family-level of the nasal microbiota of 16 purpose-bred dogs (Source: Ericsson et al., 2016).

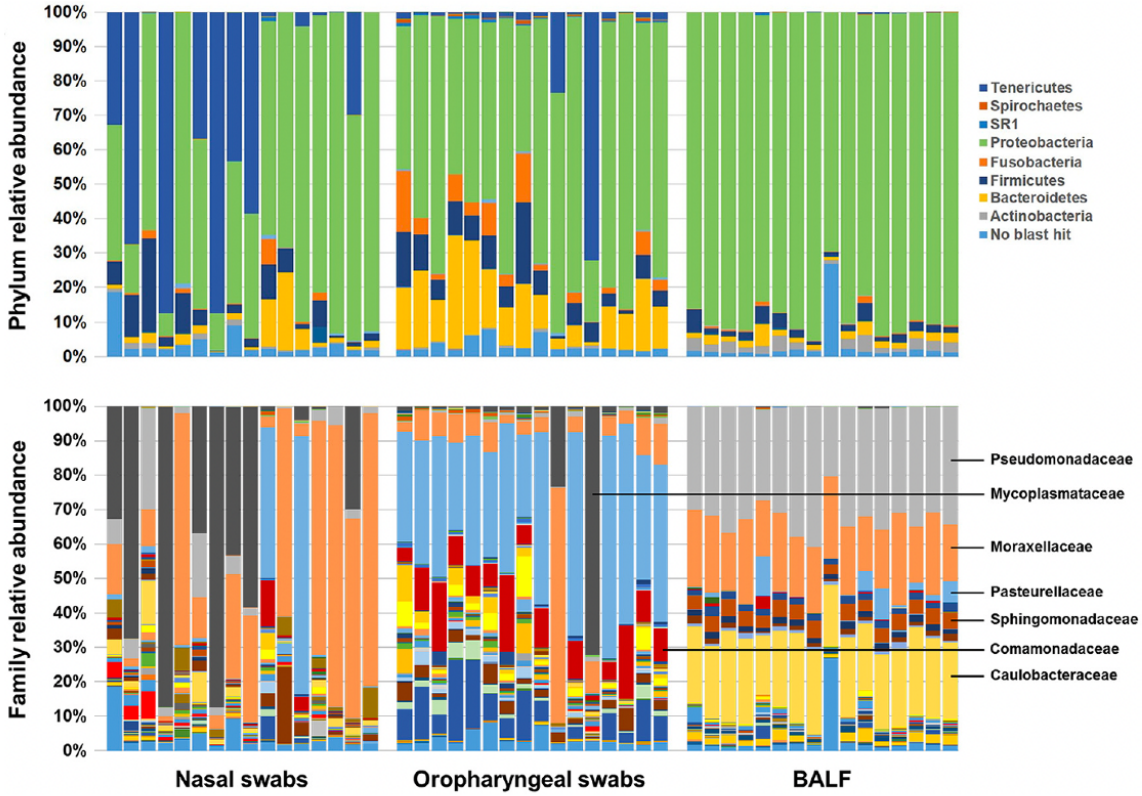
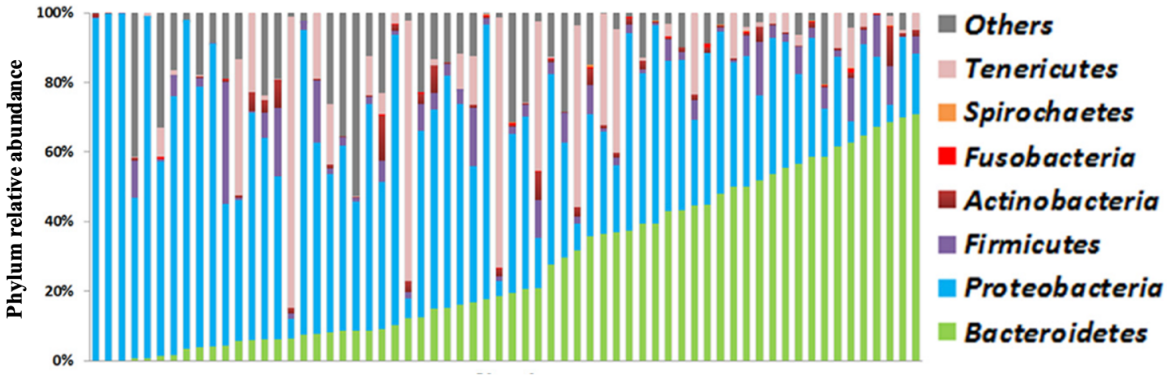


Figure 20: Most common bacterial phyla identified in nasal samples from 64 healthy client-owned dogs (Source: Isaiah et al., 2017).



Although the same major bacterial taxa have been consistently identified across studies (Rodrigues Hoffmann et al., 2014; Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017), notable variations exist in the reported results. These discrepancies likely stem from methodological differences in case selection, sample collection, and analytical protocols. For instance, one study relied on a homogeneous cohort of purpose-bred female Beagle dogs (Ericsson et al., 2016), whereas others included more heterogeneous populations of client-owned dogs from various geographic regions (Rodrigues Hoffmann et al., 2014; Isaiah et al., 2017; Tress et al., 2017). While most studies collected nasal swabs under general anesthesia, Tress et al. (2017) sampled awake dogs, which may have increased the risk of contamination from the skin microbiota.

Sampling techniques also varied. In the study by Ericsson et al. (2016), the swab was inserted halfway into the nasal cavity, whereas Isaiah et al. (2017) inserted it approximately half an inch. In the remaining studies (Rodrigues Hoffmann et al., 2014; Tress et al., 2017), the sampling site was not precisely defined but appeared to involve superficial swabbing at the level of the nostrils rather than deeper nasal sampling. Nevertheless, a recent study demonstrated that the bacterial community composition of the anterior nasal cavity remains comparable when samples are collected at depths of 15 mm and 25 mm in healthy awake dogs, suggesting that modest differences in sampling depth are unlikely to substantially influence microbiota profiles (Salamon et al., 2026). Additionally, different hypervariable regions of the 16S rRNA gene were targeted for sequencing, including V1–V3 (Rodrigues Hoffmann et al., 2014), V4 (Ericsson et al., 2016; Tress et al., 2017), and V4–V6 (Isaiah et al., 2017). These methodological inconsistencies likely contribute to the observed variability across studies.

Other potential confounding factors have also been explored. Sex and breed did not appear to significantly influence the composition of the nasal microbiota (Rodrigues Hoffmann et al., 2014; Isaiah et al., 2017). In dogs, minor changes (increased Shannon diversity index of the microbiota of the nasal mucosa) have a been reported in dogs older than 9 years in one study (Tress et al., 2017), and no changes in another (Isaiah et al., 2017). Geographic location may play a role: a comparison of dogs of the same breed residing in Alabama and California revealed significant differences in both alpha- and beta-diversity, suggesting an environmental influence (Isaiah et al., 2017). Inter-individual and temporal variability has also been documented (Rodrigues Hoffmann et al., 2014; Isaiah et al., 2017). Finally, no significant differences were found when comparing the nasal microbiota of working dogs with different olfactory detection roles (Isaiah et al., 2017).

3.3 The nasal microbiota in healthy humans

Distinct microbial communities, referred to as microbial niche-specificity, have been identified across various anatomical sites of the human body, with specific signature taxa occupying particular microenvironments. Despite this site-specificity, the relative representation of taxa can vary considerably between individuals (The Human Microbiome Project Consortium, 2012). In healthy humans, the nasal cavity harbors a rich and diverse bacterial community, predominantly composed of members of the phyla Actinobacteria, Firmicutes, Proteobacteria, and Bacteroidetes (Ramakrishnan et al., 2013). At the genus level, microbial profiles show strong inter-individual variability, often dominated by *Propionibacterium* (also referred to as *Cutibacterium*), *Staphylococcus*, *Corynebacterium,* or *Moraxella* (Ramakrishnan et al., 2013; Bassis et al., 2014; Zhou et al., 2014). Although identified at lower abundances, it has also been hypothesized that the genus *Burkholderia* operates as a central bacteria regulating the microbial network in the nose of healthy subjects (Wagner Mackenzie et al., 2017).

Microbial niche-specificity also extends to the different anatomical subregions of the nasal cavity, each characterized by unique physicochemical properties. These include the anterior nares, middle meatus, paranasal sinuses, and nasopharynx, each supporting distinct microbial communities (de Steenhuijsen Piters et al., 2015; Dickson et al., 2017).

Numerous factors are thought to influence the composition of the nasal microbiota. In early life, the mode of delivery and breastfeeding status have been shown to shape the initial microbial colonization (Dominguez-Bello et al., 2010; Shilts et al., 2016). In adults, environmental exposures, tobacco smoke inhalation, immune system maturation, surgical interventions, antibiotic therapy, and intranasal medications may all contribute to community composition shifts (Morris et al., 2013; Man et al., 2017; Ramakrishnan et al., 2018). Age-related changes have also been reported. In elderly individuals, reduced niche-specificity between nasal subsites has been observed, suggesting a potential decline in the compartmentalization of microbial communities with advancing age (Whelan et al., 2014; Stearns et al., 2015; de Steenhuijsen Piters et al., 2016).

4 The nasal microbiota in chronic nasal diseases

4.1 General concepts

The microbiome is thought to play a major role in the development and maintenance of health through complex microbe-microbe and host-microbe interactions.

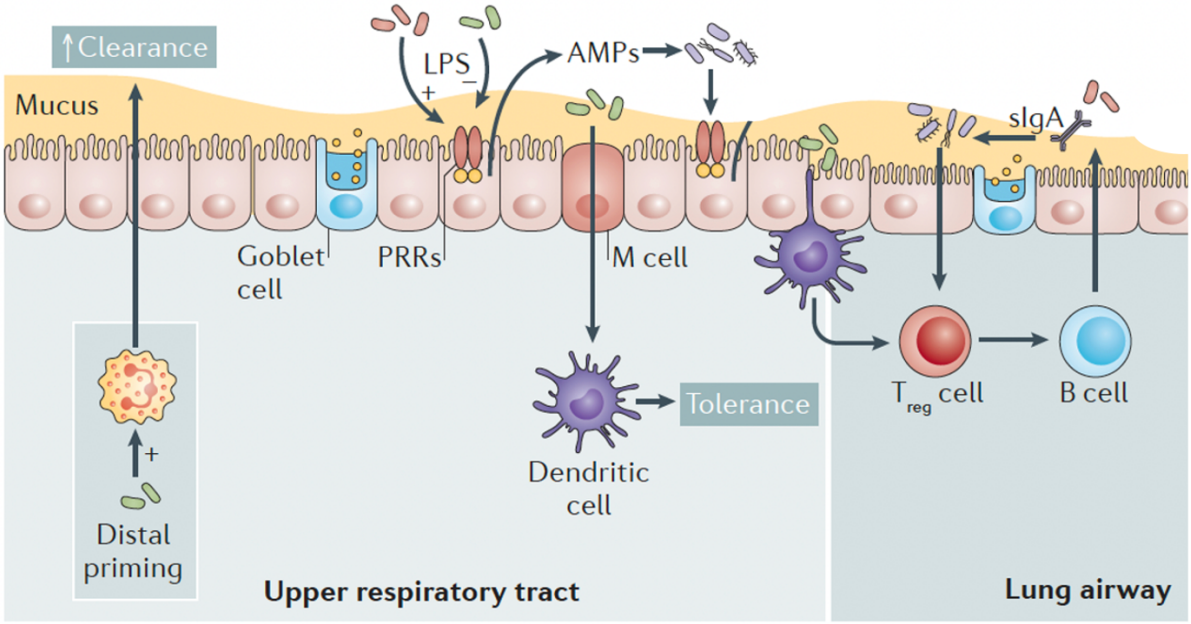
4.1.1 The microbiome as a gatekeeper of health

The microbiota plays a pivotal role in protecting the host from pathogen colonization. Several mechanisms have been proposed to explain this phenomenon, known as colonization resistance. One key strategy involves resource competition: members of a diverse, stable, and healthy microbial community likely consume most available nutrients, leaving few resources for invading pathogens to exploit (Man et al., 2017). Another important mechanism is the production of antimicrobial compounds. For instance, *Corynebacterium accolens* can metabolize host-derived triacylglycerols into antipneumococcal free fatty acids, which may inhibit colonization by *Streptococcus pneumoniae* in the nasal cavities of children (Bomar et al., 2016). Additionally, *Staphylococcus epidermidis* has been shown to prevent colonization by *Staphylococcus aureus*, a well-known nasal pathobiont, by secreting serine proteases that can disrupt pre-existing *S. aureus* biofilms (Iwase et al., 2010). Beyond direct antagonism, certain commensal microbes enhance host immunity. In the upper respiratory tract, for example, *Staphylococcus epidermidis* may promote the activity of alveolar macrophages through extracellular signaling, thereby facilitating the clearance of pathogens (Brown et al., 2017). Similarly, prior exposure to *Haemophilus influenzae* appears to prime neutrophils, enhancing their capacity to kill *S. pneumoniae* (Lysenko et al., 2007).

Some commensal species have been identified as particularly important for maintaining microbial balance and ecosystem stability. These so-called "keystone species" exert disproportionately large effects on their environment. *Corynebacterium* spp., for example, are considered potential keystone members of the upper respiratory tract microbiota in humans (Man et al., 2017). It has been suggested that, in certain contexts, the host may actively support the growth of beneficial microbes, such as those producing antimicrobial molecules, as a strategic defense against pathogens (Man et al., 2017). Furthermore, host–microbiota interactions are known to shape

immune system development, influencing immune maturation, tolerance, and resistance to bacterial infection, as illustrated in Figure 21.

Figure 21: Host-microbiota crosstalk in the human respiratory tract (Source: adapted from Man et 2017).



Most of the host-microbiota interaction takes place at the mucosal surface.
The first line of defense against invading organisms is set by the mucus lining the respiratory epithelium. The mucus traps microbial pathogens which are cleared though ciliary action towards the oral cavity. The mucus contains glycoproteins (mucin secreted by goblet cells) which accommodate resident micro-organisms and prevent infection (Linden et al. 2008). The next line of defense is the epithelial cell layer which separates the resident microbiota from the lamina propria. Resident micro-organisms prime immune cells (epithelial cells, neutrophils and dendritic cells) locally or systemically, leading to clearance of pathogens. Locally, the host will respond to microbial colonization through the release of antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA). These sIgA can bind and form complexes with the commensal bacteria, which selectively presents the bacterial components to tolerogenic dendritic cells. Epithelial cells (such as microfold or M cells that constitute the Mucosa-Associated Lymphoid Tissue), macrophages and dendritic cells have pattern recognition receptors (PRRs) to sense the microbiota by recognizing specific lipopolysaccharides (LPS). Alveolar dendritic cells can also directly sample luminal micro-organisms initiating adaptive immune response through T_{reg} and B cells. These pathways are central for the maintenance of immune tolerance. This tolerant milieu contributes to the normal development and maintenance of resident bacterial communities.

4.1.2 The microbiome and its role in disease

The delicate balance between immune tolerance toward resident beneficial microbiota and defense against invading pathogens can be disrupted in various ways. As previously discussed, the microbiota is believed to modulate both directly and indirectly the tolerogenic mechanisms that

help restrain excessive inflammatory responses. When these mechanisms fail, immune-mediated diseases, including autoimmune, allergic, or autoinflammatory disorders, may emerge. Evidence suggests that neonatal colonization by commensal microbes plays a critical role in the development of both innate and adaptive immunity, thereby shaping the host's capacity for inflammatory regulation later in life (Olszak et al., 2012). These and similar findings have led to the formulation of the "hygiene hypothesis," which posits that reduced microbial exposure during early life, particularly during the neonatal period, may predispose individuals to immune-mediated diseases in later stages of life.

Bacteria have historically been categorized as either "good" or "bad" based on their effects on host health. However, in recent decades, it has become evident that certain bacterial strains can behave as harmless commensals under specific conditions but may become harmful in others. The term pathobiont has been introduced to describe such species. This concept acknowledges the existence of a balanced state, in which pathobionts coexist symbiotically with the host without causing disease. However, when this balance is disrupted, due to impaired host immune defenses and/or alterations in the microbial community (dysbiosis), these organisms may shift toward pathogenic behavior, potentially contributing to disease (Jochum and Stecher, 2020). In human medicine, *Staphylococcus aureus* is one of the most well-known examples of a nasal pathobiont (Brugger et al., 2016).

The term dysbiosis is frequently used in literature addressing the bacterial microbiota in the context of health and disease. It refers to a compositional and/or functional disruption of the resident microbial community that impairs its ability to maintain homeostasis and support host functions. Dysbiosis may manifest through one or more non-mutually exclusive alterations: the loss of beneficial microorganisms (including keystone species), the overgrowth of pathobionts or opportunistic organisms, and a global reduction in microbial diversity (DeGruttola et al., 2016). According to the "dysbiosis hypothesis", such imbalances in microbial communities may contribute to disease pathogenesis and exacerbate clinical symptoms. This hypothesis has been implicated in a wide array of chronic conditions, including inflammatory bowel disease and chronic rhinosinusitis (Copeland et al., 2018; Zhang et al., 2022).

4.2 The nasal microbiota in dogs with chronic nasal diseases

4.2.1 Using cultural methods

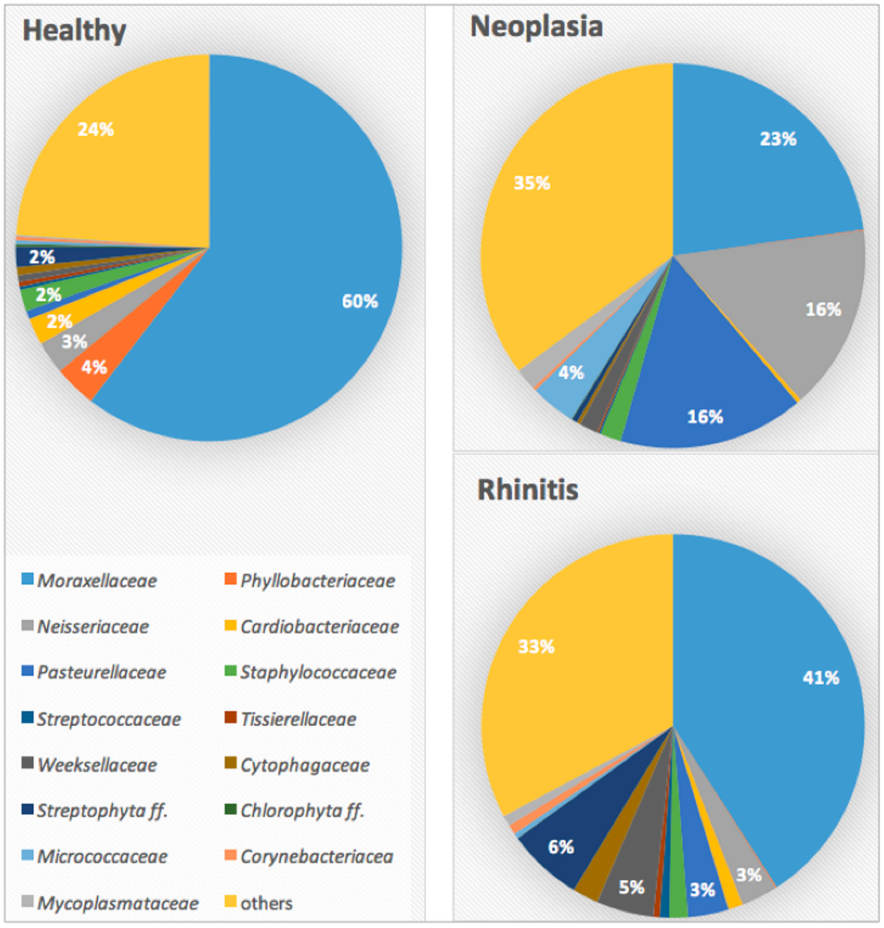
The earliest studies investigating the nasal bacterial population in dogs with nasal diseases, conducted prior to the use of sequencing techniques, relied on culture-based methods (Lobetti et al., 2009, Plickert et al., 2014, Rösch et al., 2019, Niedenführ et al., 2024). Mixed growth of multiple species was common. Most frequently isolated bacteria were *Staphylococcus* spp. (average 50.1% (min. 37.2%-max. 57%)), *Pasteurella* spp. (12.8% (4.4-18.6)), *Escherichia coli* (12% (3.5-18.7)), *Streptococcus* spp. (7.5% (3.1-14.9)) (Plickert et al., 2014; Rösch et al., 2019; Niedenführ et al., 2024). Among Staphylococcus species, *Staphyloccus pseudintermedius* and *Staphylococcus aureus* were the most frequently represented species (Niedenführ et al., 2024). Other less commonly isolated species were *Pseudomonas* spp., *Corynebacterium* spp., *Neisseria* spp., *Klebsiella* spp. and *Moraxella* spp. (Plickert et al., 2014; Niedenführ et al., 2024).

4.2.2 Using DNA sequencing methods

Nasal biopsies from dogs with chronic idiopathic rhinitis, nasal neoplasia, and FR have been evaluated for bacterial and fungal DNA using PCR techniques (Windsor et al., 2006). No significant differences in bacterial DNA loads were observed among the different disease groups. Furthermore, no DNA from bacterial pathogens commonly implicated in upper respiratory tract infections across species—such as *Bartonella*, *Mycoplasma*, or *Chlamydia*—was detected in any of the samples. The first, and so far only, study investigating the nasal microbiota of dogs with nasal neoplasia and chronic idiopathic rhinitis using 16S rRNA gene-based amplicon sequencing was published in 2017 (Tress et al., 2017, Figure 22). In this study, the neoplasia group included dogs diagnosed with a variety of tumor types: adenocarcinoma (n=6), osteosarcoma (n=3), squamous cell carcinoma (n=2), esthesioneuroblastoma (n=2), transitional cell carcinoma (n=1), and lymphoma (n=1). A significant shift in nasal microbial community composition was found between healthy dogs and those with nasal neoplasia, whereas no statistically significant differences were detected between healthy dogs and those with chronic idiopathic rhinitis, nor between chronic idiopathic rhinitis and neoplasia

groups. At the taxonomic level, *Moraxella* spp. showed a notable decrease, while members of the *Pasteurellaceae* family were significantly increased in both chronic idiopathic rhinitis and neoplasia cases. These findings suggest that nasal dysbiosis is associated with canine nasal disease. However, whether such microbial alterations contribute to the pathogenesis of the disease or simply arise as a consequence of underlying pathological processes remains unclear. One hypothesis is that immune dysregulation associated with the primary disease may allow for the overgrowth of specific bacterial taxa. Conversely, as previously discussed, dysbiosis itself may initiate or exacerbate disease. For instance, toxigenic strains of *Pasteurella multocida* have been shown to influence immune cell differentiation and function (Kubatzky et al., 2013), potentially contributing to disease progression.

Figure 22: Mean values of most common bacterial families in the nasal cavity of healthy dogs, dogs with nasal neoplasia and chronic rhinitis (Source: Tress et al., 2017).



4.3 The nasal microbiota in humans with chronic nasal diseases

In human medicine, several studies have demonstrated notable differences in the nasal bacterial communities of healthy individuals compared to those with chronic rhinosinusitis (CRS) (Ccami-Bernal et al., 2024). Patients with CRS exhibit decreased microbial diversity and a reduced abundance of health-associated bacterial taxa, alongside an increased presence of pathobionts such as *Staphylococcus aureus*. A consistent overrepresentation of anaerobic bacteria has also been reported. This may reflect selective pressure from antimicrobial therapies, which favor the growth of anaerobes, as well as the development of low-oxygen niches within biofilms or mucus plugs (Cho et al., 2020).

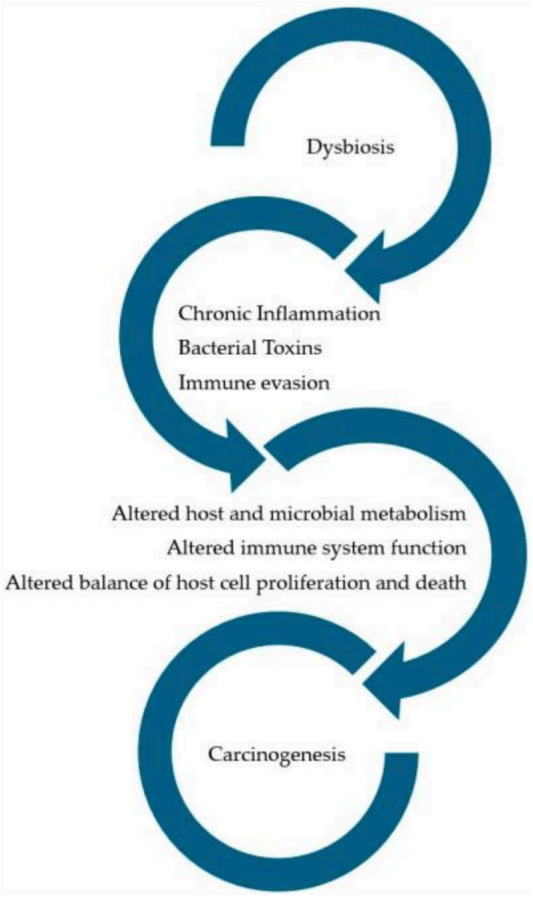
The underlying mechanisms contributing to CRS pathogenesis appear heterogeneous and remain incompletely understood. In one study, a more detailed immunological profiling revealed an exaggerated immune response to commensal bacteria, suggesting that in some cases, CRS may result from an inappropriate immune hyperreactivity (Aurora et al., 2013). Other contributing factors likely include impaired mucociliary clearance, defects in the sinonasal epithelial barrier, formation of anaerobic microenvironments, and overgrowth of pathobionts or opportunistic pathogens (Cho et al., 2020; Ccami-Bernal et al., 2024).

Recent work has also explored the therapeutic potential of microbiota-modulating strategies in the treatment of CRS with nasal polyps, proposing that such approaches may complement current anti-inflammatory therapies and improve clinical outcomes (Gomez-Garcia et al., 2024). If these strategies prove effective in human medicine, it is conceivable that similar approaches could offer therapeutic benefits in canine patients. However, research into the nasal microbiota of dogs with chronic idiopathic rhinitis is still in its early stages, and further investigation is required before any targeted microbiota-based treatments can be proposed.

Regarding nasal neoplasms, emerging evidence suggests that disturbances in the nasal microbiota may contribute to the development of these malignancies, particularly squamous cell carcinoma, via mechanisms such as chronic inflammation, immune dysregulation, and impaired cellular homeostasis (Figure 23). In one study, Shi et al. (2021) examined the nasal microbiota in patients with natural killer/T-cell lymphoma of nasal type. They observed a distinct microbial profile in these patients compared to individuals with chronic rhinosinusitis or healthy controls. These findings not only offer insights into the possible pathogenic mechanisms underlying such aggressive

neoplasms but also raise the prospect of microbiota-targeted interventions as potential preventive or therapeutic strategies.

Figure 23: Potential implications of dysbiosis contributing to carcinogenesis (Source: Loperfido et al., 2024).



Objectives

Fungal rhinosinusitis is a frequent reason of consultation in canine medicine. The pathophysiology of this disease is incompletely understood and unsatisfactory response to treatment is not uncommon. A local dysimmunity, allowing the ubiquitous *Aspergillus fumigatus* to develop is suspected, but the possible implication of the nasal microbiome has not been investigated yet. The rationale underlying this work was the hypothesis that the nasal bacterial microbiota might play a role in the development, progression, or recurrence of fungal rhinosinusitis in dogs. We chose to focus on the bacterial microbiota, as current methodologies still present significant limitations for the comprehensive analysis of the mycobiota and virobiota. Moreover, increasing evidence highlights the role of the nasal bacterial microbiota in the pathogenesis, progression, and therapeutic response of various chronic nasal conditions in humans, particularly chronic rhinosinusitis and allergic rhinitis.

Before relying on DNA sequencing to characterize the nasal bacterial communities, we first aimed to evaluate the information that conventional culture methods can provide. In clinical practice, nasal swab culture remains the simplest and most widely accessible diagnostic technique for veterinarians, despite ongoing uncertainty regarding its accuracy and clinical relevance. In practice, dogs with chronic nasal diseases such as fungal rhinosinusitis, chronic idiopathic rhinitis and nasal neoplasia are also commonly treated with antimicrobial treatment (local or systemic) based or not on the results of nasal swab culture. Consequently, the **first objective** of this work was to compare the bacterial communities identified through conventional agar-plate culture with those obtained using 16S rRNA gene amplicon sequencing from matched nasal swab samples collected in dogs with chronic nasal disease. This comparison sought to assess the degree of concordance between culture-dependent and culture-independent methods and to determine how reliably traditional culture reflects the broader bacterial diversity revealed by sequencing.

To investigate the nasal microbiota in dogs with nasal disease, a knowledge of the core nasal microbiota in healthy dogs, and by which factors the nasal microbiota could be influenced, was needed first. Few publications were available in client-owned healthy dogs describing the core nasal microbiota using DNA sequencing methods (Tress et al., 2017; Isaiah et al., 2017). Breeds of dogs are differently predisposed to chronic nasal diseases according to their skull conformation. Indeed, fungal rhinosinusitis affect young to middle-aged mesocephalic and dolichocephalic dogs while brachycephalic dogs are never affected. The **second objective** was to determine the core nasal microbiota in healthy dogs predisposed and not predisposed to fungal rhinosinusitis, and to identify possible influence of other intrinsic or extrinsic factors.

As a **third objective**, we then aimed to assess whether a specific dysbiosis of the nasal microbiota was associated with dogs with fungal rhinosinusitis, compared to the nasal microbiota of healthy breed-matched dogs and dogs with another chronic nasal disease, i.e. chronic idiopathic rhinitis. An additional objective of this study was to observe if the nasal microbiota differed in patients according to the outcome of the fungal rhinosinusitis, and the evolution of the microbiota after cure.

Experimental section

Experimental section

Study 1 :

Comparison of culture and culture-independent methods in dogs with nasal discharge

Preamble

Secondary bacterial rhinitis is frequently associated with any chronic nasal diseases such as chronic idiopathic rhinitis, nasal tumor and fungal rhinosinusitis (Windsor et al., 2004). In general practice, secondary bacterial rhinitis is commonly treated with antibiotics based on a swab culture. Antibiotics are probably being overused in this context, and the usefulness of culture-oriented treatment is questionable. While culture methods have long been a cornerstone in veterinary microbiology, their limitations, including low sensitivity to fastidious or slow-growing organisms, call into question their reliability as a sole diagnostic tool. In human medicine, studies have demonstrated discrepancies between culture-dependent and culture-independent methods, with the latter uncovering significant microbial diversity not captured through traditional diagnostics. Similar observations are now emerging in veterinary medicine, but data remain limited.

To what extent nasal swab culture reflects the bacterial communities in dogs with nasal disease is unknown. The use of DNA sequencing could provide a more accurate profile of the nasal microbiota, highlighting the potential for improved diagnostic accuracy and tailored antimicrobial therapy in canine bacterial rhinitis. Study 1 compares the use of conventional culture and 16S rRNA gene amplicon sequencing in dogs with nasal discharge clinically suspected to have a bacterial component. This study also contributes to the growing body of work investigating the role of dysbiosis in chronic upper respiratory tract conditions and supports the development of more accurate diagnostic and therapeutic strategies in canine patients.

This study compared standard agar culture with 16S rRNA gene amplicon sequencing on nasal swabs from 29 dogs with nasal discharge. Agreement between methods was limited, with culture frequently failing to detect dominant taxa identified by sequencing, while some cultured bacteria represented only minor or absent components of the nasal microbiota. Sequencing revealed nasal dysbiosis in most dogs, independent of recent antimicrobial treatment. Overall, conventional culture did not reliably reflect the bacterial communities present, indicating that reliance on culture alone may be misleading when assessing suspected bacterial rhinitis in dogs.

Experimental section

Study 1 :

Comparison of culture-dependent and -independent bacterial detection results on nasal swabs in dogs with nasal discharge

JSAP. 2024;65(6):376-386

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JSAP JOURNAL OF SMALL ANIMAL PRACTICE

ORIGINAL ARTICLE

Comparison of culture-dependent and -independent bacterial detection results on nasal swabs in dogs with nasal discharge

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Objectives: The role of bacterial communities in the pathophysiology of canine nasal disease is still unclear. How and when to treat dogs with suspected secondary bacterial rhinitis and on which test to rely before making a decision to treat with antimicrobials has not been established. The objective is to compare the results of bacterial identification using agar-plate cultures and 16S rRNA gene amplicon sequencing in dogs with nasal discharge suspected to be of bacterial origin.

Materials and Methods: Twenty-nine client-owned dogs presented for investigation of nasal disease were included in the study. Paired swabs were collected from the same affected nasal cavity. One swab was streaked on 4 agar media (Columbia Blood Agar, MacConkey, Chapman and Edward's). The other swab was stored in a sterile cryotube at −80°. Extracted DNA underwent a polymerase chain reaction targeting the V1-V3 region of the 16S rRNA gene.

Results: At least one of the species detected by amplicon sequencing with a relative abundance of >10% was also identified by culture in 14 cases (48.3%), in association with marked predominance of one taxon (>80% relative abundance) in six of 14 cases. In 12 dogs (41.4%), the cultured isolates were rare or undetected components of the corresponding sequence libraries. A negative culture in the face of bacterial predominance (>50% relative abundance) of a potentially pathogenic bacteria detected by sequencing occurred in 17% (n=5) of cases; however, the use of other agar media may have decreased this percentage.

Clinical Significance: Standard culture does not reliably predict the bacterial profile detected by 16S rRNA gene amplicon sequencing.

Journal of Small Animal Practice (2024); **65,** 376–386
DOI: 10.1111/jsap.13734
Accepted: 20 March 2024; Published online: 9 April 2024

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INTRODUCTION

Acute or chronic nasal discharge is a common reason for consultation in canine medicine. Frequent primary nasal diseases diagnosed in dogs include chronic idiopathic rhinitis, fungal rhinitis/rhinosinusitis, nasal/nasopharyngeal neoplasia, nasal/nasopharyngeal foreign body and oronasal defect (Meler et al., 2008; Plickert et al., 2014; Rösch et al., 2019; Tasker et al., 1999; Windsor & Johnson, 2006). In practice, dogs with nasal discharge are commonly treated with antimicrobial treatment (local or systemic) based or not on the results of nasal swab culture. However, the usefulness of this complementary exam as well as the use of systemic antimicrobials in such cases is questionable. Indeed, primary nasal bacterial infections rarely occur in dogs (Hawkins et al., 2008; Lee-Fowler & Reinero, 2012; Meler et al., 2008; Rösch et al., 2019; Tasker et al., 1999; Windsor & Johnson, 2006) although bacterial infections frequently complicate primary conditions (Lee-Fowler & Reinero, 2012; Windsor & Johnson, 2006). The primary cause of nasal discharge has to be determined before considering the use of antimicrobials in dogs (Lee-Fowler & Reinero, 2012). Moreover, culture results may not reflect the actual infection. Indeed, the major infection-causing organism may be difficult to grow on conventional culture media and the microbiota may be of more interest than a single colony type grown in a given culture medium. Furthermore, a cultivable flora also exists in the nose of healthy dogs (Bailie et al., 1978; Clapper & Meade, 1963; Smith, 1961). As a result, the cultured colony type(s) might not be the one(s) responsible for the infection.

Until recently, our understanding of normal nasal microbiota was based on publications of the mid-19 century, in which nasal passages of healthy dogs were cultured (Bailie et al., 1978; Clapper & Meade, 1963; Smith, 1961). The recent contribution of results obtained with next-generation sequencing methods has given a more precise and exhaustive idea of the nasal microbiota in healthy dogs (Banks et al., 2020; Ericsson et al., 2016; Isaiah et al., 2017; Rodriguez et al., 2019; Tress et al., 2017; Vangrinsven et al., 2021). Based on 16S rRNA gene amplicon sequencing, a species-rich bacterial community was detected in the nose of healthy dogs (Banks et al., 2020; Ericsson et al., 2016; Isaiah et al., 2017; Rodriguez et al., 2019; Tress et al., 2017; Vangrinsven et al., 2021) with different alterations of this community in dogs with nasal diseases (Tress et al., 2017; Vangrinsven, 2021).

To what extent nasal swab culture reflects the bacterial communities in dogs with nasal disease is unknown. The primary aim of this study was to compare the results obtained with agar-plate cultures and 16S rRNA gene-based amplicon sequencing using matched samples of nasal swabs.

MATERIAL AND METHODS

Study population

Dogs presented for investigation of a nasal disease associated with acute or chronic nasal discharge and with a clinical suspicion of bacterial infection were prospectively included. The suspicion of bacterial infection was based on the presence of a continuous or intermittent mucopurulent-like nasal discharge. Pre-treatment with antimicrobials was not an exclusion criterion.

Sampling method

This study was approved by the animal ethical committee (N° 16-1854, October 27, 2016) and all samples were obtained with the consent of the owners.

Clinical data were recorded, general anaesthesia was performed and two consecutive samples were collected in the same nasal cavity before starting rhinoscopic examination. Dogs were premedicated with butorphanol (0.2 mg/kg; Butomidor®, Richter Pharma) intravenously in combination with medetomidine (5 µg/kg; Medetor®, CP-Pharma) followed by propofol (Propovet®, Zoetis) on demand for induction. The dogs were intubated and placed in sternal recumbency. First an eSwab (Copan®, eSwab™, 490 CE.A, Brescia, Italy) was introduced into the distal third of the most affected nasal cavity followed by the introduction of a FLOQswab (Copan®, FLOQSwabs™, 553C, Brescia, Italy) at the same site in the same nasal cavity. The swabs were introduced into the nasal cavity through a sterile speculum which maintained the nostril open and prevailed the swab of being in contact with the skin and nose. Three gentle circular movements were performed with each swab before withdrawal of the swabs through the speculum. The eSwab was immediately re-inserted in his sterile tube and sent for culture. The end of the sew of the FLOQswab was cut with sterile scissors and stored at −80° in a sterile cryotube until batched DNA extraction. Direct and retrograde rhinoscopy was performed in all dogs. Other diagnostic tests (CT of the head, fungal culture, nasal biopsy with histopathologic evaluation, dental probing) were performed at the clinician's discretion depending on the clinical suspicion.

Agar-plate culture method and bacterial identification

The eSwabs were sent to the microbiology laboratory of the same institution site for bacterial culture and isolation, within 6 hours. Specimens were plated and streaked onto MacConkey agar (selective for enterobacteria, Prolabochemical), Mannitol salt agar (selective for staphylococci, VWR), Columbia Blood Agar (non-selective medium, bioMerieux), Edward agar (selective for streptococci and enterococci, Oxoid) and were incubated overnight in aerobic conditions at 37°C. If no growth was observed, the incubation was extended to 48 hours. If no significant growth was observed after 48 hours, culture was reported negative. Growth was categorised based on the number of colonies: 0 colonies=no growth, 1 to 10 colonies=1+, 10 to 50 colonies=2+, 50 to 100 colonies 3+ and >100 colonies=4+. Colonies were counted at first on each selective agar medium and compared to the growth on the Columbia Blood Agar medium. Suspect colonies were pre-identified on the basis of colony shape, Gram staining, bacterial cell shape and catalase and oxidase production (Markey

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et al., 2013). Bacterial identification was performed by matrix-assisted laser desorption ionisation/time of flight (MALDI-TOF) mass spectrometry on a Microflex LT platform (Bruker Daltonik, Bremen, Germany) (Descy et al., 2010).

Molecular-based method: extraction, sequencing and data analysis

Total bacterial DNA was extracted from the nasal swabs and two negative control swabs using the DNEasy Blood and Tissue kit (QIAGEN Benelux BV; Antwerp, Belgium) following the manufacturer's recommendations. Spectrophotometry (NanoDrop, Thermo Scientific) was used for total DNA concentration measurement.

Bacterial 16S rRNA gene amplicons were generated via amplification of the V1-V3 hypervariable regions of the 16S rRNA gene using the following primers (with Illumina overhand adapters): forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGGCAC-3′) as previously described (Ngo et al., 2018). The DNA was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second polymerase chain reaction (PCR) round for indexing, using the Nextera XT index primers 1 and 2. A final quantification, by quantitative (q)PCR, of each sample in the library was performed using the KAPA SYBR" FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalisation, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA) (Ravi et al., 2018). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run (*Acinetobacter baumannii*, *Actinomyces odontolyticus*, *Bacillus cereus*, *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, *Clostridium beijerinckii*, *Clostridioides difficile*, *Cutibacterium acnes*, *Deinococcus radiodurans*, *Enterococcus faecalis*, *Escherichia coli*, *Helicobacter pylori*, *Lactobacillus sakei*, *Lactococcus lactis*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Staphylococcus aureus* and *Streptococcus agalactiae*).

Alignment and clustering were done with MOTHUR software package (v1.41.0) with an operational taxonomic unit (OTU) clustering distance of 0.03 and based on the SILVA database (V1.32) of full-length 16S rRNA sequences. Vsearch (Rognes et al., 2016) algorithm was used for chimera detection.

After DNA extraction from nasal swab samples, quantification of the total bacterial flora was performed with a quantitative real-time PCR targeting the V2-V3 region of the 16S rRNA gene with the following primers: forward (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse (5′-ATTACCGCGGCTGCTGG-3′). The standard curve was based upon 10-fold dilution of a quantified PCR product. This PCR product was purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Leiden, The Netherlands), and quantified with PicoGreen targeting double-stranded DNA (Promega).

Data and statistical analyses

Bacterial richness, evenness and alpha-diversity were obtained with MOTHUR (v1.41.0) at species level using the Chao1 index, Simpson index-based measure and the inverse Simpson's index respectively. Beta-diversity was estimated with AMOVA (analysis of molecular variance; 10,000 iterations) and beta-dispersion was assessed with HOMOVA (analysis of molecular variance homogeneity; 10,000 iterations).

The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion were compared between the dogs treated with antimicrobials, ongoing or recent, and untreated dogs using MOTHUR and a Mann–Whitney test (XLstat 2019.3.2, Addinsoft, Paris, France). Differences were considered significant for a P-value <0.05.

Comparison between culture- and molecular-based results

Results were distributed in three tables according to the agreement between the two methods. Good and moderate agreement were attributed when at least one of the most abundant sequenced taxa (all taxa with a relative abundance >10%) was cultured (Table 2). When, among the most abundant sequenced taxa, the one taxon with the highest relative abundance was detected by culture, the agreement was considered good. When, among the most abundant sequenced taxa, the one taxon with the highest relative abundance was not detected by culture, but other lower abundance taxa were detected by culture, the agreement was considered moderate.

Poor agreement was attributed when none of the most abundant sequenced taxa were identified by culture while at least one of them was expected to grow on the selected media (cf. material and methods) (Table 3). A third table (Table 4) regroups the results of samples for which the selected growth conditions did not allow the growth of the most abundant species detected by sequencing. An agreement category could therefore not be established for these samples.

RESULTS

Study population

Twenty-nine client-owned dogs were included between December 2019 and January 2021. All dogs were presented for the investigation of a nasal disease. Clinically all dogs had continuous or intermittent muco-purulent-like nasal discharge, which was sometimes associated with other clinical signs of nasal disease such as sneezing, reverse sneezing, hyperkeratosis, ulceration, depigmentation, pain and decreased or increased permeability of the nasal passages (Table 1).

Four dogs were treated with systemic antimicrobials at the time of sampling while in five other dogs, the treatment had been interrupted within the 15 days before sampling. Three dogs were under non-steroidal anti-inflammatory therapy at the time of sampling and in another dog this treatment was stopped 5 days before sampling. Two dogs were receiving an oral antifungal treatment at the time of sampling. Sinonasal aspergillosis was diagnosed in 11 dogs, idiopathic lymphoplasmacytic rhinitis in eight dogs and nasal neoplasia in three dogs. Other primary nasal problems were the presence of nasal foreign body (among which

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Table 1. Clinical data

	Breed	Age (years)	Sex	Nasal discharge (day of sampling)	Diagnosis	Treatment (day of sampling)
1	Border Collie	5	M	Seromucous	Sinonasal aspergillosis	Ophthalmic terramycin
6	Standard poodle	4.7	M	Mucopurulent	Sinonasal aspergillosis	None
7	Rottweiler	4.1	M	Purulent/ haemorrhagic	Sinonasal aspergillosis	Non-steroidal anti-inflammatory drug
10	Rottweiler	3.9	F	Mucopurulent	Sinonasal aspergillosis	Itraconazole
11	Rottweiler	4.2	M	Mucohaemorrhagic	Sinonasal aspergillosis	Amoxicillin clavulanic acid
12	Golden retriever	5.1	M	Serohaemorrhagic	Sinonasal aspergillosis	Itraconazole
20	Border Collie	5	F	Seromucous	Sinonasal aspergillosis	None
22	Newfoundland	7.2	M	Serohaemorrhagic	Sinonasal aspergillosis	Doxycycline
25	Mixed-breed	11	F	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis	None
3	Dachshund	0.7	M	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis with nasal foreign body	None
23	Italian pointing dog	1.4	M	Mucopurulent/ haemorrhagic	Sinonasal aspergillosis with nasal foreign body	None
21	Rhodesian ridgeback	10.2	F	Mucopurulent	Lymphoplasmacytic rhinitis (sequel sinonasal aspergillosis)	None
2	Akita Inu	5.9	M	Purulent	Lymphoplasmacytic rhinitis	None
5	Parson jack russel	11.9	M	Mucopurulent	Lymphoplasmacytic rhinitis	Meloxicam
17	Husky	7.1	M	Seromucous	Lymphoplasmacytic rhinitis	None
18	Jack Russel	9.9	M	Purulent	Lymphoplasmacytic rhinitis	None
26	Mixed-breed	8.7	M	Mucopurulent	Lymphoplasmacytic rhinitis	None
27	Münsterländer	4.6	M	Seromucous	Lymphoplasmacytic rhinitis	None
29	Jack Russel	10.9	F	Mucopurulent	Lymphoplasmacytic rhinitis	Amoxicillin clavulanic acid until 2 days ago
8	Weimaraner	6.9	F	Mucopurulent/ haemorrhagic	Nasal neoplasm (uncharacterised)	Meloxicam+amoxicillin clavulanic acid until 5 days ago
16	Mixed-breed	15.2	F	Mucous/haemorrhagic	Nasal adenocarcinoma	None
28	Weimaraner	6.9	F	Haemorrhagic	Nasal adenocarcinoma	None
4	Medium poodle	13.1	M	Seromucous	Oronasal defect, hard palate tumour	Doxycycline until 1 week ago
15	Mixed-breed	1	M	Mucohaemorrhagic	Nasal foreign body	Meloxicam, local NaCl
19	Mixed-breed	11	M	Mucopurulent	Nasal foreign body	Marbofloxacin until 10 days ago
13	Labrador	10.3	F	Purulent	Mucocutaneous pyoderma nasal planum	Amoxicillin clavulanic acid
9	Beauceron	2.3	F	Mucopurulent	Lower airway disease	None
14	Cocker spaniel	1.4	M	Mucopurulent	Lower airway infection	Amoxicillin clavulanic acid until 9 days ago
24	Brussels Griffon	11.7	F	Purulent	Lower airway infection	Amoxicillin clavulanic acid

When "none" is indicated in treatment section: no treatment within the last 2 weeks

two with sinonasal aspergillosis), oronasal defect and disease of the planum nasale. In three dogs, nasal disease was associated with bacterial bronchopneumonia.

Culture-based method

Three dogs had a negative culture on the four media; none of them had been treated with antimicrobials during the last 2 weeks before sampling.

Bacterial species isolated from each dog are shown in Tables 2-4. In the group of dogs with recent or ongoing antimicrobial treatment, six of nine dogs (66.7%) had ≤1 cultured colony type. In the group of dogs without recent or ongoing antimicrobial treatment, eight of 20 dogs (40%) had ≤1 cultured colony type.

The most commonly isolated species among all 29 samples included *Staphylococcus pseudintermedius* (isolated in 12 dogs; 41.4%), *Staphylococcus epidermidis* (3; 10.3%), *Klebsiella pneumoniae* (3; 10.3%), *Bordetella bronchiseptica* (2; 6.9%), *Streptococcus equi* (2; 6.9%), *E. coli* (2; 6.9%), *Neisseria dumasiana* (2; 6.9%) and *S. aureus* (2; 6.9%).

The remaining occasionally isolated species were *Neisseria canis*, *Staphylococcus sciuri*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*, *Morganella morganii*, *Kluyvera ascorbata*, *P. aeruginosa*, *Enterobacter cloacae/xiangfangensis*, *Mannheimia haemolytica*, *Paenibacillus* sp., *Rothia nasimurium*, *Proteus mirabilis* and *Bacillus licheniformis*. In one dog (dog 15), the identification of one colony type (around 50 colonies of a coccobacillus Gram– Oxydase+ Lactose– growing on the MacConkey agar) was inconclusive by MALDI-TOF mass spectrometry.

Discrimination using MALDI-TOF mass spectrometry between members of the *E. cloacae* complex to which *E. cloacae and Enterobacter xiangfangensis* belong is known to be weak (Godmer et al., 2021).

16S rRNA gene amplicon sequencing method

From 3,625,695 raw reads in the 29 nasal swabs, we obtained 3,386,574 reads after cleaning (length and sequence quality). Finally, we retained 290,000 reads [10,000 reads per sample, minimal read length is 450 nucleotides with 0 ambiguous nucleotide (Kozich et al., 2013)] to adjust for uneven sequencing depth across samples. Suspected contaminants were removed by filtering them from the OTU. At the end of the analysis, we

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obtained 15,840 OTUs among which 975 phylotypes at species level and 410 phylotypes at genus level. The two negative control swabs yielded no results.

Between 13 and 225 distinct OTUs were detected in the nasal samples. In dogs without antimicrobial treatment (n=20) the mean ±standard deviation of unique OTUs was 69 ±57 and in dogs with recent or ongoing antimicrobial treatment (n=9) it was 81 ±53. The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion did not significantly differ between treated/recently treated and untreated dogs.

A total of 25 phyla were identified throughout all nasal samples among which Proteobacteria followed by Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria were the most frequently encountered. The most abundant families were *Enterobacteriaceae*, *Staphylococcaceae*, *Moraxellaceae*, *Porphyromonadaceae*, *Bacteroidaceae* and *Streptococcaceae* (Fig 1). Together they represent 57.4% of the total bacterial taxa. Among these families, the most abundant genera were *Staphylococcus* (mean relative abundance 16.5%), *Escherichia-Shigella* (12.8%), *Moraxella* (7.1%), *Porphyromonas* (6.2%), *Bacteroides* (5.2%) and *Streptococcus* (4.8%).

Comparison between culture- and molecular-based results

Taxa with a total relative abundance >50% (dominant), 10% to 50% (major) and <10% (minor) detected by sequencing were also found in culture in 46.7%, 20.0% and 0.4% of the cases respectively. Conversely, among the organisms that were cultured, 34.8% represented a major or dominant taxa by amplicon sequencing.

In nine samples (31%, samples 1, 2, 11, 13, 16, 18, 20, 24 and 28), a good agreement between the two methods was observed (Table 2). In most cases, this was associated with one overrepresented taxon (major or dominant taxon). Six samples in this group had a positive culture for different species of *Staphylococcus* (*S. aureus*, *S. pseudintermedius*, *S. sciuri* and *S. haemolyticus*) and amplicon sequencing detected the same species with a very high relative abundance (RA >80%) in four samples. Other samples were dominated by *S. equi* (90.3% RA) or *E. coli* (81.1% RA).

A moderate agreement between culture and sequencing results was present in five samples (17.2%, samples 9, 15, 21, 23 and 26, Table 2). In this group, samples showed a more heterogeneous profile without dominant taxa detected by sequencing. In three samples (9, 21 and 26), bacteria were cultured but not detected with sequencing. These were *S. epidermidis* (growth 1+), *N. canis* (1+) and *N. dumasiana* (2+).

A poor agreement between culture and sequencing results was assigned in eight samples (27.6%, Table 3). Heterogeneous profiles without dominant taxa were also observed. In all samples, *E. coli* was sequenced (minimum relative abundance: 10%, maximum relative abundance: 37.8%) but not cultured. In five cases, *E. coli* was associated with *Bacteroides fragilis* and/or *Lachnoclostridium* sp.

In four samples (4, 5, 7 and 29), species were isolated with culture but were undetected by sequencing. These species were *Klebsiella aerogenes* (growth 1+), *S. pseudintermedius* (growth 1+, isolated in three of the four samples), *P. mirabilis* (growth 1+) and *E. cloacaelxiangfangensis* (growth 3+). In three samples (8, 22, 29), cultured colony types were detected by sequencing but these only belonged to minor taxa, none of the most abundant species detected by sequencing were isolated by culture.

Among the moderate and poor agreement groups, taxa of the Pasteurellaceae family were detected by sequencing in five different samples (5, 18, 21, 26, 29), with a minimum and maximum relative abundance of 11.5% and 30.3%, respectively. In all five cases, however, taxa of this family were not cultured.

For seven samples, the growth requirements for the most abundant species detected by sequencing were not met (12, 14, 17, 19 and 27) or unknown (3 and 10, Table 4). In all cases, one dominant taxon was detected: *Moraxella* sp. (n=2), *Defluviitaleaceae* unclassified (n=1), *Porphyromonas cangingivalis* (n = 1), Gammaproteobacteria unclassified (n=1), *Actinomyces canis* (n=1), *Mycoplasma cynos* (n = 1). For five of the seven samples, all the cultured colony types, with a growth varying from 1+ to 3+, were not detected by sequencing.

DISCUSSION

The current study aimed to compare the results of bacterial identification from paired nasal swabs using agar-plate cultures and 16S rRNA gene amplicon sequencing. A moderate to good agreement and a poor agreement were observed in 48.3% and 27.6% of the samples, respectively. In 24% of the cases, the conditions required for the growth of the dominant species detected by sequencing were inadequate or unknown.

These discrepancies illustrate that standard culture does not reliably predict the bacterial profile detected by 16S rRNA gene amplicon sequencing.

Based on what has been described in the literature in healthy dogs (Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017; Vangrinsven et al., 2021), most of the dogs in this study had a nasal microbiota profile compatible with dysbiosis. Meaning that normally dominating taxa are underrepresented and substituted by normally low-abundance species. Although the type of profile associated with a bacterial infection has not yet been clearly defined, an unusual high abundance of pathogen-related taxa detected in sequencing analyses has been reported to be characteristic of bacterial infection (Singh et al., 2015).

In healthy dogs, the nasal microbiota has been described as being dominated by the phylum Proteobacteria representing between 50% and 80% of the total bacterial taxa in previous studies (Ericsson et al., 2016; Isaiah et al., 2017; Rodrigues Hoffmann et al., 2014; Tress et al., 2017; Vangrinsven et al., 2021) and largely dominated by the genus *Moraxella* (Tress et al., 2017; Vangrinsven et al., 2021). In the present study, Proteobacteria was also the dominating phylum, but consisting mainly of the genus *Escherichia-Shigella*. The other predominant genus was *Staphylococcus*. These two genera are among the most commonly isolated by culture in dogs with nasal disease (Meler et al., 2008). They are also encountered in healthy dogs

Table 2. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples with a moderate to good agreement

Dog	Strain	Culture			16S rDNA sequencing			
	MALDI-TOF mass spectrometry	Media	Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
13	*Staphylococcus aureus*	MSA	4+	*Staphylococcus aureus*	87.7	*Staphylococcus aureus*	87.7
28 A	*Streptococcus equi*	EDW	4+	*Streptococcus equi*	90.3	*Streptococcus equi*	90.3
B	*Klebsiella pneumoniae*		3+	*Klebsiella pneumoniae*	0.24		
16 A	*Escherichia coli*	MCC	4+	*Escherichia coli*	81.1	*Escherichia coli*	81.1
B	*Morganella morganii*	MCC	2+	Undetected			
20 A	*Staphylococcus pseudintermedius*	MSA	4+	*Staphylococcus pseudintermedius*	16.6	*Kluyvera ascorbata*	61.8
B	*Kluyvera ascorbata*	MCC	1+	*Kluyvera ascorbata*	61.8	*Staphylococcus pseudintermedius*	16.6
1 A	*Pseudomonas aeruginosa*	MCC	3+	*Pseudomonas aeruginosa*	2.6	*Staphylococcus pseudintermedius*	97.1
B	*Staphylococcus pseudintermedius*	MSA	1+	*Staphylococcus pseudintermedius*	97.1		
11	*Staphylococcus pseudintermedius*	MSA	3+	*Staphylococcus pseudintermedius*	85.5	*Staphylococcus pseudintermedius*	85.5
24	*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	29.8	*Staphylococcus pseudintermedius*	29.8
2	*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	98.5	*Corynebacterium* sp.	15.1
						Staphylococcus pseudintermedius	98.5
18 A	*Staphylococcus sciuri*	MSA	2+	*Staphylococcus sciuri*	21.9	*Staphylococcus sciuri*	21.9
B	*Klebsiella pneumoniae*	MCC	1+	*Klebsiella pneumoniae*	5.1	*Pasteurella canis*	11.5
C	*Staphylococcus haemolyticus*	MSA	1+	*Staphylococcus haemolyticus*	11.0	*Staphylococcus haemolyticus*	11.0
						Acinetobacter soli	10.1
9 A	*Bordetella bronchiseptica*	MCC	4+	*Bordetella bronchiseptica*	20.9	*Bacilli* unclassified	32.3
						Bordetella bronchiseptica	20.9
B	*Staphylococcus epidermidis*	MSA	1+	Undetected		*Escherichia coli*	13.6
21 A	*Streptococcus equi*	EDW	1+	*Streptococcus equi*	20.8	*Pasteurellaceae* unclassified	23.3
B	*Neisseria canis*	COL	1+	Undetected		*Streptococcus equi*	20.8
						Escherichia coli	18.3
						Bacteroides fragilis	10.7
23	*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	14.3	*Escherichia coli*	20.6
						Staphylococcus pseudintermedius	14.3
						Moraxella sp.	12.9
						Bacteroides fragilis	11.9
						Lachnoclostridium sp.	10.3
26 A	*Neisseria dumasiana*	COL	2+	Undetected		*Pasteurella canis*	30.3
B	*Escherichia coli*	MCC	1+	*Escherichia coli*	10.7	*Escherichia coli*	10.7
15 A	*Neisseria dumasiana*	COL	4+	*Neisseria* sp.	10.6	*Porphyromonas cangingivalis*	46.9
B	Unidentified*	COL	3+			*Porphyromonas gulae*	11
						Neisseria sp.	10.6

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar, EDW Edward agar
*Unconclusive identification by MALDI-TOF mass spectrometry

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Table 3. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples with a poor agreement

Dog	Strain	Culture			16S rDNA sequencing			
		MALDI-TOF mass spectrometry		Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
8		*Staphylococcus pseudintermedius*	MSA	1+	*Staphylococcus pseudintermedius*	6.6	*Escherichia coli*	32.1
							Lachnoclostridium sp.	15.3
							Bacteroides fragilis	13.4
22		*Moraxella canis*	COL	1+	*Moraxella canis*	1.2	*Escherichia coli*	35.9
							Bacteroides fragilis	12.2
							Lachnoclostridium sp.	12.0
29	A	*Staphylococcus epidermidis*	MSA	4+	*Staphylococcus epidermidis*	0.05	*Pasteurella canis*	13.6
	B	*Enterobacter cloacae/xiangfangensis**	MCC	3+	Undetected		*Escherichia coli*	13.5
7		*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	37.2
							Lachnoclostridium sp.	14.0
							Bacteroides fragilis	10.2
5		*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	25.4
							Pasteurellaceae unclassified	14.4
							Bacteroides fragilis	11.8
4	A	*Klebsiella aerogenes*	MCC	1+	Undetected		*Mycoplasma canis*	16.6
	B	*Staphylococcus pseudintermedius*	MSA	1+	Undetected		*Escherichia coli*	10.8
6	C	*Proteus mirabilis*	MCC	1+	Undetected		*Escherichia coli*	37.8
		No growth					*Bacteroides fragilis*	19.7
							Lachnoclostridium sp.	13
25		No growth					*Conchiformibius* sp.	31.6
							Escherichia coli	10

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar
*Weak discrimination between these two species with MALDI-TOF mass spectrometry

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Table 4. Comparison of culture and predominant taxa for sequencing results in nasal cavities: samples for which growth conditions (culture) of the most abundant species (sequencing) were inadequate or unknown

Dog	Strain	Culture			16S rDNA sequencing			
		MALDI-TOF mass spectrometry		Growth	Closest 16S rRNA match (compared to culture results)	Relative abundance %	Most abundant species (>10% relative abundance) in sample	Relative abundance %
17	A	*Mannheimia haemolytica*	COL	3+	Undetected		*Moraxella* sp.	91.7
	B	*Staphylococcus epidermidis*	MSA	2+	Undetected			
3	A	*Paenibacillus* sp.	COL	2+	Undetected		*Defluviitaleaceae* unclassified	88.7
	B	*Staphylococcus pseudintermedius*	MSA	1+	Undetected			
	C	*Klebsiella pneumoniae*	MCC	1+	Undetected			
12		*Staphylococcus saprophyticus*	MSA	2+	Undetected		*Porphyromonas cangingivalis*	90.2
19		*Rothia nasimurium*		1+	Undetected		*Moraxella* sp.	78.0
10	A	*Staphylococcus aureus*	MSA	1+	Undetected		Gammaproteobacteria unclassified	97.9
	B	*Bacillus licheniformis*	COL	1+	Undetected			
27		No growth					*Actinomyces canis*	76.7
14	A	*Staphylococcus pseudintermedius*	MSA	2+	*Staphylococcus pseudintermedius*	0.7	*Mycoplasma cynos*	84.8
	B	*Bordetella bronchiseptica*	MCC	2+	*Bordetella bronchiseptica*	7.5		

COL Columbia blood agar, MCC MacConkey agar, MSA Mannitol salt agar

but are mostly around tenfold less abundant (Ericsson et al., 2016; Tress et al., 2017; Vangrinsven et al., 2021). These results are in agreement with previous studies demonstrating alterations of the nasal microbiota in dogs with nasal diseases (Tress et al., 2017; Vangrinsven, 2021).

The main difficulty with culture interpretation of the nasal cavities is the high degree of contamination by the rich and diverse normal commensal nasal flora. Moreover, the close contact between nasal passages and the external environment in association with the dog sniffing/nose-licking behaviour makes contamination by environmental and oral flora also possible. As a result, a positive culture cannot automatically be the synonym of nasal bacterial infection.

So far, bacteria isolated by nasal swab culture were considered to be healthy flora when there was only a sparse growth of bacteria that have been reported cultured from the nasal passages of healthy dogs (Lobetti, 2009; Plickert et al., 2014). Bacteria have been considered pathogenic if there was a pure and heavy growth and if they were bacteria not previously reported cultured from the nasal passages of healthy dogs (Lobetti, 2009). Based on the current results, however, these criteria do not seem to be applicable for all cases. A first obstacle is the absent or weak growth of some bacteria by culture, such as *A. canis*, *P. cangingivalis*, *M. cynos* and *Moraxella* sp. among others, while they were detected at a high relative abundance by sequencing. For these bacteria, growth conditions were not ideal or even inadequate. *Moraxella* sp. need CO_2 to allow and support growth, *Porphyromonas* sp. are strict anaerobic bacteria, *Actinomyces* sp. grow better in anaerobiosis and *Mycoplasma* sp. do not develop on agar medium. For the unclassified taxa, *Defluviitaleaceae* and Gammaproteobacteria unclassified, adequate growth conditions are difficult to predict. The second obstacle is the heavy growth (3+ or 4+) of bacterial species which were undetected or were sequenced with a low relative abundance such as *E. cloacae/xiangfangensis* (undetected), *K. pneumoniae* (RA 0.24%), *P. aeruginosa* (RA 2.6%), *S. epidermidis* (RA 0.05%) and *M. haemolytica* (undetected). One explanation could be related to the sampling method that we used, which consisted of two consecutive swabs. The material collected with swab 1 and used for culture could differ from the material collected with swab 2 which was used for sequencing. For undetected species, deeper sequencing of specimens may also have increased the sensitivity for molecular detection. Excessive growth of a weakly abundant species or selection for fast-growing bacteria over pathogens are other potential explanations for these discrepancies between the two methods. Assuming that pathogens are more likely to be a major or dominant species, the dilution of the sample before inoculation on the culture media could be an alternative to detect only major/dominating flora.

In the present study, poor culture specimen handling and misidentification are very unlikely since the specimens were handled immediately, by the same operator in the same laboratory and MALDI-TOF MS is a very accurate identification technique. In practical conditions, however, such obstacles render culture interpretation, as a sole method to detect nasal bacterial infection, challenging. It must also be remembered that identification of particular organisms with either approach does not imply that this organism is the causative agent of disease, which further complicates result interpretation.

More intriguing were the sequenced bacterial species for which growth requirements were met but still bacteria were not isolated by culture, which was repeatedly the case for *E. coli*. A possible explanation could be, again, the fact that two consecutive swabs were used. Other possibilities include competition for nutrients with other bacterial species, detected bacteria that are dead or inability of culture to replicate the nasal conditions. In all but one of these cases, there was no growth on the MacConkey agar excluding competition with other species for nutrients on the media where *E. coli* is

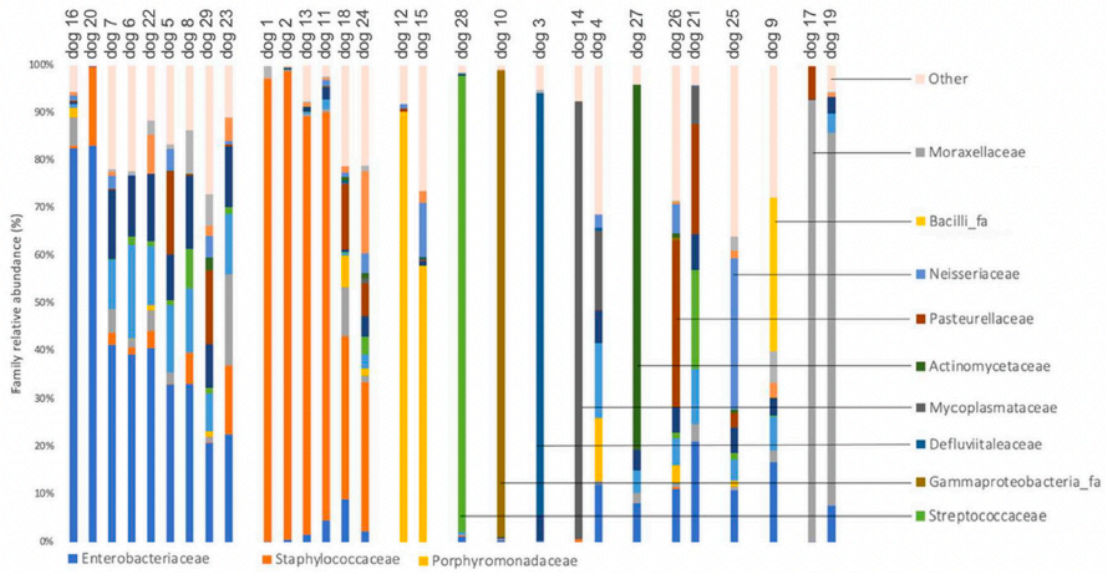

FIG 1. Relative abundance at family level. Samples were grouped based on major families

expected to grow. In five cases, the uncultured *E. coli* was associated with other bacteria typically found in the faecal flora such as *Bacteroides fragilis* and *Lachnoclostridium* sp. This could reflect a previous inspiration of faeces with bacteria that are dead and therefore do not grow. However, previous studies in healthy dogs (Isaiah et al., 2017; Rodriguez et al., 2019;Tress et al., 2017; Vangrinsven et al., 2021), with presumed similar sniffing behaviour, failed to show similar findings, which does not support this hypothesis.

In 5 cases, taxa of the *Pasteurellaceae* family were also detected by sequencing but not cultured. These taxa should grow on the Columbia agar +5% sheep blood medium in aerobic conditions. However, these were not dominant taxa and *Pasteurella* species grow best with CO_2 or in microaerophilic conditions, the absence of CO_2 during incubation could potentially explain their absence in the culture results.

Bacteria with higher abundance and growing easily on selected media were more likely to be identified by culture. If we assume that pathogens, once established, multiply and become dominant or major taxa, culture should be able to highlight them, at least when they grow on the selected media. Yet, the concept that low-abundance bacteria (minor taxa) may play a significant role in disease processes has also been put forward in human beings (de Cena et al., 2021). Current knowledge in terms of canine nasal microbiota is not yet advanced enough to know if such microbes play a role in canine nasal diseases, but if they did, culture would probably not be sensitive enough to detect them.

In two dogs, a high abundance of *Moraxella* sp. was observed resembling the type of profile that has been described in healthy dogs (Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017; Vangrinsven et al., 2021). Yet, clinical symptoms were similar to the other dogs of the study and secondary bacterial infection was suspected. In these two dogs culture results were positive for *M. haemolytica+S. epidermidis* and *R. nasimurium*, results that, taken separately, could have led to antimicrobial treatment while this treatment would have been considered unnecessary based on sequencing results. Moreover, as mentioned earlier, culture of *Moraxella* sp. was not attempted while it would be interesting to detect its presence. Indeed, when *Moraxella* sp. is the dominant species, bacterial infection and significant dysbiosis are unlikely since this is the profile most often found in the nose of healthy individuals (Tress et al., 2017; Vangrinsven et al., 2021).

Among the high-abundance species detected by sequencing, which could reflect bacterial infection, only *S. pseudintermedius* was detected more than once. When present, it was detected with culture, albeit not always with a significant growth. For most of the others (*M. cynos*, *P. cangingivalis* and *A. canis*), as standard growth conditions are not adequate, targeted PCR could be an alternative. But such a PCR panel would probably be quite broad, difficult to determine and not very practical for use. Alternatively, a PCR for *Moraxella* sp. with a low cycle threshold would likely reflect a healthy nasal flora without dysbiosis.

Like it can be observed in the sample of dogs that have been included in this study, systemic antimicrobials are regularly prescribed by veterinarians in dogs with nasal disease in which secondary bacterial infection is suspected. The usefulness of systemic antimicrobials in this context is however unclear and, in the authors opinion, the influence of systemic antimicrobials on the nasal microbiota is likely to be negligible. The total bacterial flora, intrinsic diversity values, beta-diversity and beta-dispersion did not significantly differ between dogs treated with antimicrobials, ongoing or recent, compared to untreated dogs, suggesting

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a limited effect of such treatments on the nasal flora. This would be in accordance with previous studies suggesting that the ability of systemic antimicrobials to affect the nasal bacterial flora may be less than assumed (Hauser et al., 2016; Jain et al., 2018; Tress et al., 2017). The number of colony types was lower in treated dogs possibly reflecting the effect of antibiotic treatment on culture results. However, the present study was not designed to assess the effect of antimicrobial treatment on the nasal microbiota and nasal swab culture results so no final conclusions can be drawn based on these results.

As mentioned earlier, the use of two consecutive swabs may have influenced the results and is one of the limitations of this study. Alternatively, the material of one single swab could have been suspended in an adequate medium then aliquoted for culture on one hand and PCR on the other. Another limitation is the absence of specific growth conditions to improve culture of microbes with particular growth requirements. However, since the goal of this study was to reproduce routine clinical conditions, conventional culture media were used. Propidium monoazide could have been used to mask the DNA of dead bacteria before amplification and limit the bias of this DNA in the sequencing results. However, this method does not mask 100% of the dead bacteria with a variating effectiveness from one taxon to another, making interpretation of clinical samples challenging. A group of healthy dogs was not included in this study because previous studies describing the nasal microbiota in healthy dogs yielded comparable main results. One of these previous publications (Vangrinsven et al., 2021) was conducted by the same team using identical swabs, sampling technique, DNA extractions sets, primer sets, bioinformatic pipelines and database. However, data were not processed together in one unique table which could have led to some differences but likely minor ones.

In conclusion, the present results do not support the routine use of standard nasal swab for the diagnostic workup in dogs with nasal diseases. First it should be kept in mind that, whatever the method used, the true clinical implication of the identified bacteria(s) is difficult to establish. Then, various observations in the present study are interesting to point out because they reveal discrepancies between culture and sequencing results which further complicates the interpretation of nasal swab culture results. Identification by sequencing of dominant or major species potentially contributing to nasal disease and requiring specific growth conditions is not a rare event. Positive culture, even with a pure and heavy growth, does not invariably mean that this colony type is a dominant or even major taxa. When amplicon sequencing and culture results are interpreted together, a more comprehensive and interpretable information is available. Nasal swab culture can be considered under specific conditions such as in dogs with recurring purulent nasal discharge of unknown origin despite investigation of the underlying condition although interpretation of the results remains challenging. Clinical application of amplicon sequencing may be employed in these cases if culture is negative or if targeted antimicrobial therapy against the cultivable bacteria fails to produce any disease improvement.

Acknowledgements

The authors would like to thank Albert Belinda, Phan Kim-Thu and Di Biagio Eugenia for their help in samples collection and storage.

Author contributions

Emilie Vangrinsven: Conceptualization (lead); data curation (lead); formal analysis (lead); methodology (lead); visualization (equal); writing – original draft (lead); writing – review and editing (lead). **Jean Noël Duprez:** Conceptualization (supporting); data curation (equal); formal analysis (supporting); methodology (supporting); supervision (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Cécile Meex:** Data curation (equal); formal analysis (equal); methodology (equal); writing – review and editing (supporting). **Bernard Taminiau:** Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); supervision (equal); validation (equal); visualization (equal); writing – review and editing (equal). **Georges Daube:** Conceptualization (supporting); methodology (supporting); supervision (equal); validation (equal); writing – review and editing (supporting). **Frédéric Billen:** Conceptualization (equal); methodology (supporting); supervision (equal); writing – review and editing (supporting). **Jacques Mainil:** Conceptualization (equal); methodology (supporting); supervision (equal); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Cecile Clercx:** Conceptualization (equal); methodology (equal); supervision (lead); validation (lead); visualization (equal); writing – review and editing (equal).

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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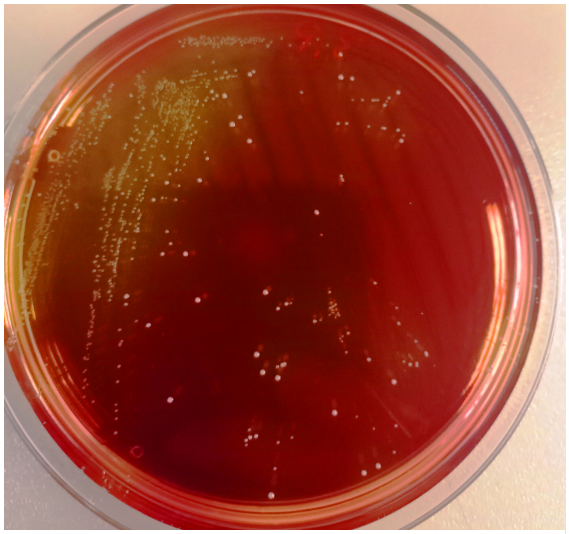
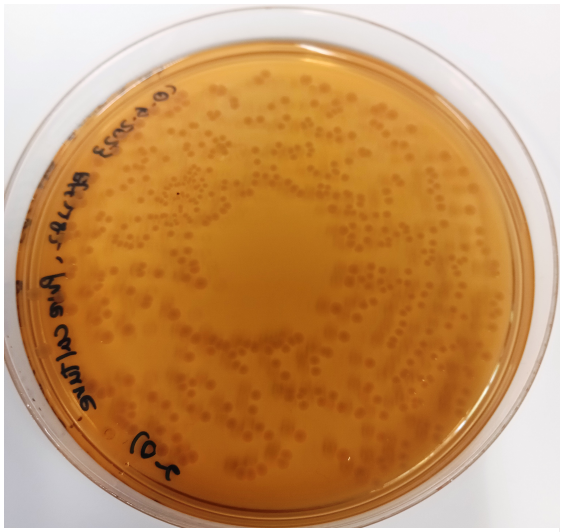
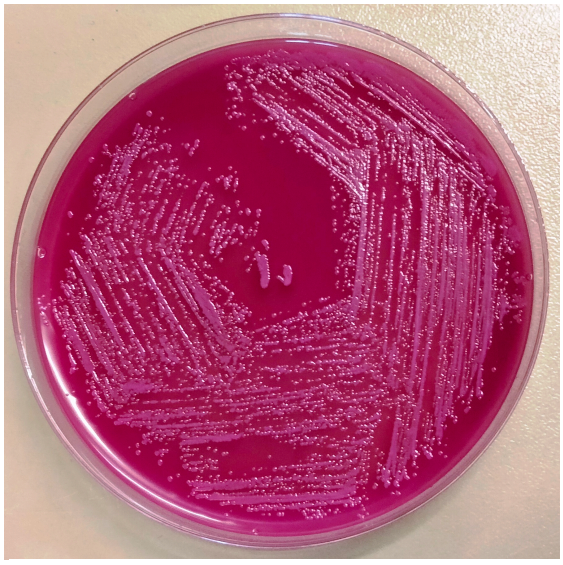
Figure 21: Several of our Petri dishes illustrating the growth of different bacterial species (Source: Images courtesy of Jean-Noël Duprez, University of Liège, Belgium).



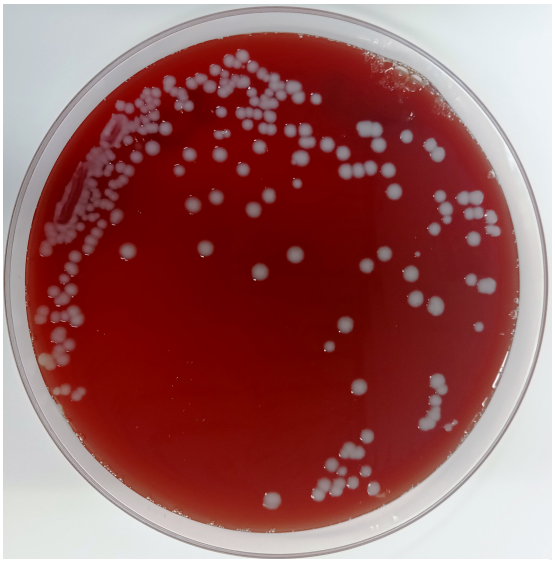
Mannitol salt agar (Chapman),mixed culture of *Staphylococcus aureus* (yellow) and *Staphylococcus epidermidis* (white without halo)



MacConkey agar, pure culture of *Bordetella bronchiseptica*



MacConkey agar, pure culture of *Escherichia coli*



Columbia blood agar, pure culture of *Pasteurella multocida*

Experimental section

Study 2 :

Variations in facial conformation are associated with differences in nasal microbiota in healthy dogs

Preamble

In dogs, *Aspergillus fumigatus* infection is a common cause of nasal disease in meso- or dolichocephalic breeds (Peeters and Clercx 2007, Valdes et al., 2020). In contrast, brachycephalic breeds, with their unique nasal cavity structures, are rarely affected by chronic nasal diseases such as fungal rhinosinusitis. In human beings, recent reports suggest that the microbiota can modulate the host immune response to invading fungal micro-organisms (Romani et al., 2015; Gonçalves et al., 2017; Libertucci and Young 2019) which could represent a novel therapeutic approach or an interesting point of view to the understanding of the pathophysiology of the disease.

The upper airway microbiome in healthy humans is shaped by both extrinsic factors (such as birth delivery mode, feeding type, exposure to tobacco smoke, and air pollution) and intrinsic factors including genetics, mucosal immunity, and age (Bosch et al., 2016; Biesbroek et al., 2014; Yu et al., 2017; Ahmed et al., 2019; Liu et al., 2015; Igartua et al., 2017; Whelan et al., 2014). In dogs, the nasal cavities host complex bacterial communities which have been reported to be influenced by similar extrinsic and intrinsic factors, including age, body weight, and geographical location (Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017). Differences in facial anatomical conformation and consequently in breathing/airflow pattern might result in different nasal microbial composition. Accordingly, the aim of study 2 was to describe and compare the nasal microbiota of healthy dogs across breeds categorized by body and facial conformation.

Using 16S rRNA gene sequencing, this study characterized the nasal microbiota of 46 healthy dogs and assessed the impact of facial conformation. Brachycephalic dogs had a distinct nasal microbiota with significantly higher richness and alpha-diversity, reduced abundance of Proteobacteria (notably *Moraxella*), and increased Actinobacteria and Firmicutes compared with other breed groups. Breed-related facial conformation was the main factor influencing microbiota composition, while age, sex, and environment had minimal effects. These results establish reference data for the healthy canine nasal microbiota and support a potential link between anatomy and microbial ecology.

Experimental section

Study 2 :

Variations in facial conformation are associated with differences in nasal microbiota in healthy dogs

BMC Vet Res. 2021;17(1):361

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Vangrinsven *et al. BMC Veterinary Research* 2021, **17**(1):361
https://doi.org/10.1186/s12917-021-03055-w

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Variations in facial conformation are associated with differences in nasal microbiota in healthy dogs

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Abstract

Background: Extrinsic and intrinsic factors have been shown to influence nasal microbiota (NM) in humans. Very few studies investigated the association between nasal microbiota and factors such as facial/body conformation, age, and environment in dogs. The objectives are to investigate variations in NM in healthy dogs with different facial and body conformations. A total of 46 dogs of different age, living environment and from 3 different breed groups were recruited: 22 meso—/dolichocephalic medium to large breed dogs, 12 brachycephalic dogs and 12 terrier breeds. The nasal bacterial microbiota was assessed through sequencing of 16S rRNA gene (V1-V3 regions) amplicons.

Results: We showed major differences in the NM composition together with increased richness and α-diversity in brachycephalic dogs, compared to meso—/dolichocephalic medium to large dogs and dogs from terrier breeds.

Conclusion: Healthy brachycephalic breeds and their unique facial conformation is associated with a distinct NM profile. Description of the NM in healthy dogs serves as a foundation for future researches assessing the changes associated with disease and the modulation of NM communities as a potential treatment.

Keywords: Nasal cavity, Microbiota, Dogs, Breed, Facial conformation

Background

Studies investigating the composition of the microbial communities of the nasal cavities in healthy individuals using culture-independent molecular methods have arisen over the last decade in humans [1–6] and more recently in companion animal veterinary medicine [7–11].

In healthy humans, both extrinsic and intrinsic factors have been reported to shape an individual's upper airway microbiome. Extrinsic exposures reported to influence the nasal microbiota (NM) composition include delivery at birth mode [12] and feeding type [13] in infants, and exposure to tobacco smoke [14] and air pollution [15] in adults.

Intrinsic factors like host genetics [16] and associated host mucosal immunity [17] as well as age [18] also seem to modulate the NM. Moreover, in specific anatomic sites in the upper respiratory tract as well as within the nasal cavities, distinct micro-niches containing specialized bacterial communities have been described, demonstrating intra-patient spatial variation of the NM [2].

Studies in humans suggest that the NM is a key factor in maintaining respiratory health by affecting both resistance to pathogens and immunological responses [19–21]. While a greater mucosal diversity may play a role in limiting inflammation and protecting against infections [22–24], potential microbial species known as "keystone species" may also have a beneficial effect on the ecosystem's balance, function and health [21, 25, 26].

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In dogs, complex bacterial communities dominated by the Moraxellaceae family have been detected in the nasal cavities of healthy animals [7, 9, 10]. Spatial variations between nasal and oropharyngeal swabs [7, 9], temporal variations [9], changes associated with geographical location [9] as well as variations associated with older age and lower bodyweight [10] have been reported suggesting that extrinsic and intrinsic factors also influence upper airway bacterial composition in dogs.

Variable predispositions for chronic nasal disease are observed between different dog breeds. Indeed, chronic lymphoplasmacytic rhinitis (LPR) and sinonasal aspergillosis (SNA) are almost exclusively encountered in middle-aged, medium to large meso- or dolichocephalic canine breeds [27, 28]. In contrast, brachycephalic breeds, in which extreme breeding selection has progressively led to very distinct anatomical features of the nasal cavities [29, 30] are rarely affected by chronic inflammatory or infectious nasal diseases. Anatomical abnormalities described in the nasal cavities of brachycephalic dogs include marked global reduction of the size of the nasal cavities, stenosis of the nares and vestibulum, malformed rostral and caudal aberrant turbinates. Those abnormalities are associated with intranasal contact points, reduced lumen and increased collapsibility of the nasopharynx and missing paranasal sinuses. They cause abnormal breathing patterns, differences in airflow distribution and increased resistance to airflow [31–33]. Very few studies investigated the association between the NM and the facial/body conformation [10] or breed [9]. One study [10] compared the NM in dogs with different cephalic indexes (meso- and dolichocephalic) and failed to observe differences in nasal microbial composition. In the same study, dogs with a body weight of less than 10 kg had a significantly higher Shannon diversity index and species richness than dogs with a body weight over 10 kg. In another study [9], no differences in nasal bacterial communities were reported in dogs from different large dolichocephalic breeds.

We hypothesized that differences in facial anatomical conformation and consequently in breathing/airflow pattern might result in different nasal microbial composition. Therefore, the aim of this study was to describe and compare the composition of the NM in healthy dogs of various breeds, categorized into 3 groups according to their body and facial conformation.

Results

Study population

Forty-six client-owned dogs were recruited between November 2017 and July 2018. Groups DL (meso—/dolichocephalic dogs of medium to large breeds), B (brachycephalic breeds) and T (terrier breeds) contained 22, 12 and 12 dogs respectively.

Breed, age, sex and bodyweight distribution

Group DL included mixed breed ($n=5$), Belgian Shepherd ($n=4$), Australian Shepherd (n = 4), Labrador ($n=2$), Beauceron ($n=2$), Border Collie ($n=2$), Golden retriever ($n=1$), Dalmatian (n = 1), and White Swiss Shepherd (n = 1). French Bulldog ($n=6$), English Bulldog ($n=2$), Pug (n = 2) and Cavalier King Charles Spaniel (n = 2) composed the group B. Group T was composed of 4 breeds: Jack Russel terrier ($n=7$), Yorkshire terrier ($n=3$), West Highland white terrier ($n=1$) and Pinscher (n = 1). All dogs were healthy and from different households. The distribution of age, bodyweight and sex among groups is reported in Table 1. Dogs of group B were younger (more dogs in age class 1) compared to group T (more dogs in age class 2 and 3; $p=0.02$). Median age for all dogs was 6 years with one 6 months old dog in group B and one 8 months old dog

Table 1 Distribution of age, bodyweight, sex, and living environment among the 3 breed groups of healthy dogs

	Group DL ($n=22$)	Group B ($n=12$)	Group T (n = 12)
Age mean (min-max)	5.8 (0.8–11.3)	3.9 (0.6–9.2)	6.8 (2.6–10.8)
Age classes n, %	class 1: 5, 23%	class 1: 5, 42%	class 1: 0, 0%
	class 2: 13, 59%	class 2: 6, 50%	class 2: 10, 83%
	class 3: 4, 18%	class 3: 1, 8%	class 3: 2, 17%
Bodyweight mean (min-max), kg	28 (14.8–41)	13.1 (6.8–21.4)	7.1 (2.9–10)
Female n, %	14, 64%	5, 42%	7, 58%
City n, %	7, 32%	4, 33%	3, 25%

Age class 1: < 3 years, class 2: 3–8 years and class 3: > 8 years

Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds

Group B = brachycephalic dogs of small breeds

Group T = mesocephalic or dolichocephalic terrier dogs of small breeds

in group DL, one dog of 10.8 years in group T and two dogs of 10.4 and 11.3 years in group DL. The remaining dogs were between 1 and 10 years of age. As expected, all three groups had marked differences in bodyweight associated with their breed type. Gender distribution was comparable in the 3 groups.

Living environment and diet

Distribution of living environment was similar between groups (Table 1). Contact with tobacco smoke and food type were not taken into account because no dog lived in close contact with tobacco smoke (smokers were not smoking inside the house or in a place where the dog could be in regular contact with tobacco smoke) and because all dogs were fed with commercial dry food, at the exception of 4 of them (1 dog in group DL and 2 dogs in group T were eating a mix of commercial dry food and home-made diet; 1 dog in group B was eating a home-made diet). For these parameters, groups were too small to perform statistical analysis.

Most common taxa

Good's coverage index was superior to 99% in all dogs with a median value of 99.81% (99.70–99.88%) indicating an equal and adequate sampling effort for all dogs. At the finest taxonomic resolution, a total of 755 operational taxonomic units (OTUs) were found throughout all samples. The most common taxa at phylum and family level in healthy dogs from different breed groups are illustrated in Fig. 1. A total of 20 phyla were identified in the samples of the nasal cavities of healthy dogs, the most abundant being Proteobacteria followed by Tenericutes, Actinobacteria, Firmicutes and Bacteroidetes. Other phyla such as Fusobacteria and Patescibacteria represented less than 1% of the mean relative abundance.

The most common taxon at the family level was Moraxellaceae representing 41.6% of the total taxa: this family was detected in all samples at percentages ranging from 0.04 to 94.8%. Pseudomonadaceae, unclassified family of the Mollicutes class, Cardiobacteriaceae, unclassified family of the Pseudomonadales order, Microbacteriaceae, Streptococcaceae and Staphylococcaceae constituted the major part of the other frequently detected families although some of them were present in large amounts in only a few animals. *Moraxella* was the predominant genus in the majority of samples. Together with the genera *Pseudomonas*, *Suttonella*, *Leucobacter*, and unclassified genera of the class Mollicutes and of the order Pseudomonadales they represented 80.1% of all taxa throughout all samples.

Influence of age, sex, living environment and breed groups (DL, B and T) on the nasal microbiota

Constrained ordination

Redundancy analysis (RDA) based on the table of values at species level showed that first breed group (Adjusted R^2 0.045 for group B versus group DL and T) and then to a lesser extent age classes (Adjusted R^2 0.006 for age class 1 versus age class 2 and 3) contributed to the variability of the microbiota (explaining together 12.9% of the variance).

Richness, evenness and α-diversity

A similar RDA was performed based on richness (Chao1 index), evenness (Simpson index) and α-diversity (inverse Simpson index). Only breed groups contributed to the constraint (Adjusted R^2 0.074, explaining 11% of the variance for group B versus group DL and T).

There were no differences in richness, α-diversity or evenness across the different age groups, sexes or living environments when these parameters were evaluated individually. Among the breed groups, Group B had a significantly higher species α-diversity and richness compared to group DL and T (Fig. 2). Evenness did not differ significantly between breed groups.

Beta-diversity

When using an analysis of molecular variance (AMOVA) at species level, the β-diversity was not different between the age ($p=0.29$), the sex ($p=0.63$), or the living environment ($p=0.65$) groups. The analysis of the molecular variance homogeneity (HOMOVA) at species level also did not reveal a significant difference between these groups (age, $p=0.86$; sex, $p=0.49$; living environment, $p=0.72$). This was also suggested by the non-metric multidimensional scaling (NMDS) plot of microbial communities at species level based on a Bray-Curtis dissimilarity matrix which failed to reveal any clustering for these three exposures. In contrast, NMDS plot revealed a distinct separation between group B and the 2 other groups DL and T (Fig. 3). A significant difference in bacterial community composition was verified by an AMOVA analysis ($p=0.02$) and pairwise comparison demonstrated that group B was different compared to groups DL and T (group B vs group DL $p=0.017$; group B vs group T $p=0.008$; group DL vs group T $p=0.511$). The analysis of the molecular variance homogeneity (HOMOVA) did not reveal a significant difference between breed groups ($p=0.08$).

Bacterial composition in the nasal cavity of dogs from different breed groups

Based on the results of RDA, richness, evenness, α-diversity and β-diversity, breed group is the dominant variable influencing the nasal microbiota within the variables that were evaluated. Further investigation of

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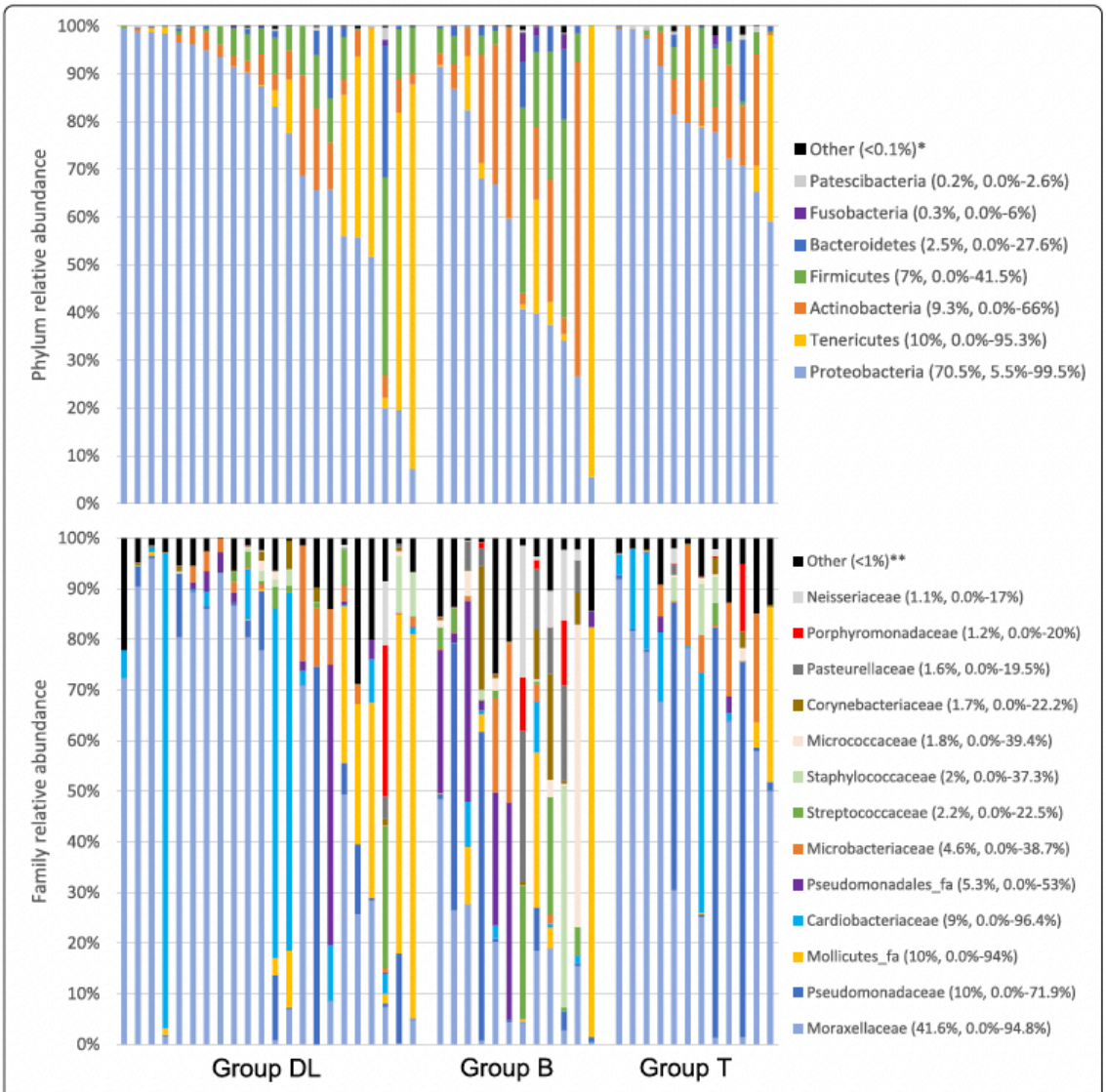


(mean, min-max)
*13 remaining phyla representing <0.1% of mean relative abundance
**145 remaining families representing <1% of mean relative abundance
Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds
Group B = brachycephalic dogs of small breeds
Group T = mesocephalic or dolichocephalic terrier dogs of small breeds
Each bar chart represents one dog, samples are shown in the same order (for phylum and family) within each group

Fig. 1 Composition of nasal microbiota at phylum and family level in healthy dogs from 3 different breed groups

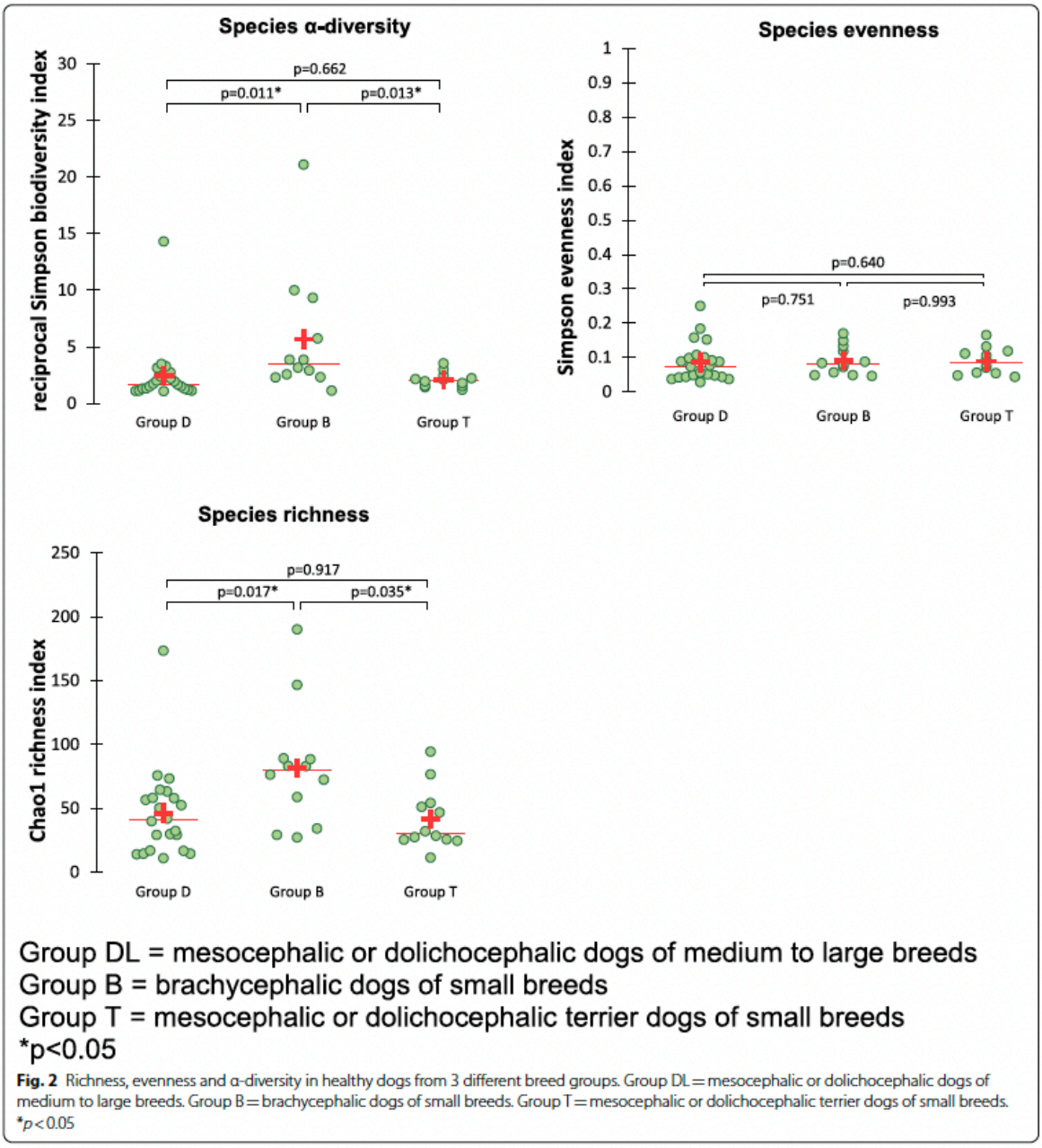


Fig. 2 Richness, evenness and α-diversity in healthy dogs from 3 different breed groups. Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds. Group B = brachycephalic dogs of small breeds. Group T = mesocephalic or dolichocephalic terrier dogs of small breeds. *$p < 0.05$

bacterial composition was therefore performed by comparing the breed groups.

Most common taxa

The total bacterial flora quantified by 16S rRNA gene based qPCR did not significantly differ between groups of different breeds (Fig. 4).

Despite the fact that the community composition at the phylum level showed a dog-to-dog variation within each group (Fig. 1), the most abundant phylum throughout the 3 groups was Proteobacteria since it was the most predominant (more than 50%) in all dogs of group T, 6/12 dogs of group B and 19/22 dogs of group DL.

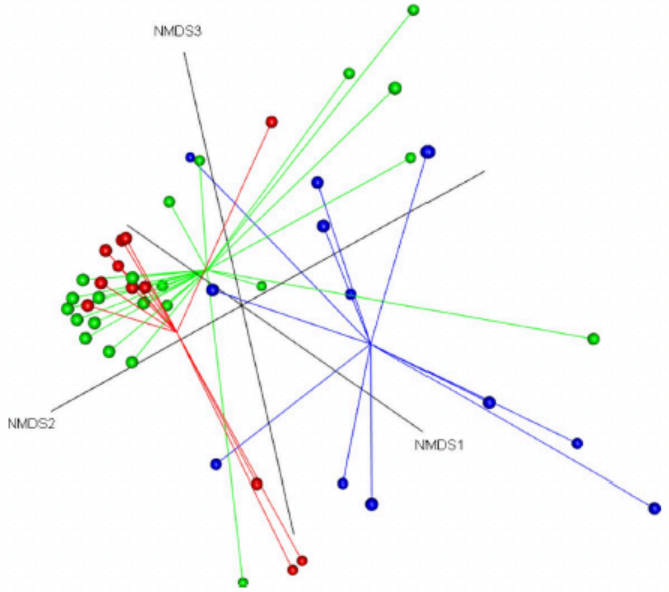
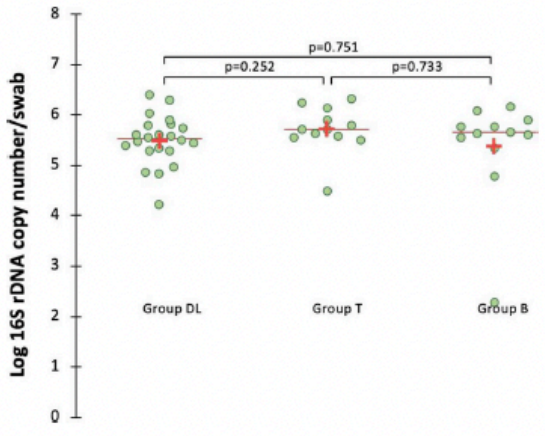


Fig. 3 Non-metric multidimensional scaling (NMDS) ordination of nasal microbiota communities using Bray-Curtis in healthy dogs from 3 different breed groups. Significant clustering was observed between breed groups. (green, group DL; blue, group B; red, group T). Samples are represented by dots and the lines connecting dots are vectors defining the centromere of the group. Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds. Group B = brachycephalic dogs of small breeds. Group T = mesocephalic or dolichocephalic terrier dogs of small breeds. Stress value = 0.1255474

Linear discriminant analysis (LDA) effect size (LEfSe) scores indicated bacterial taxa that were mainly present in group B. The genera *Haemophilus* (family Pasteurellaceae), *Parvimonas* (family Clostridiales_Family_XI), unclassified genus within the family Pasteurellaceae, *Fusobacterium* (family Fusobactericiaea), *Gemella* (family Bacillales_Family_XI), *Rothia* (family Micrococcaceae), *Conchiformibius* (family Neisseriaceae), *Abiotrophia* (family Aerococcaceae) and *Streptococcus* (family Streptococcaceae) were significantly more represented in dogs from the group B. At species level 8 taxa were enriched in group B while only one taxon was associated with group T (Fig. 5).

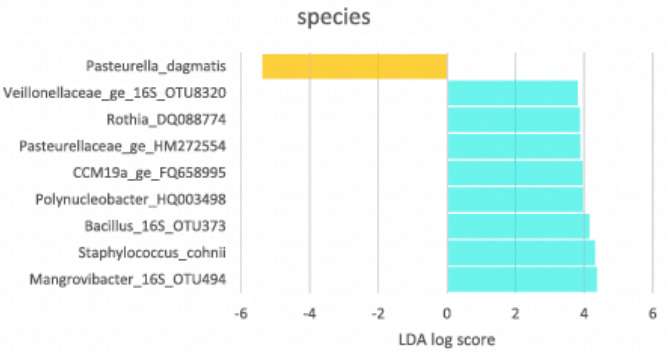
Differences in relative abundances

Mean relative abundances of taxa that significantly differ between groups can be found in Table 2. At the phylum level the proportion of Proteobacteria was significantly lower in group B, in comparison with DL and T, while the proportion of Actinobacteria, Firmicutes and other Proteobacteria was increased. In the phylum Proteobacteria, the genera *Moraxella* (family Moraxellaceae) and *Suttonella* (family Cardiobacteriaceae) were significantly lower in group B. Genera of other Proteobacteria that were more frequently observed in group B are an unclassified genus within the Pseudomonadales order as well as *Conchiformibus* (family Neisseriaceae) and an unclassified genus of the Pasteurellaceae family. Among the phylum Actinobacteria, the genera *Corynebacterium* (family *Corynebacteriaceae)* and *Rothia* (family Micrococcaceae) were more abundant in group B, although this was not significant. Among the phylum Firmicutes, *Streptococcus* (family *Streptococcaceae*), *Staphylococcus* (family Staphylococcaceae), *Abiotrophia* (family Aerococcaceae) and *Gemella* (family Bacillales_Family_XI) were more abundant (however not significantly) in group B.



Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds
Group T = mesocephalic or dolichocephalic terrier dogs of small breeds
Group B = brachycephalic dogs of small breeds

Fig. 4 Total bacterial flora in healthy dogs from 3 different breed groups. Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds. Group T = mesocephalic or dolichocephalic terrier dogs of small breeds. Group B = brachycephalic dogs of small breeds



Nine differentially abundant bacterial species were detected (p = 0.05, LDA score > 3.0) at species level. Of these, 8 bacterial groups were significantly over-represented in brachycephalic dogs (blue) and one bacterial group was over-represented in dogs of terrier breeds (yellow).

Fig. 5 Linear discriminant analysis (LDA) effect size (LEfSe) at species level of Illumina sequencing datasets based on 16S rRNA gene sequences. Nine differentially abundant bacterial species were detected ($p = 0.05$, LDA score > 3.0) at species level. Of these, 8 bacterial groups were significantly over-represented in brachycephalic dogs (blue) and one bacterial group was over-represented in dogs of terrier breeds (yellow)

The phylum Tenericutes (almost exclusively composed by an unclassified species of the class Mollicutes) was less abundant in group T compared to the two other groups. The abundance of the genus *Pseudomonas* (family Pseudomonadaceae) was increased in group T although this was mainly caused by 3 individuals (Fig. 1).

The genus *Staphylococcus*, in group B, was mainly composed of the species *Staphylococcus pseudintermedius* (3.07% (0–36.74%)). In contrast, the percentage of the species *Staphylococcus aureus* was slightly lower in group B (0.01%) compared to the two other groups (0.33% (0–4.55%) in group DL, 0.41% (0–4.97%) in group T).

The similarities and differences in bacterial composition between the 3 groups were visualized using a Venn diagram based on the occurrences at species level (Fig. 6) and showed that 74 OTU were shared between all groups

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Table 2 Bacterial groups at > 1% mean relative abundance among the 3 breed groups of healthy dogs at phylum, family and genus level

Taxon	Group DL			Group B			Group T		
Phylum **Family** ***Genus***	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**
Proteobacteria	73.5	27.6	**A*** **	53.3	25.6	**B**	81.1	12.8	**A**** **
Moraxellaceae	48.9	38.1	**A**** **	16.4	15.7	**B**	53.5	30.1	**A**** **
Moraxella	48.9	38.1	**A**** **	16.3	15.7	**B**	53.0	30.7	**A**** **
Pseudomonadaceae	6.8	13.0	**A**	10.0	19.2	**AB**	16.0	27.1	**B** **
Pseudomonas	6.8	13.0	NS	10.0	19.2	NS	15.8	26.8	NS
Pseudomonadales_fa	3.1	10.0	**A** **	14.0	19.1	**B**	0.6	1.2	**A*** **
Pseudomonadales_ge	3.1	10.0	**A** **	14.0	19.1	**B**	0.6	1.2	**A** **
Cardiobacteriaceae	12.8	26.7	**A** **	1.9	3.0	**B**	8.8	14.2	**AB**
Suttonella	12.8	26.7	**A** **	1.9	3.0	**B**	8.8	14.2	**AB**
Pasteurellaceae	0.2	0.6	NS	5.8	6.3	NS	0.2	0.5	NS
Pasteurellaceae_ge	0.1	0.5	NS	4.7	5.6	NS	0.2	0.5	NS
Neisseriaceae	0.4	1.7	NS	3.2	5.5	NS	0.3	0.8	NS
Conchiformibius	0.4	1.7	NS	3.1	5.3	NS	0.3	0.7	NS
Burkholderiaceae	0.4	0.8	NS	1.0	2.1	NS	0.5	1.1	NS
Gammaproteobacteria_fa	0.7	0.8	NS	0.4	0.5	NS	1.0	1.0	NS
Gammaproteobacteria_ge	0.7	0.8	NS	0.4	0.5	NS	1.0	1.0	NS
Actinobacteria	4.7	5.2	**A***	18.2	19	**B**	8.8	14.2	**AB**
Corynebacteriaceae	0.6	1.2	NS	4.9	7.7	NS	0.6	1.0	NS
Corynebacterium_1	0.2	0.4	NS	2.6	5.6	NS	0.3	0.8	NS
Corynebacterium	0.4	1.2	NS	1.8	5.2	NS	0.2	0.7	NS
Micrococcaceae	0.2	0.5	NS	6.1	16.2	NS	0.4	0.7	NS
Rothia	0.1	0.3	NS	6.0	16.2	NS	0.1	0.4	NS
Microbacteriaceae	2.9	4.7	NS	5.7	12.0	NS	6.5	8.7	NS
Leucobacter	2.4	4.6	NS	5.7	12.0	NS	6.4	8.7	NS
Tenericutes	12.8	23	NS	11.4	25.8	NS	3.7	10.8	NS
Mollicutes_fa	12.6	23	**A**	11.4	25.8	**AB**	3.7	10.7	**B***
Mollicutes_ge	12.6	23	NS	11.4	25.8	NS	3.7	10.7	NS
Firmicutes	6.2	8.7	NS	12.3	14.5	NS	3.6	4.2	NS
Streptococcaceae	1.7	4.1	NS	4.8	7.1	NS	0.3	1.1	NS
Streptococcus	1.7	4.1	NS	4.8	7.1	NS	0.3	1.1	NS
Staphylococcaceae	1.3	2.8	NS	3.3	10.3	NS	1.6	3.1	NS
Staphylococcus	1.2	2.8	NS	3.3	10.3	NS	1.6	3.1	NS
Aerococcaceae	0.8	3.1	NS	1.6	3.2	NS	0.0	0.1	NS
Abiotrophia	0.7	3.0	NS	1.5	3.1	NS	0.0	0.1	NS
Carnobacteriaceae	1.1	2.6	NS	0.0	0.2	NS	0.0	0.0	NS
Bacillales_Family_XI	0.2	0.8	NS	1.0	1.9	NS	0.0	0.1	NS
Gemella	0.2	0.8	NS	1.0	1.9	NS	0.0	0.1	NS
Clostridiales_Family_XI	0.8	1.7	NS	0.5	0.9	NS	1.0	1.7	NS

Table 2 (continued)

Taxon	Group DL			Group B			Group T		
Phylum **Family** ***Genus***	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**	**Mean rel. Freq. (%)**	**SD (%)**	**Tukey's multiple comparisons test ($p < 0.05$)**
Helcococcus	0.7	1.7	NS	0.4	0.8	NS	1.0	1.7	NS
Bacteroidetes	2.5	6.3	NS	3.3	4.3	NS	1.7	3.5	NS
Porphyromonadaceae	0.9	4.1	NS	1.7	3.3	NS	1.0	3.3	NS
Porphyromonas	0.9	4.1	NS	1.7	3.3	NS	1.0	3.3	NS
Weeksellaceae	1.3	3.3	NS	0.8	1.0	NS	0.5	0.9	NS
Fusobacteria	0.1	0.2	NS	1.0	1.8	NS	0.2	0.6	NS

Mean relative percentages (mean rel. freq) and standard deviation (SD) of the most abundant bacterial groups, annotated to the level of phylum, family and genus, based on sequencing of the V1-V3 region of the 16S rRNA gene

Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds

Group B = brachycephalic dogs of small breeds

Group T = mesocephalic or dolichocephalic terrier dogs of small breeds

A,B: Operational taxonomic units not sharing a common letter between breed groups differ significantly. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; NS not significant

which represents 16.9, 18.7 and 35.6%, of groups DL, B, and T respectively. These 74 OTUs represent the vast majority (92.21%) of the total bacterial population. The relative proportion of unique OTUs to each group was comparable with a value around 45–50% for the 3 groups (group DL: 51.9%, group B: 47.1%, group T: 43.8%). These unique OTUs represent a small proportion (together around 3%) of the total bacterial population. As a result, the differences associated with group B were more related to differences in abundance of shared OTUs than to the presence of unique OTUs.

Discussion

The focus of this study was to investigate variations in the NM in healthy dogs of different breeds with different facial and body conformations. To this end the NM of 46 dogs of 3 different breed groups were compared. We showed major differences in the NM composition together with increased richness and α-diversity in brachycephalic dogs, compared to meso−/dolichocephalic medium to large dogs and dogs from terrier breeds. Additionally, no specific correlations with sex or environment were determined. The influence of age remains however unclear.

In the present study, the nasal microbial population was largely dominated by the phylum Proteobacteria and the family Moraxellaceae, in accordance with what has been described in previous studies using 16S rRNA gene amplicon sequencing for bacterial analysis [7, 9, 10]. The phylum Proteobacteria has been found to dominate in association with Tenericutes and Bacteroidetes [7, 9] or in association with Firmicutes and Bacteroidetes [10] while in the current study it was associated with Tenericutes and Actinobacteria followed by Firmicutes and Bacteroidetes. The Actinobacteria phylum predominated in the group of brachycephalic dogs, likely explaining the difference with other studies in which this breed type was not sampled.

The group of brachycephalic dogs in this study was also characterized by a higher species α-diversity and richness, a decrease in Proteobacteria (mainly a decrease in *Moraxella* and *Suttonella*) associated with an increase in Actinobacteria (mainly an increase in *Corynebacterium* and *Rothia*), Firmicutes (mainly an increase in *Streptococcus* and *Staphylococcus*) and other Proteobacteria (mainly an increase in unclassified genus of the order Pseudomonadales, unclassified genus of the family Pasteurellaceae, and *Conchiformibius*). Different reasons could explain these changes.

A first hypothesis rests on the probable occurrence of an important reflux of oropharyngeal secretions in brachycephalic breeds [34]. Indeed, in humans, an association between severe obstructive sleep apnea, local inflammation and alterations in NM has been demonstrated [35]. Such association was presumed to be due to recurrent obstruction during sleep causing reflux of oropharyngeal secretions that otherwise would be swallowed in a healthy subject. Human oral commensals, such as *Streptococcus*, *Veillonella* and *Porphyromonas* were frequently identified in men with obstructive sleep apnea. Dogs of brachycephalic breeds frequently have at least a certain degree of upper respiratory obstruction and

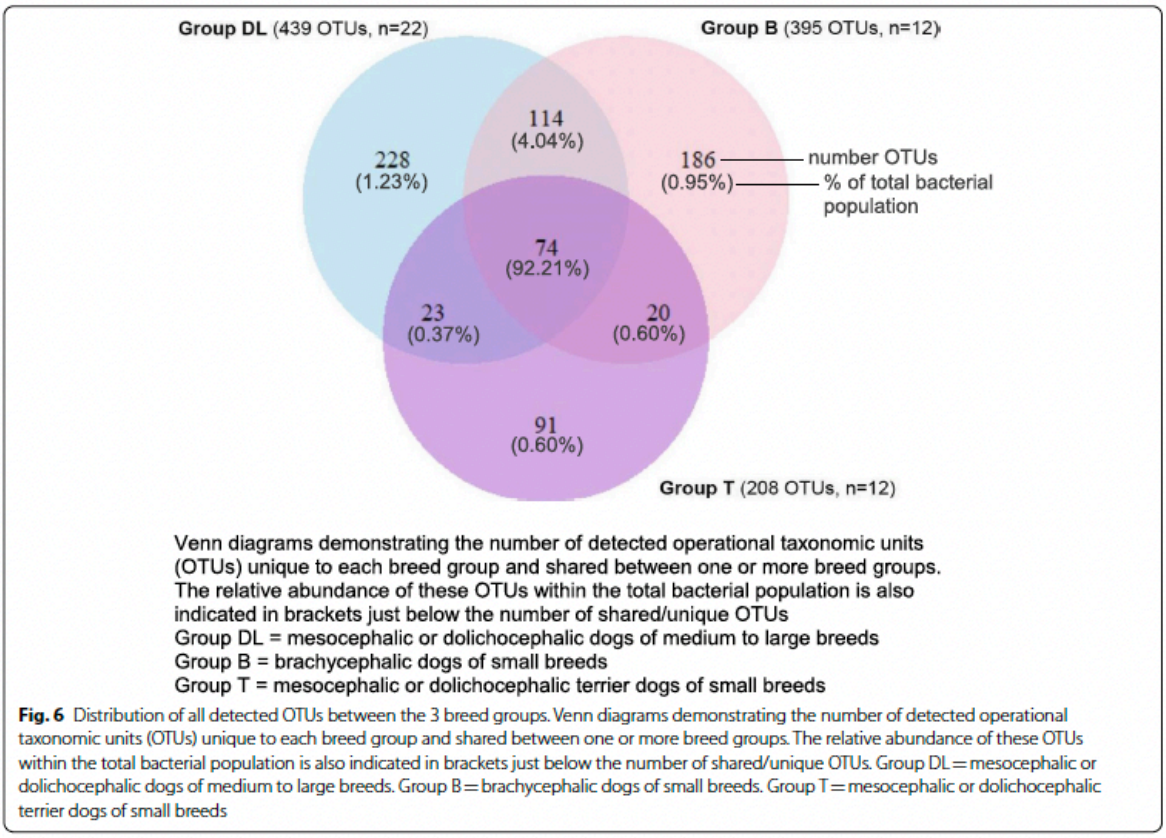

Fig. 6 Distribution of all detected OTUs between the 3 breed groups. Venn diagrams demonstrating the number of detected operational taxonomic units (OTUs) unique to each breed group and shared between one or more breed groups. The relative abundance of these OTUs within the total bacterial population is also indicated in brackets just below the number of shared/unique OTUs. Group DL = mesocephalic or dolichocephalic dogs of medium to large breeds. Group B = brachycephalic dogs of small breeds. Group T = mesocephalic or dolichocephalic terrier dogs of small breeds

in the English bulldog obstructive sleep apnea has been described and even evaluated as a model for obstructive sleep apnea in humans [36]. In dogs, the oropharyngeal microbiota has been shown to be associated with higher abundance of *Porphyromonas* sp. (family Porphyromonadaceae), Pasteurellaceae, *Conchiformibius* sp. (family Neisseriaceae), with less *Moraxella* sp. (Moraxellaceae) and Pseudomonadaceae compared to the nasal cavities [7, 9]. Interestingly, these features corresponded to some of the variations observed in the NM of brachycephalic dogs in this study supporting the hypothesis of contamination of the NM by the oropharyngeal populations.

Another hypothesis to explain differences in NM composition in brachycephalic dogs is related to the changes in airflow distribution and increased airflow resistance described in brachycephalic breeds [32, 33]. Indeed, in one study in men investigating changes in bacterial communities after sinus surgery [37], greater airflow was suggested to cause reductions in temperature and humidity creating a cooler and drier postoperative ecosystem, with an effect on bacterial composition. According to this hypothesis, changes in intranasal temperature and humidity in brachycephalic dogs could be related to distinct intranasal microenvironment in these breeds compared to meso- and dolichocephalic dog. Unfortunately, intranasal temperature and relative humidity have not yet been analyzed in dogs.

Alternatively, the differences in NM found in brachycephalic dogs could also be associated with the reduced length of the nasal cavities in brachycephalic dogs, and the closer proximity between the nares and the deeper intranasal sites. In humans *Corynebacterium* [38], *Propionibacterium* and *Staphylococcus* genera [2] have been shown to dominate in the anterior nares compared with deeper intranasal sites. Such differences have been suspected to be secondary to niche-specific micro-environmental conditions such as pH, humidity, temperature and epithelium type [2, 6, 39], with humidity and moisture representing more favorable environmental factors for *Corynebacterium* and *Staphylococcus* species on this mucosal site [2]. In dogs, compared to the other sites of the skin, the nares were shown to be colonized by Moraxellaceae (genus *Moraxella*) in combination with Oxalobacteraceae (genus *Ralstonia*),

Corynebacteriaceae (genus *Corynebacterium*) and Staphylococcaceae (genus *Staphylococcus*) [40]. Therefore, the higher predominance of *Staphylococcus* (phylum Firmicutes) and Corynebacteriaceae (phylum Actinobacteria) found in the nasal cavities of brachycephalic breeds could be associated with the closer location between the sampled site and the nares.

The presence of bacterial population of the nares in the NM of brachycephalic dogs could also be linked to contamination of the swab by the microbiota of the nares and surrounding skin, due to the reduced nasal passage in these breeds. However, such a contamination has been minimized by the use of a sterile speculum for introduction of the swab into the nasal cavity.

Finally, as only one of these hypotheses could hardly explain all the variations observed in the NM of brachycephalic dogs compared to the other breeds, most likely the differences in NM in brachycephalic dogs could be multifactorial and be initiated by more than one of these mechanisms.

As previously described [7, 9, 10] *Moraxella* was the most abundant genus in the family Moraxellaceae in this study. Despite their numerical dominance, the genus *Moraxella* and associated family Moraxellaceae were significantly lower in brachycephalic breeds compared to the 2 other breed groups. In dogs, the abundance of *Moraxella* in the nasal passages has been reported to be decreased in dogs with nasal disease (chronic rhinitis and nasal neoplasia) [10]. Whether this is a cause or consequence remains to be discovered and the exact role of *Moraxella* in the nasal cavities is still unknown. The decreased amount of *Moraxella* observed in brachycephalic dogs in this study is not in favor of this alteration being at the origin of nasal diseases, as brachycephalic dogs are less prone to these pathologies compared with the other breed groups [41]. Consequently, the decreased amount of *Moraxella* reported in dogs with nasal disease is more likely due to local changes in microenvironment secondary to the disease. This is consistent with our hypothesis that the lower abundance of Moraxellaceae in brachycephalic dogs in this study could be due to selection pressure by other taxa, such as those colonizing the nares or those brought by oropharyngeal reflux, and/or due to local changes in microenvironment as detailed earlier.

In young children, profiles dominated by *Moraxella* and *Dolosigranulum* combined with *Corynebacterium* form a stable nasal microbiome associated with lower rates of respiratory infections. With the exception of *Moraxella catarrhalis* which is described to be associated with bronchiolitis, otitis and chronic rhinosinusitis [19, 25, 42, 43], *Moraxella* might be a keystone bacterium in infants.

In dogs, the vast majority of Moraxellaceae found in this study, besides the species *Moraxella canis*, were not resolved beyond genus level. As a result, the potential presence of *M. catarrhalis* in healthy dogs is unknown and the lower abundance of *Moraxella* in brachycephalic dogs makes the hypothesis of *Moraxella* as a keystone bacterium less likely in dogs.

Patterns of microbiota sharing have been described between humans and companion animals. Humans and pets living in the same household seem to share more microbiota with each other than humans and pets living in different households [44]. Although *Staphylococcus aureus is one* of the most well-described pathobiont of the nasal cavities in men [45], its level of carriage was low in this study, particularly in brachycephalic breeds. Interestingly, an inverse correlation between the genus *Corynebacterium*, a family that was more abundant in brachycephalic breeds, and *S. aureus* has been reported in some studies of adult NM [2, 46]. However, conclusions cannot be drawn as inter-species interactions are very complex and have not been studied yet in the nasal cavities of dogs. The carriage of *Staphylococcus pseudintermedius* in companion animals has also received attention as it can also be an opportunistic pathogen [47]. In opposition to *S. aureus*, *S. pseudintermedius* was more prevalent in brachycephalic breeds. These informations could be of value when evaluating dog breeds as a potential source of Staphylococcus carriage.

Differences in NM due to age have been described in humans, but mostly between infants and adults, possibly in association with maturation of the immune system [43] and in elderly, where a nasal community shifting toward oropharyngeal population would occur [18]. In dogs, minor changes (increased Shannon diversity index of the microbiota of the nasal mucosa) have a been reported in dogs older than 9 years in one study [10], and no changes in another [9]. In the present study, age (< 3 years) appeared as being one of the variables participating to the constraint, along with breed group in the RDA analysis performed on the table of values at species level. Other analyses (RDA based on the intrinsic diversity as well as the different individual analyses) however failed to show a correlation with the age classes. Dogs of group B were significantly younger than dogs of group T (but not than dogs of group DL). As a result, the effect of age could be due to the fact that dogs of group B are younger or age could enhance the breed effect. Breed and age cannot be completely dissociated in this study cohort.

The effect of growing and ageing was not specifically addressed by including significant numbers of immature and/or very old dogs. Nevertheless, in dogs, young age has not been described as influencing the NM and beside one dog of 6 months in group B, all dogs were older than

1 year. Altogether, shifts of the NM in association with age at first sight seem to be negligible and the influence of growth or immune system immaturity on the NM of group B was unlikely to explain the differences with group T and DL.

Differences in NM between individuals living in rural and industrial locations have been reported, the latter being associated with exposure to pollution which has been hypothesized to cause microbiota alteration [15]. In dogs, one study also suggested that the NM could differ based on location [9]. Dogs of the same breed were compared between Alabama and California and differences in α- and β-diversity were observed.

Differences between dogs living in rural and industrial regions were not observed in the present study. However, in contrast to the two studies cited above, differences in geographic locations and associated environmental conditions among the dogs of this cohort were probably quite limited since all dogs were living in Belgium which is a small country with few environmental differences.

The sampling protocol described in this study aimed to limit contaminations by the nares and surrounding skin by introducing the swab through a sterile speculum and during anesthesia. Performing sampling during anesthesia allowed proper swabbing of the nasal mucosa of deeper subsites. This protocol as well as the swab that was used (Copan®, FLOQSwabs™, 553C, Brescia, Italy) seem suitable to sample the nasal microbial population as it yielded sufficient material. This is in agreement with other studies showing that swab samples are representative of the microbiome in the nasal cavities in healthy subjects [48].

Comparison across studies is made difficult by the potential introduction of bias due to variations in sampling technique, sampling site, DNA extraction, different variable regions of the 16S rRNA gene and the taxonomic database used to characterize the bacterial microbiota. It is possible that some of the differences with other publications in dogs are due to variations in sampling method. In one study the nasal mucosa was sampled in awake animals without speculum, which likely prevented sampling deep subsites and could have led to contamination by the nares and surrounding skin [10]. In another study, samples were performed in anesthetized dogs at midway between the tip of the nose and the medial canthus without speculum [7]. And finally, in yet another, swabs were sampled without anesthesia and at a distance of half an inch from the nares, so again more in the cranial part of the nasal cavities [9]. In all these studies a different region of the 16S rRNA gene was sequenced (V4 or V4-V6) and different databases were used.

Limitations in this study include the absence of information concerning anatomical spatial organization of the NM in groups DL and T as well as the absence of oropharyngeal and nostril swabs which would have allowed comparing the microbiota in the 3 adjacent niches, and the possible impact of reflux of oropharyngeal secretions and/or nares contamination on the NM. Statistical comparison of different types of diet was not performed due to small group size. The vast majority of them were fed with dry commercial food and although from many different brands, these diets were probably much more similar to each other, compared with the tremendous variations in diets seen in humans. The influence of other non-measured environmental parameters (season and crowding conditions for example) cannot be excluded. Even if age and living environment were not individually associated with significant changes of the NM in this study, it cannot be excluded that the combination of multiple factors (age, diet, environment) could have influenced the results. Finally, in addition to the bacterial composition, the nasal cavities host a complex viral and fungal community that has not been taken into account but could also influence the bacterial microbiota.

Conclusion

In conclusion, healthy brachycephalic breeds and their unique facial conformation is associated with a distinct nasal microbial profile. Whether this finding is associated with the lack of predisposition for nasal diseases in brachycephalic breeds is unknown. Results of the present study contribute to the current knowledge of the composition of healthy NM in dogs which is a necessary foundation for future research aimed at identifying the impact of various perturbations, e.g., antibiotics, vaccines, infections, diseases, on the ecology of canine nasal microbial communities.

Methods

Study population

Client-owned healthy dogs were prospectively recruited and categorized into 3 groups including meso−/dolichocephalic dogs of medium to large breeds (group DL), brachycephalic dogs of small breeds (group B) and meso−/dolichocephalic dogs of small terrier breeds (group T). Dogs from the DL group were selected to represent a population of dogs particularly predisposed to SNA or LPR, dogs from group B were recruited as they are not predisposed to chronic nasal diseases and have a very distinct body and facial conformation. Finally, terrier dogs were selected to represent small breed dogs without the typical facial conformation of brachycephalic dogs and as being less predisposed breeds to SNA or LPR compared to dogs from the DL group.

All dogs were exempt of clinical signs and had a normal physical examination and normal hematology and

biochemistry blood work. Dogs receiving antimicrobial, anti-inflammatory or immunosuppressive medication were excluded from the study. In order to investigate a possible effect of age or environmental living conditions on the NM, all dogs included were subsequently re-classified according to their age (< 3 years (age class 1), 3–8 years (age class 2) and > 8 years (age class 3)) and their environmental living conditions (living either in a city, considered as an environment with high pollution load, or in the countryside, considered as an environment with low pollution load). Owners were also asked about exposure of their pet to tobacco smoke and type of food provided.

Sample collection

This study was approved by the ethical committee of the University of Liege (approval number: 1854) and all samples were obtained with informed and written owner consent.

Dogs were premedicated with butorphanol (0.2 mg/kg; Butomidor®, Richter Pharma) intravenously in combination with medetomidine (5 µg/kg; Medetor®, CP-Pharma). Propofol (Propovet®, Zoetis) on demand was used for induction without intubation. Under general anesthesia the nostril was maintained open using a sterile speculum, and a sterile swab (Copan®, FLOQSwabs™, 553C, Brescia, Italy) was introduced through the speculum up to the distal third of the right nasal cavity (Fig. 7). Three gentle complete circular movements were used to brush the mucosa before withdrawal of the swab through the speculum. The top of the swab was then cut, stored in a sterile cryotube and banked at −80 °C until further analyses.

DNA extraction and high throughput sequencing

Total bacterial DNA was extracted from all nasal swabs at the same time with the DNeasy Blood and Tissue kit (QIAGEN Benelux BV; Antwerp, Belgium) following the manufacturer's recommendations. Spectrophotometry (NanoDrop, Thermo Scientific) was used for total DNA concentration measurement.

Bacterial 16S rRNA gene amplicons were generated via amplification of the V1-V3 hypervariable regions of the 16S rRNA gene using the following primers (with Illumina overhand adapters): forward (5′-GAGAGT TTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCG GCTGCTGGCAC-3′). The DNA was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second polymerase chain reaction (PCR) round for indexing, using



Fig. 7 Sample collection. A sterile swab (Copan®, FLOQSwabs™, 553C, Brescia, Italy) was introduced through a sterile speculum up to the distal third (dashed line) of the nasal cavity. Three complete circular movements were used to brush the nasal mucosa before withdrawal of the swab through the speculum

the Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, USA). A final quantification, by quantitative PCR, of each sample in the library was performed using the KAPA SYBR" FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run.

Quantification of the total bacterial flora was performed with a quantitative real-time PCR targeting the V2-V3 region of the 16S rRNA gene after DNA extraction from samples. The quantitative real-time PCR were performed on the ABI 7300 real-time PCR system with Takyon™ ROX SYBR® Mastermix dTTP Blue reagents (Eurogentec, Seraing, Belgium) for a total reaction volume of 20 µl. The amplification was carried out with 40 cycles of a 95 °C denaturation phase followed by a 60 °C annealing phase. The PCR results were expressed in number of copies of the 16S rRNA gene per swab. The standard curve was based upon 10-fold dilution of a quantified PCR product. This PCR product targeting the V2-V3 region of the 16S rRNA gene was purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Leiden, The Netherlands) and quantified with PicoGreen targeting double-stranded DNA (Promega) before use.

Data analysis

Alignment and clustering were done with MOTHUR software package (v1.41.0) with an OTU clustering distance of 0.03 and based on the SILVA database (V1.32) of full-length 16S rRNA gene sequences. Vsearch [49] algorithm was used for chimera detection.

From 6,082,571 raw reads, we obtained 5,596,282 reads after cleaning (length and sequence quality). Finally, we retained 5000 reads (median 4997 reads per sample) to adjust for uneven sequencing depth across samples. Suspected contaminants were removed by filtering them from the OTU table.

Statistical analysis

Statistical analyses were performed using XLstat (2019.3.2, Addinsoft, Paris, France), Rstudio (v1.1.463) vegan package [50] and MOTHUR (v1.40). Differences were considered significant for a p-value < 0.05.

The distribution of age classes was compared between the 3 breed groups (DL, B and T) using a Kruskal-Wallis test.

A redundancy analysis (RDA) on values at species level and on intrinsic diversity values (richness, evenness, alpha-diversity) were performed to evaluate the relationships between the nasal microbiota and the different potential explanatory variables (age, sex, living environment and breed group) that could influence/shape it. Forward selection was conducted to select significant variables using the "ordiR2step" function (with adjusted R^2 coefficient) from the vegan package [50].

Individual analyses for the same variables were then carried out. Bacterial richness (Chao1 index), evenness (Simpson index-based measure), α-diversity (inverse Simpson's index) and Good's coverage index were obtained with MOTHUR at species level. They were compared between the 3 breed groups (DL, B and T), males and females, the 3 age classes and the 2 categories of living environment using a Kruskal-Wallis or Mann-Whitney test. Beta-diversity (bacterial community composition) at species level was assessed with MOTHUR using a dissimilarity matrix of Bray-Curtis then estimated with AMOVA (10,000 iterations) and β-dispersion was assessed with HOMOVA (10,000 iterations) in MOTHUR. NMDS plots were performed based on a Bray-Curtis dissimilarity matrix at the species level to represent the global bacterial composition (β-diversity) between groups (vegan 3D [51] and rgl [52] package). The total bacterial flora was compared between the 3 breed groups using a Kruskal-Wallis test. LEfSe was performed to detect differences in bacterial composition between breed groups at species level with MOTHUR (significant for an LDA score > 3.0 [53]). Statistical differences in the relative abundance at phylum, family, genus and species level between breed groups were assessed with a two-way ANOVA followed by a Tukey's multiple comparison test. A Venn diagram based on the occurrences at species level was also obtained with R.

All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de Bioproject ID PRJNA656294.

Abbreviations

NM: Nasal microbiota; LPR: Chronic lymphoplasmacytic rhinitis; SNA: Sinonasal aspergillosis; OTU: Operational taxonomic unit; AMOVA: Analysis of molecular variance; rRNA: ribosomal ribonucleic acid; NMDS: Non-metric multidimensional scaling; qPCR: Quantitative polymerase chain reaction; PCR: Polymerase chain reaction; HOMOVA: Homogeneity of molecular variance; LDA: Linear discrimination analysis; LEfSe: Linear discriminant analysis effect size; rDNA: Ribosomal deoxyribonucleic acid; B: Brachycephalic breeds; T: Terrier breeds; DL: Meso- or dolichocephalic medium to large breeds.

Acknowledgements

The authors would like to thank Albert Belinda, Phan Kim-Thu and Romijn Sylvain for their help in samples collection and storage.

Authors' contributions

EV and CC conceived and designed the study. EV, AF, FB, BT, GD and CC participated in data acquisition for the study. EV, AF and BT analyzed the data. EV, AF and BT performed the statistical analyses. EV and CC interpreted the results. EV and CC wrote the manuscript and the authors read and approved the final version of the manuscript.

Funding
No funding was obtained for this study.

Availability of data and materials
All sample raw reads associated with this study have been deposited at the National Center for Biotechnology Information (NCBI) under the accession number PRJNA656294.

Declarations

Ethics approval and consent to participate
Samples from healthy domestic dogs were collected according to a protocol approved by the Ethical Committee of the University of Liège (protocol #1854) and with the informed consent of the owners.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 30 August 2020 Accepted: 19 October 2021
Published online: 24 November 2021

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Experimental section

Study 3 :

Assessment of the nasal microbiota in dogs with fungal rhinitis before and after cure and in dogs with chronic idiopathic rhinitis

Preamble

Study 3 extends the investigation of the canine nasal microbiota in dogs with nasal disease to explore the potential for microbiome-based diagnostic and therapeutic strategies. In humans, microbiota dysbiosis is considered a key factor in the pathogenesis of chronic inflammatory respiratory diseases (Lal et al., 2017; Gan et al., 2021; Lee et al., 2018; Abreu et al., 2012; Hoggard et al., 2017; Copeland et al., 2018; Cho et al. 2020), with specific bacterial species, biofilms (Galli et al., 2008), and "keystone species" (Copeland et al., 2018) playing significant roles in maintaining mucosal health and preventing pathogen colonization. This suggests that modulating the microbiome could offer novel therapeutic strategies to manage upper respiratory tract infections and inflammation. However, the role of nasal microbiota in the pathophysiology of chronic nasal diseases in dogs, such as fungal rhinitis and chronic idiopathic rhinitis, is largely unexplored. Only one study has reported on nasal microbiota alterations in dogs with nasal neoplasia or chronic rhinitis (Tress et al., 2017). Furthermore, no data exists on nasal microbiota alterations in dogs with fungal nasal disease, and the potential for bacterial dysbiosis to influence fungal growth or disease recurrence remains unknown.

This study evaluated nasal microbiota changes in dogs with fungal rhinitis and chronic idiopathic rhinitis compared with healthy controls using 16S rRNA sequencing. Both diseases were associated with marked dysbiosis, mainly characterized by a decrease in Proteobacteria, particularly *Moraxella*, and increased microbial diversity, with disease-specific microbial signatures. Microbiota composition often remained altered after fungal rhinitis cure, and antimicrobial or antifungal treatments showed no significant impact. These findings highlight a strong association between nasal dysbiosis and chronic canine nasal diseases but do not allow determination of whether these alterations represent a cause or a consequence of disease

Experimental section

Study 3 :

Assessment of the nasal microbiota in dogs with fungal rhinitis before and after cure and in dogs with chronic idiopathic rhinitis

BMC Microbiol. 2023;23(1):104

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Vangrinsven *et al. BMC Microbiology* (2023) 23:104
https://doi.org/10.1186/s12866-023-02828-7

BMC Microbiology

RESEARCH Open Access

Assessment of the nasal microbiota in dogs with fungal rhinitis before and after cure and in dogs with chronic idiopathic rhinitis

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Abstract

Background Pathogenesis of canine fungal rhinitis is still not fully understood. Treatment remains challenging, after cure turbinate destruction may be associated with persistent clinical signs and recurrence of fungal rhinitis can occur. Alterations of the nasal microbiota have been demonstrated in dogs with chronic idiopathic rhinitis and nasal neoplasia, although whether they play a role in the pathogenesis or are a consequence of the disease is still unknown. The objectives of the present study were (1) to describe nasal microbiota alterations associated with fungal rhinitis in dogs, compared with chronic idiopathic rhinitis and controls, (2) to characterize the nasal microbiota modifications associated with successful treatment of fungal rhinitis. Forty dogs diagnosed with fungal rhinitis, 14 dogs with chronic idiopathic rhinitis and 29 healthy control dogs were included. Nine of the fungal rhinitis dogs were resampled after successful treatment with enilconazole infusion.

Results Only disease status contributed significantly to the variability of the microbiota. The relative abundance of the genus *Moraxella* was decreased in the fungal rhinitis (5.4 ± 18%) and chronic idiopathic rhinitis (4.6 ± 8.7%) groups compared to controls (51.8 ± 39.7%). Fungal rhinitis and chronic idiopathic rhinitis groups also showed an increased richness and α-diversity at species level compared with controls. Increase in unique families were associated with fungal rhinitis (Staphyloccaceae, Porphyromonadaceae, Enterobacteriaceae and Neisseriaceae) and chronic idiopathic rhinitis (Pasteurellaceae and Lactobacillaceae). In dogs with fungal rhinitis at cure, only 1 dog recovered a high relative abundance of Moraxellaceae.

Conclusions Results confirm major alterations of the nasal microbiota in dogs affected with fungal rhinitis and chronic idiopathic rhinitis, consisting mainly in a decrease of *Moraxella*. Besides, a specific dysbiotic profile further differentiated fungal rhinitis from chronic idiopathic rhinitis. In dogs with fungal rhinitis, whether the NM returns to its pre-infection state or progresses toward chronic idiopathic rhinitis or fungal rhinitis recurrence warrants further investigation.

Keywords Nasal cavity, Dog, Nasal, Microbiota, Aspergillosis, Fungal rhinitis, Mycotic rhinitis, Chronic idiopathic rhinitis

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Background

With advances in culture-independent technologies, the role of the upper respiratory tract microbiota in health and disease has become an intense area of research in human medicine [1–6] and to a much lesser extend in canine medicine [7–12].

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Fungal rhinitis secondary to infection with *Aspergillus fumigatus* is a common cause of nasal disease in dogs while it is uncommon in humans. Why young otherwise healthy dolichocephalic dogs have their nasal cavity and sinus invaded by the fungus is not yet totally understood, despite extensive investigation [13–15]. The host itself but also the fungus have been described as contributing to local dysimmunity. The host develops a Th1 type mucosal immune response along with the up-regulation of proinflammatory cytokines and chemokines [16, 17]. In return the fungus reacts with in-host adaptive genetic and phenotypic changes which could enable growth of the pathogen and contribute to the suppression of the local immune system [15, 18].

In human beings, reports over the past five years suggest that the microbiota can modulate the host immune response to invading fungal micro-organisms [19–21]. The microbiota is expected to influence immune homeostasis through host-to-microbe and microbe-to-microbe interactions [4, 22–24]. It has been shown, by correlating changes in metabolite profiles with microbiota metagenomic composition, that certain bacterial species contribute to host-fungal symbiosis and mucosal homeostasis in humans with *Aspergillus*-related lung disease [25]. Formation of sinonasal bacterial biofilms of multiple species (such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*) have been reported to damage epithelia sufficiently for the establishment of fungal biofilms [26] while the release of extracellular molecules by *P. aeruginosa* has been shown to stimulate the growth of *A. fumigatus* [27]. It can therefore be suspected that, in the nasal cavities of dogs with fungal rhinitis, the microbiota is able to influence the immunological response to fungi, the clinical fungal disease severity, as well as the response to treatment.

Chronic idiopathic rhinitis is a common heterogeneous disease characterized by lymphoplasmacytic to mixed inflammation of the sinonasal cavities without any identifiable cause [28]. In humans, disruption of the microbiota homeostasis has been described as being the primary driver or at least exacerbating factor for nasal chronic inflammatory diseases such as chronic idiopathic rhinitis [2, 6, 24, 29–32] and allergic rhinitis [2, 6]. In the pathophysiology of chronic idiopathic rhinitis in humans, two other interesting notions are the importance of bacterial biofilms [33] as well as the concept of keystone species maintaining a stable and healthy state by providing resistance to colonization by pathogens [31].

Altogether, this suggests that specific bacterial strains, as well as factors influencing the microbial composition and/or modulating microbial disturbances may be an untapped source of therapeutics to mitigate the severity of upper respiratory tract infections and/or inflammation. However, the role of nasal microbiota (NM) alterations in the pathophysiology of canine chronic nasal diseases has been very little studied since only one single study describes alterations of the NM in dogs with nasal neoplasia or chronic rhinitis [10].

So far, data relative to the NM in dogs with fungal nasal disease are not available. Therefore, whether bacterial dysbiosis exist in fungal rhinitis, and whether it may be one of the factors able to either initiate or entertain the local fungal development, or allow recurrence, is unknown. Likewise, all therapeutic protocols for fungal rhinitis described are based on attempts to eliminate the fungus [13, 34, 35] instead of targeting the relationship between the host and the fungus, and a possible approach based on NM modulation has not been considered yet.

For all these reasons, further knowledge concerning the alterations of the NM in canine chronic nasal diseases such as fungal rhinitis and chronic idiopathic rhinitis is needed. Improved understanding of the relationships between the microbiota, host responses and non-native microorganisms would help to develop future therapeutic approaches acting to prevent the invasion of pathogenic microorganisms. A first step is to characterize any specific dysbiosis associated with both chronic idiopathic rhinitis and fungal rhinitis. Therefore, the aim of this study was to describe and compare the NM in dolichocephalic dogs with fungal rhinitis (at diagnosis and cure in a subpopulation of dogs) and in dogs with chronic idiopathic rhinitis versus a control population of healthy dogs.

Results

Study population

Eighty-three client-owned dogs were recruited and divided into 3 groups: 29 in the control group, 40 dogs in the fungal rhinitis (FR) group and 14 in the chronic idiopathic rhinitis (CR) group (Table 1). A first batch (7 healthy, 9 FR and 8 CR dogs) was collected and sequenced in 2017 [36], a second batch (22 healthy dogs and 17 dogs with FR) in 2018 and finally a last group was analyzed in 2020 (6 dogs with CR and 14 dogs with FR among which 9 dogs were sampled twice: at diagnosis and at cure). The data of the three sequencing sets were gathered in one table and processed together in one unique table. Before this step the same swabs, DNA extraction sets and primers sets were used and samples were manipulated in the same lab by the same technician.

In the control group, breeds included were Labrador retriever ($n=4$), Belgian shepherd ($n=4$), Border collie ($n=4$), Australian shepherd ($n=4$), Beauceron ($n=3$), Golden retriever ($n=3$), Alaskan malamute ($n=2$), German shepherd ($n=1$),

Table 1 Characteristics of the groups according to the disease status

	Control group	CR group	FR group
Number	29	14	40
Age (years)	7 (0.8–11.3) [a]	9 (1–14.3) [a]	6.4 (1.2–14.3) [a]
Gender	12 males, 17 females	6 males, 8 females	27 males, 14 females
Weight (kg)	30 (14.8–47.8) [a]	24 (6–36) [a]	30.2 (3.7–55) [a]
Antibiotic treatment	/	1 (topical)	11 (systemic)
Antifungal treatment	/	/	10

FR: fungal rhinitis, *CR*: chronic idiopathic rhinitis

[a] Median (min–max)

Doberman ($n=1$), Dalmatian ($n=1$), White Swiss shepherd ($n=1$), and mixed-breed ($n=1$). Included dogs had a normal general examination and bloodwork and were not receiving any treatment within one month before sampling.

Breeds included in the FR group were Border collie ($n=5$), rottweiler ($n=3$), American Staffordshire terrier ($n=3$), mixed-breed ($n=3$), Labrador ($n=2$), Bull Terrier ($n=1$), Greater Swiss Mountain dog ($n=1$), Golden retriever ($n=11$), Cocker spaniel ($n=1$), Great Dane ($n=1$), Australian shepherd ($n=1$), giant poodle ($n=1$), Rhodesian ridgeback ($n=1$), Beauceron ($n=1$), Siberian husky ($n=1$), German shepherd ($n=1$), Dobermann ($n=1$), Jack Russel ($n=1$) and Dachshund ($n=1$). At the time of sampling, 11 dogs were receiving systemic antimicrobials (Table 1), 10 dogs were treated with oral antifungal drugs, 4 with non-steroidal anti-inflammatory drugs and 2 with steroids, within the 2 previous weeks.

Breeds included in the CR group were mixed-breed ($n=3$), Siberian husky ($n=2$), Jack Russel terrier ($n=2$), Dalmatian ($n=1$), American Staffordshire terrier ($n=1$), poodle ($n=1$), Bernese mountain dog ($n=1$), Barzoï ($n=1$), Dachshund ($n=1$) and Border collie ($n=1$). One dog was treated with topical antimicrobial therapy (thiamphenicol) and saline at the time of sampling (Table 1). All the other dogs did not receive anti-inflammatory or antimicrobial treatment for at least two weeks before sampling albeit this was not an exclusion criterion.

Nasal microbiota analysis

At the finest taxonomic level 4,887 operational taxonomic units (OTUs) were present throughout all samples. The Good's coverage of all samples was higher than 96% with median 99.3% (96.6%-99.9%) indicating that the sequencing depth was sufficient for reliable analysis of these nasal microbial community samples.

The distribution of age, sex and bodyweight according to disease status is reported in Table 1. Age did not differ significantly between groups ($p=0.15$).

Healthy dogs

The most common taxa at phylum level were Proteobacteria (mean relative percentage 54.1%, min 1.0%-max 99.9%), Firmicutes (15.5%, 0.1–96.8%), Tenericutes (8.7%, 0.0–81.5%) and Actinobacteria (7.8%, 0.0–83.7%), representing 97% of the bacterial population in this group (Table 2). Beside the family Moraxellaceae, three dogs had a high relative abundance (>50%) of Cardiobacteriaceae (phylum Proteobacteria) and two dogs a high relative abundance of an unclassified family of the Mollicutes class (phylum Tenericutes). Among the phylum Proteobacteria, the genus *Moraxella* represented the most abundant taxon with a mean relative percentage at 51.8%.

Dogs with fungal rhinitis at diagnosis and cure

At diagnosis, the most common taxa at phylum level were Firmicutes (mean relative percentage 35.1%, min 0.0%-max 99.8%) followed by Proteobacteria (34.2%, 0.1–98.6%) and Bacteroidetes (13.4, 0.0–60.4).

Out of the 40 dogs with FR, 9 were resampled at the time of cure. Median time to achieve cure in this subpopulation was 4.4 weeks (2.9–14). Six, 2 (dogs 5 and 6) and 1 (dog 2) dogs achieved cure after 1, 2 and 3 infusion protocols (Fig. 1).

The most common taxa at phylum level at the time of cure was equally distributed compared to diagnosis with Firmicutes (42.4%, 0.4–99.7%), Proteobacteria (27.0%, 0.1–99.3%) and Bacteroidetes (16.1%, 0.1–96.8%).

Dogs with chronic idiopathic rhinitis

At phylum level the most common taxa were Firmicutes (mean relative percentage 39.1%, min 0.1%-max 92.3%), Proteobacteria (30.3%, 0.7–75.4%), Bacteroidetes (13.4%, 0.3–40.7%) and Actinobacteria (12.5%, 0.0–97.8%).

Table 2 Bacterial groups at > 1% mean relative abundance among the control, FR and CR groups at phylum, family and genus level

Taxon	Control group ($n = 29$)				CR group ($n = 14$)				FR group ($n = 40$)			
Phylum Family *Genus*	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p < 0.05$)	Detected in n dogs	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p < 0.05$)	Detected in n dogs	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p < 0.05$)	Detected in n dogs
Proteobacteria	71.6%	29.3	A	29	30.3%	22.2	B***	14	34.2%	29.8	B***	42
Moraxellaceae	52.0%	39.5	A	29	5.4%	9.6	B***	13	6.3%	18.0	B***	36
Moraxella	51.8%	39.7	A	29	4.6%	8.7	B***	11	5.4%	18.0	B***	32
Neisseriaceae	0.4%	1.4	A	12	7.1%	9.9	AB	13	7.7%	11.6	B*	34
Conchiformibius	0.3%	1.4	A	8	4.4%	8.7	AB	12	6.7%	11.3	B*	28
Neisseria	0.0%	0.1	A	3	2.6%	6.2	B*	9	1.0%	2.3	AB	25
Enterobacteriaceae	0.9%	2.8	NS	15	3.6%	6.1	NS	13	9.2%	21.9	NS	36
Escherichia_Shigella	0.3%	1.1	NS	11	1.7%	2.8	NS	13	7.0%	18.1	NS	35
Proteus	0.0%	0.0	NS	1	1.4%	5.4	NS	2	0.8%	5.1	NS	7
Pasteurellaceae	0.1%	0.4	A***	11	10.5%	14.5	B	13	1.6%	2.5	A***	29
Pasteurella	0.0%	0.0	A**	1	6.0%	13.4	B	11	0.1%	0.3	A**	19
Pasteurellaceae_ge	0.1%	0.4	A**	6	3.9%	7.5	B	9	1.2%	2.3	A*	26
Cardiobacteriaceae	9.8%	23.9	A*	19	0.1%	0.2	AB	6	0.4%	1.6	B	15
Suttonella	9.8%	23.9	A*	19	0.0%	0.0	AB	2	0.4%	1.6	B	7
Pseudomonadaceae	6.1%	12.5	NS	26	0.6%	1.1	NS	13	6.2%	20.4	NS	31
Pseudomonas	6.1%	12.5	NS	25	0.6%	1.1	NS	13	6%	20.4	NS	30
Firmicutes	10.3%	17.3	A	29	39.1%	27.2	B**	14	35.1%	31.9	B**	41
Peptostreptococcaceae	0.0%	0.1	NS	5	0.6%	0.9	NS	10	1.0%	2.5	NS	31
Lactobacillaceae	1.6%	5.3	A	10	19.6%	23.9	B*	14	9.4%	18.4	AB	28
Lactobacillus	1.6%	5.3	A	10	19.6%	23.9	B*	14	9.4%	18.4	AB	28
Lachnospiraceae	0.1%	0.2	A	8	0.8%	1.3	AB	11	1.1%	1.7	B*	32
Bacillales_Family_XI	0.1%	0.7	A***	15	1.3%	1.6	B	11	0.3%	0.5	A***	28
Gemella	0.1%	0.7	A***	1	1.3%	1.6	B	9	0.3%	0.5	A***	14
Streptococcaceae	4.0%	12.4	A	22	7.7%	6.0	B**	14	5.1%	6.3	AB	35
Lactococcus	0.4%	1.3	A	6	5.3%	6.3	B**	14	2.5%	4.4	AB	27
Streptococcus	3.6%	12.7	NS	20	2.4%	2.7	NS	12	2.6%	4.3	NS	34
Staphylococcaceae	2.1%	4.4	NS	20	4.4%	7.6	NS	11	14.1%	29.4	NS	39
Staphylococcus	2.1%	4.4	NS	19	4.4%	7.6	NS	11	13.9%	29.3	NS	39
Bacteroidetes	2.0%	5.6	A	26	13.4%	11.7	B*	14	13.4%	14.8	B***	41
Porphyromonadaceae	0.7%	3.5	A	5	4.0%	7.3	AB	10	6.5%	12.5	B*	35
Porphyromonas	0.7%	3.5	A	5	4.0%	7.3	AB	10	6.5%	12.5	B*	35

Table 2 (continued)

Taxon	Control group ($n=29$)				CR group ($n=14$)				FR group ($n=40$)			
Phylum Family *Genus*	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p<0.05$)	Detected in n dogs	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p<0.05$)	Detected in n dogs	Mean rel. freq. (%)	SD (%)	Tukey's multiple comparisons test (corrected $p<0.05$)	Detected in n dogs
Bacteroidaceae	0.0%	0.1	NS	4	0.4%	0.5	NS	8	1.3%	3.9	NS	30
Bacteroides	0.0%	0.1	NS	5	0.4%	0.5	NS	8	1.3%	3.9	NS	30
Flavobacteriaceae	0.1%	0.4	**A**	10	4.7%	11.3	**B***	10	1.1%	1.8	**AB**	29
Capnocytophaga	0.0%	0.2	NS	3	3.2%	7.5	NS	9	0.2%	0.4	NS	22
Flavobacterium	0.0%	0.2	NS	9	1.4%	4.6	NS	8	0.8%	1.5	NS	22
Prevotellaceae	0.0%	0.1	NS	8	0.5%	0.8	NS	9	1.0%	2.7	NS	28
Weeksellaceae	1%	3.0	NS	6	3.3%	5.7	NS	8	2.2%	4.0	NS	19
Elizabethkingia	0.0%	0.0	NS	1	2.3%	4.7	NS	6	1.1%	2.6	NS	16
Actinobacteria	5.4%	9.7	NS	29	12.5%	23.8	NS	14	8.5%	9.9	NS	36
Corynebacteriaceae	0.5%	1.1	NS	21	9.1%	24.2	NS	12	1.5%	3.2	NS	25
Corynebacterium_1	0.2%	0.4	NS	13	2.2%	5.0	NS	8	1.1%	3.0	NS	22
Microbacteriaceae	2.3%	4.6	NS	21	0.6%	0.9	NS	10	0.9%	1.4	NS	27
Leucobacter	2.0%	4.4	NS	19	0.4%	0.9	NS	8	0.4%	1.1	NS	15
Micrococcaceae	1.6%	7.5	NS	19	0.7%	1.5	NS	11	1.1%	2.4	NS	26
Micrococcus	1.4%	7.3	NS	8	0.3%	1.0	NS	6	0.1%	0.4	NS	14
Propionibacteriaceae	0.3%	0.8	NS	14	0.6%	0.8	NS	12	1.9%	3.5	NS	27
Cutibacterium	0.3%	0.8	NS	12	0.5%	0.6	NS	9	1.7%	3.4	NS	25
Actinomycetaceae	0.1%	0.5	**A**	5	0.8%	1.5	**AB**	10	1.0%	1.8	**B***	26
Actinomyces	0.1%	0.5	NS	5	0.8%	1.5	NS	10	1.0%	1.8	NS	26
Tenericutes	9.7%	21.3	NS	18	2%	5.4	NS	10	1.9%	6.4	NS	29
Mollicutes_fa	9.5%	20.9	**A**	15	1.8%	4.8	**AB**	8	1.0%	6.2	**B***	5
Mollicutes_ge	9.5%	20.9	**A**	15	1.8%	4.8	**AB**	8	1.0%	6.2	**B***	3
Fusobacteria	0.1%	0.3	NS	6	1.6%	2.5	NS	11	4.2%	12.5	NS	34
Fusobacteriaceae	0.0%	0.2	NS	6	1.2%	1.9	NS	11	4.0%	12.5	NS	32
Fusobacterium	0.0%	0.2	NS	6	1.2%	1.9	NS	11	4.0%	12.5	NS	32

Mean relative percentages (mean rel. freq) and standard deviation (SD) of the most abundant bacterial groups, annotated to the level of phylum, family and genus, based on sequencing of the V1-V3 region of the 16S rRNA gene

A, B: operational taxonomic units not sharing a common letter differ significantly

FR fungal rhinitis, *CR* chronic idiopathic rhinitis

* $p<0.05$;

** $p<0.01$;

*** $p<0.001$; NS not significant

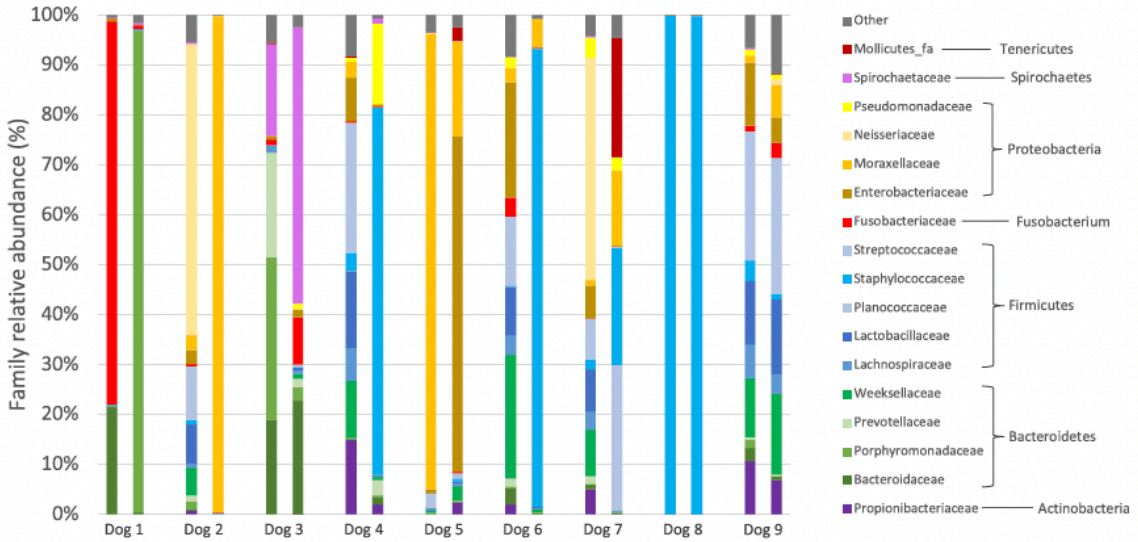

Fig. 1 Composition of nasal microbiota at family level in dogs with fungal rhinitis at diagnosis (first bar) and cure (second bar) (other: mean relative frequency < 1%)

Comparison between healthy dogs and dogs with chronic nasal diseases

The bacterial load quantified by 16S rRNA gene quantitative polymerase-chain reaction (qPCR) did not differ between the three groups.

Constrained ordination

Redundancy analysis (RDA) showed that only disease status ($p = 0.002$; Adjusted R [2] 0.142) contributed significantly to the variability of the microbiota (explaining 14.5% of the variance, Fig. 2).

Intrinsic diversity values and β-diversity

Good's coverage, species richness and α-diversity were significantly different between healthy dogs and dogs with chronic nasal diseases (Fig. 3). There was no difference in evenness. The non-metric multidimensional scaling graph of the β-diversity shows a clustering for the group of healthy dogs separating them from the diseased dogs (Fig. 4).

Differences in relative abundances: FR group versus control group

Mean relative abundances at phylum and family level are represented in Figs. 5 and 6. Table 2 shows the mean relative abundances of most abundant OTU, annotated to the levels of phylum, family and genus. The relative abundance in the Proteobacteria phylum was significantly lower in the FR group, compared with control dogs. This lower abundance in Proteobacteria was associated with a major and significant lower abundance in *Moraxella* (family Moraxellaceae) and *Suttonella* (family Cardiobacteriaceae) together with an increase of *Conchiformibius* (family Neisseriaceae). Other significant differences in the FR group compared with healthy dogs included an increase in the Firmicutes phylum with associated family Lachnospiraceae, an increase in the Bacteroidetes phylum with associated family *Porphyromonas*, an increase in Actinomycetaceae (phylum Actinobacteria), and finally a decrease in an unclassified genus of the Mollicutes class (phylum Tenericutes).

Differences in relative abundances: CR group versus control group

Similar alterations were also observed between the CR and control groups especially the decrease in Proteobacteria due to the decrease in *Moraxella* in contrast to an increase in the phyla Firmicutes and Bacteroidetes. Specific changes were also noted, such as an increase in the genera *Lactobacillus* (family Lactobaciliaceae) and *Lactococcus* (family Streptococcaceae) among the Firmicutes phylum. An increase in the genus *Neisseria* (family Neisseriaceae, phylum Proteobacteria) and the family Flavobacteriaceae (phylum Bacteroidetes) was also noted.

Differences in relative abundances: FR group versus CR group Between the FR and CR groups specifically, three significant differences were present: a higher relative abundance of *Pasteurella* and unclassified genus of the Pasteurellaceae family (both family Pasteurellaceae) as

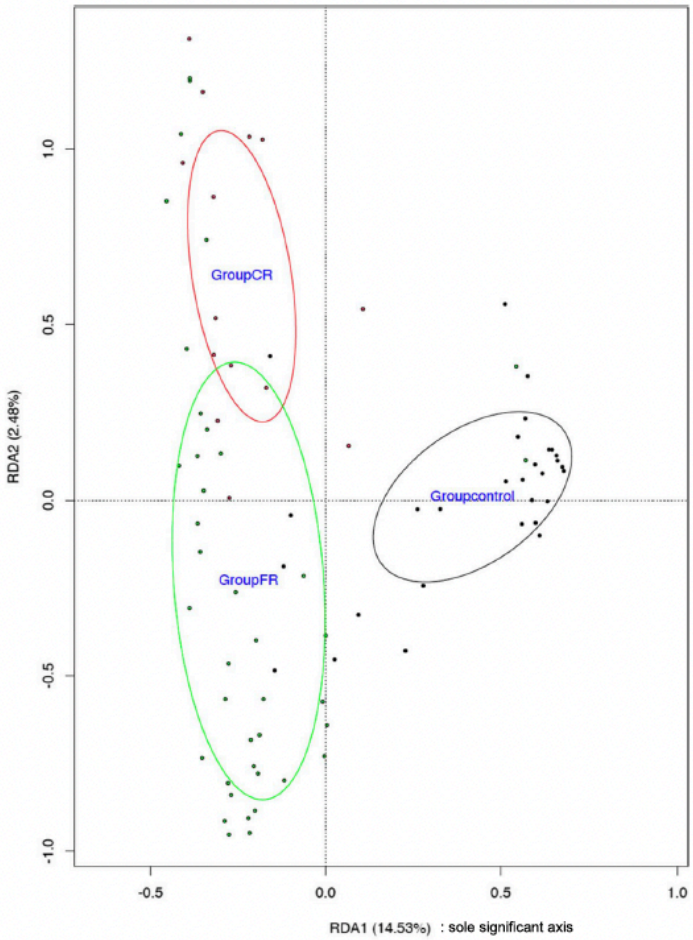

Fig. 2 Redundancy analysis at genus level for microbiota composition in dogs based on disease status. FR: fungal rhinitis, CR: chronic idiopathic rhinitis

well as *Gemella* (family Bacillales_Family_XI) in the CR group compared to the FR group.

Linear discriminant analysis effect size scores

In Fig. 7, LEfSe scores indicate bacterial taxa that were mainly present in the different groups of the study population and shows that the highest number of specific taxa are found in the FR group, followed by the CR and the control group.

Dogs with fungal rhinitis

Comparison between diagnosis and cure

The microbial composition of the 9 dogs at diagnosis and cure at family level are represented in Fig. 1. Only 1 dog (dog 2) recovered a high relative abundance of Moraxellaceae at cure. In 2 dogs (dogs 8 and 9) the NM was very similar to the one observed at diagnosis and in 5 dogs the microbiota was dominated (> 50%) by a single family: Porphyromonadaceae (dog 1), Spirochaetaceae (dog 3), Staphylococcaceae (dogs 4 and 6) and Enterobacteriaceae (dog 5). In the remaining dog (dog 7), a more heterogeneous composition was observed which was very different from its composition at diagnosis. No difference in bacterial load was observed between the two timepoints (Fig. 8). Among the intrinsic diversity values, only species evenness differed and was found to be lower at cure compared with the time of diagnosis (Fig. 8). No significant differences in relative abundances were found. NMDS plot did not show a specific pattern. Based on analysis of molecular variance (AMOVA; $p = 0.202$) and analysis of molecular variance homogeneity (HOMOVA;

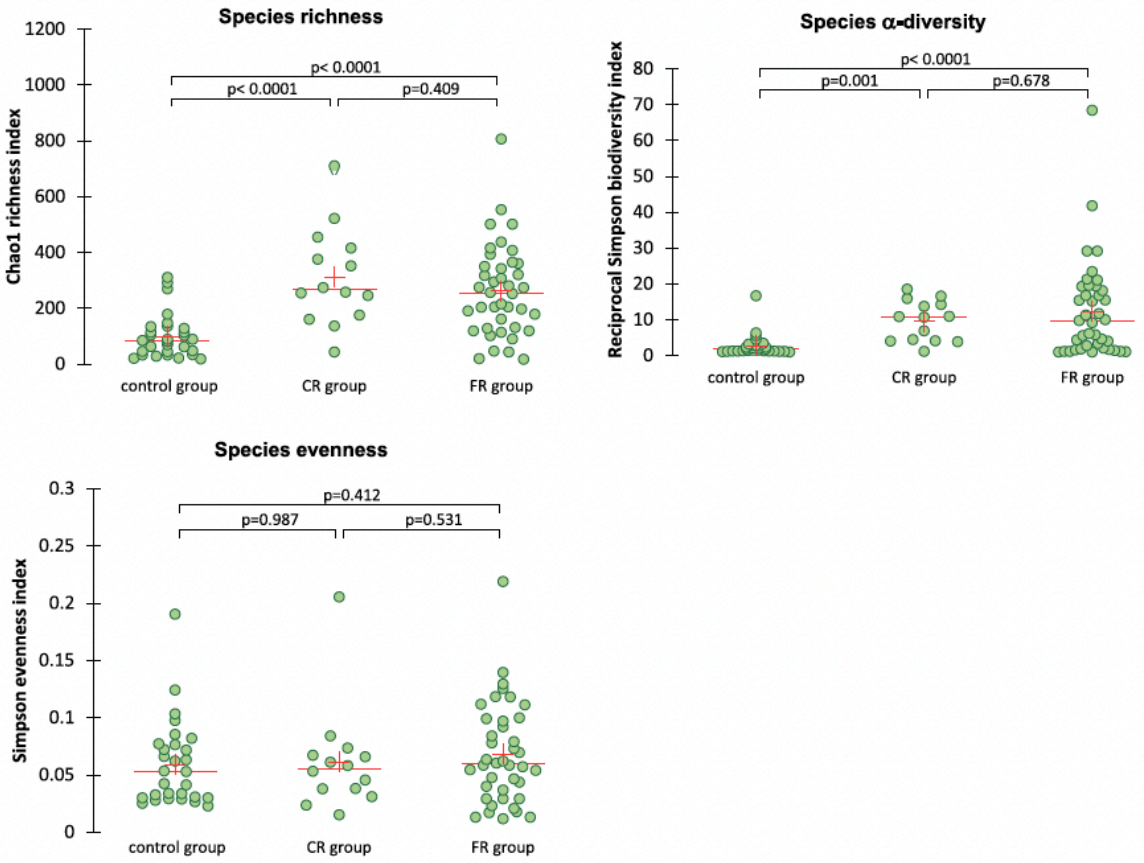

Fig. 3 Intrinsic diversity values comparing healthy dogs and dogs with chronic nasal diseases. FR: fungal rhinitis, CR: chronic idiopathic rhinitis

$p = 0.905$) beta-diversity and beta-dispersion were not different either.

Effect of treatment on the NM

Among dogs within the FR group, 11 were treated with systemic antimicrobials at the time of sampling while 29 dogs had not been receiving antimicrobials within at least the 2 previous weeks. Treated dogs were receiving amoxycillin clavulanic acid ($n = 7$), marbofloxacin ($n = 1$), marbofloxacin associated with azithromycin ($n = 1$), doxycycline ($n = 1$) or metronidazole ($n = 1$). Ten dogs were receiving an oral antifungal treatment (itraconazole, $n = 8$; ketoconazole, $n = 1$; or fluconazole, $n = 1$) at the time of sampling.

For these two types of treatments, there was no significant effect on the variance (redundancy analysis), there were no differences at the level of the intrinsic diversity values, β-diversity or relative abundances at family, genus or species level. There was also no difference in bacterial load between the dogs receiving and not receiving antimicrobial or antifungal treatment.

Discussion

The present study is the first to describe the NM in dogs with fungal rhinitis using next generation sequencing methods. Our data showed that both fungal rhinitis and chronic idiopathic rhinitis were associated with common major alterations of the NM. These alterations were characterized by a significant lower abundance in Proteobacteria, mainly due to a lower abundance in *Moraxella* while more minor differences were specific either to fungal rhinitis or chronic idiopathic rhinitis. In most dogs with cured fungal rhinitis, the NM was still different from what we consider a healthy profile. Neither antimicrobial nor antifungal treatment appears to have a significant effect on the NM in dogs with fungal rhinitis.

We showed that the NM in the healthy group was mostly dominated by the phylum Proteobacteria. This is

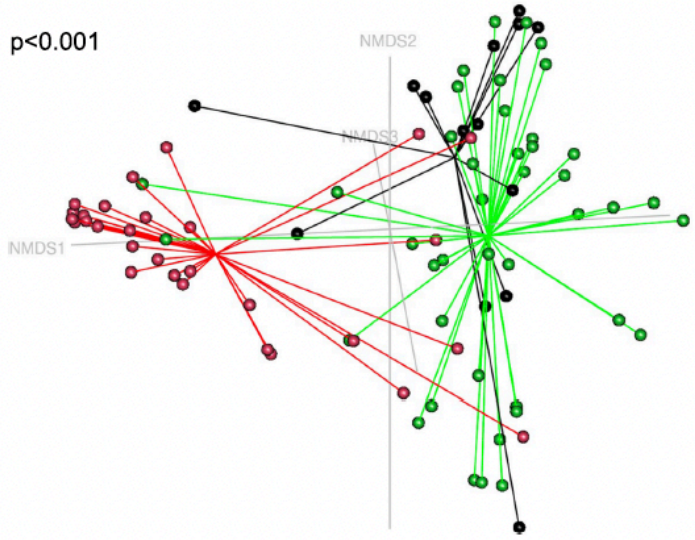

Fig. 4 Non-metric multidimensional scaling (NMDS) ordination of nasal microbiota communities using Bray–Curtis. Comparison between fungal rhinitis (FR) group (green), chronic idiopathic rhinitis (CR) group (black) and control group (red). CR group versus FR group $p = 0.054$, CR group versus control group $p < 0.001$, FR group versus control group $p < 0.001$. Stress value = 0.09800267

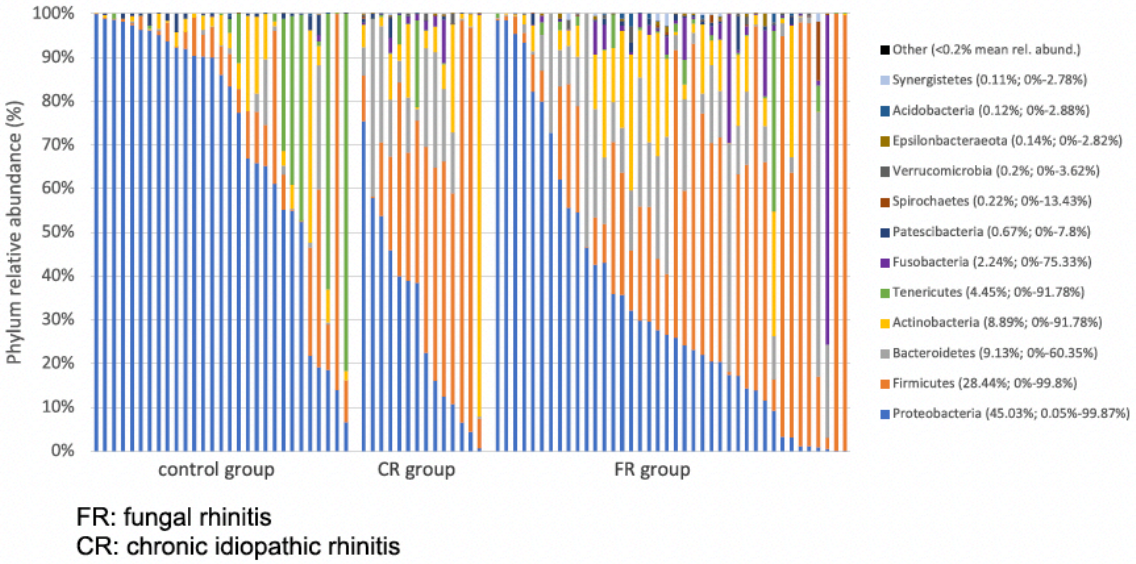

Fig. 5 Composition of nasal microbiota at phylum level in the study population. FR: fungal rhinitis. CR: chronic idiopathic rhinitis

in agreement with results of previous publications showing that the phyla Proteobacteria represents around 50 to 80% of the total bacterial population independently from age, breed or environment [8–10, 37]. Other common phyla detected in healthy dogs in this study included Firmicutes, Tenericutes, Actinobacteria, and Bacteroidetes. This is also similar to previous publications although their frequency order may vary according to the study [8–10, 37].

Like in previous studies, the Proteobacteria population was dominated by the family Moraxellaceae, and the genus *Moraxella*, followed by several other bacterial

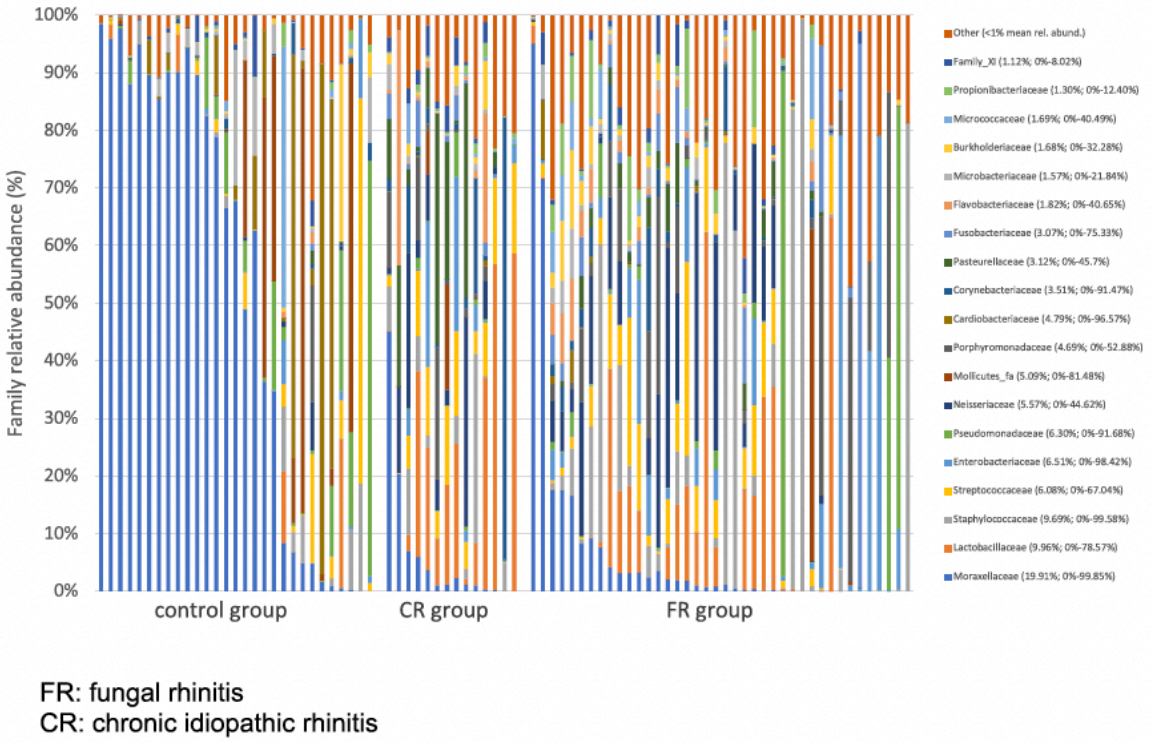

Fig. 6 Composition of nasal microbiota at family level in the study population. FR: fungal rhinitis. CR: chronic idiopathic rhinitis

families at considerably lower levels [8–10, 37]. However, not all healthy dogs had a microbial profile dominated by Moraxellaceae. Profiles dominated by Cardiobacteriaceae (phylum Proteobacteria), although in a much smaller amount, were also present. Cardiobacteriaceae was also one of the most frequently identified families in healthy dogs in previous studies [10]. Finally, some healthy dogs presented a more heterogenous profile, which was also the case in previous studies [10]. It can be hypothesized that, as it has been described in humans [1, 38], different healthy profiles exist, some of them being dominated by a particular bacterial taxon (e.g. Moraxellaceae, Cardiobacteriaceae) and others being more heterogeneous. The inter-individual variability of the nasal microbiota in healthy dogs has been described previously [9] and reflects the concept of a personalized microbiota [39, 40]. Moreover, the microbiota constantly undergoes changes of resident and transient microorganisms in response to internal and external factors [4, 12]. These factors may include the host and its local immune system, the inhaled particle-laden air, as well as atmospheric physical and chemical parameters. This is particularly true for the NM in dogs that interacts closely with the external environment which complicates the study of the NM in dogs. Based on the results of the present study and a previous study of the same group [12], facial conformation (particularly brachycephalic breeds) and disease status are two factors able to significantly influence the NM in dogs.

Both fungal rhinitis and chronic idiopathic rhinitis were associated with common major alterations of the resident nasal microbiota compared with healthy dogs. The most noticeable modification in both diseases was the marked lower relative abundance of the phylum Proteobacteria (±50% reduction) and the associated family Moraxellaceae and genus *Moraxella* (±90% reduction). Such a low relative abundance of *Moraxella* in dogs with chronic nasal diseases had already been described in the study by Tress and others [10] who compared the NM in healthy dogs to dogs with nasal neoplasia and chronic rhinitis. In children, nasopharyngeal *Moraxella*-dominated profiles have been described to be more stable and associated with a lower frequency of upper respiratory tract infections [38]. Altogether, these findings might propose *Moraxella* as a guarantor of nasal health. In dogs with chronic nasal disease, establishment of opportunistic species or overgrowth of some strains of the resident flora could overwhelm *Moraxella*, leading to a dysbiotic profile. It should be pointed out that a lower amount of *Moraxella* has also been observed in healthy brachycephalic dogs compared to other breed types [12], although to a much lesser degree compared

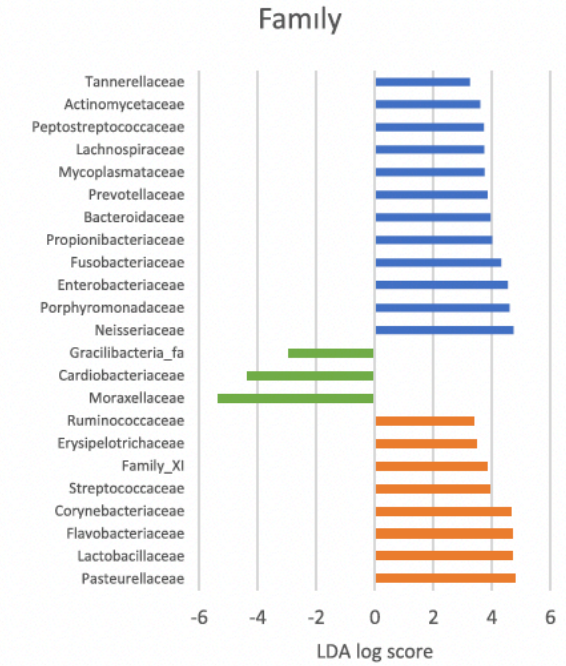

Fig. 7 Linear discriminant analysis (LDA) effect size (LEfSe) of Illumina sequencing datasets based on 16S rRNA gene sequences. Differentially abundant OTUs were detected ($p = 0.05$, LDA score > 3.0) at family level. FR group (blue), control dogs (green) and CR group (orange)

to the current data in dogs with nasal disease. This suggests that the relative abondance of *Moraxella* is at least partly dependent on facial conformation and/or air distribution strategy, rather than being exclusively associated with disease.

In dogs with nasal diseases, the inter-individual variability of the NM was obvious. This has also been described in humans with chronic rhinosinusitis and tend to complicate interpretation of results in microbiome studies [41]. Due to disruption of the normal protective mechanisms, it is expected that secondary bacterial infection can occur during nasal disease. The reasons why certain specific bacteria get the upper hand and develop is unknown. It is possible that local intranasal physical and chemical characteristics, personal nasal microbiota before the development of disease, host genetics, local host immunity and the type of nasal disease play a role.

The most noticeable alterations specific to dogs with fungal rhinitis included the higher abundance of genera such as *Staphylococcus*, *Conchiformibius*, *Escherichia_Shigella*, *Porphyromonas* and *Fusobacteria*, some taxa frequently reaching abundances of > 50% in individuals with fungal rhinitis. In healthy dogs, the same genera were also present but in small abundances, suggesting that fungal infection allows their particular development.

In dogs with CR, the most noticeable alterations were different, in particular with a higher abundance of *Pasteurella* and *Lactobacillus*, underlining the fact that FR and CR are two distinct diseases each causing unique alterations of the NM. Whether these types of dysbiosis are the consequences of the alterations associated with FR and CR or if they play an active role in the development of the disease remains to be determined.

In human beings, bacterial co-infections have been suggested to influence the development and persistence of clinical symptoms in patients with paranasal sinus *A. fumigatus* fungal balls [42, 43]. In a sheep model of sinusitis [26], inoculation of *A. fumigatus* resulted in the formation of a fungal biofilm only when co-inoculated with certain bacterial strains (*S. aureus*, *S. epidermidis*, *P. aeruginosa*). In the current study, in dogs with FR at diagnosis, genera such as *Staphylococcus* ($n = 5$), *Pseudomonas* ($n = 2$), *Porphyromonas* ($n = 3$), *Escherichia_Shigella* ($n = 3$), *Conchiformibius* ($n = 4$) and *Lactobacillus* ($n = 3$) represented the major part of the bacterial population (> 50%) in half (21/40) of the dogs, and might play an active role in the establishment, persistence and recurrence of fungal infection, either by causing epithelial inflammation and injury, or by metabolite cross-talk, and/or by modifying the immune response of the host to the fungus.

A longer follow-up in dogs with FR would allow to verify the association between these specific taxa and either resolution or recurrence of the fungal infection.

In dogs with fungal rhinitis at the time of cure, the NM was globally highly unpredictable. Amongst these 9 dogs, only one dog recovered a microbial composition with a high prevalence of Moraxellaceae. This dog needed 3 infusion protocols to reach cure, meaning he was the dog with the longest timeframe (3 months) between the collection of the two swabs. This may suggest that the NM needs more time to return to his "healthy state". Another possibility would be that in some individuals the NM returns to his pre-infection state while in others it continues to shift toward a new and different bacterial community, a scenario that has already been described in humans with chronic rhinosinusitis after sinus surgery [44].

The Pasteurellaceae and Lactobacillaceae were much more prominent in dogs with chronic idiopathic rhinitis in this study. An increase in Pasteurellaceae was earlier reported in dogs with chronic rhinitis and nasal neoplasia [10]. In the current study, 2 dogs were colonized with a high amount of *Pasteurella multocida*, which was absent in the nose of healthy dogs in the current study. This species is considered a primary pathogen in swine [45] but is also described as an opportunistic pathogen in human and veterinary medicine. The role

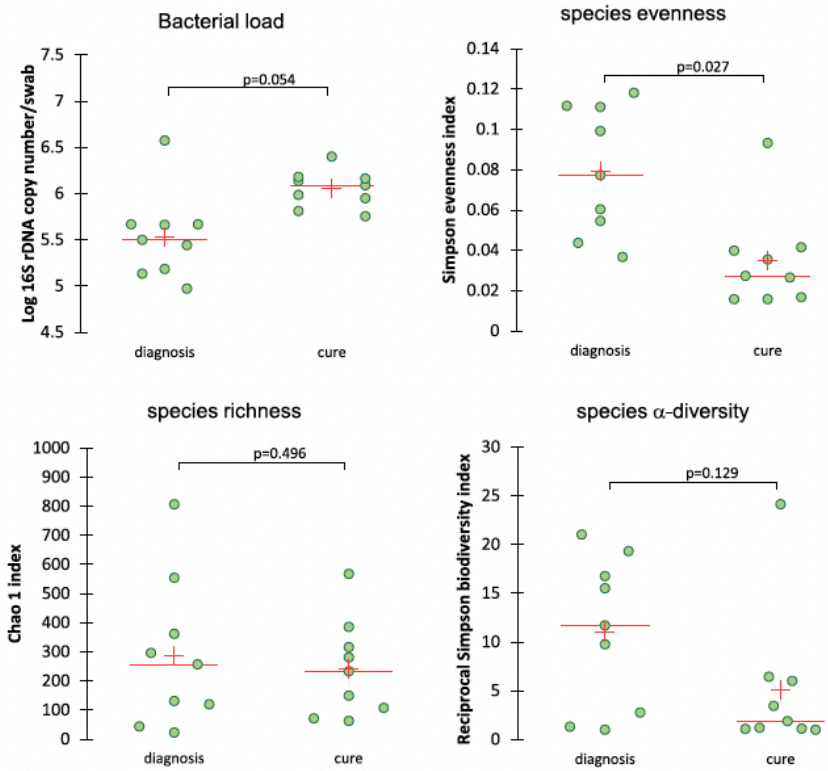

Fig. 8 Bacterial load and intrinsic diversity values in dogs with fungal rhinitis at diagnosis and cure

of *P. multocida* as a primary or opportunistic pathogen in dogs with chronic idiopathic rhinitis is currently unknown but deserves to be considered.

Lactobacilli are commensals of the gastrointestinal and female genital tract [46, 47] also used as probiotic strains [48, 49] or feed additives [50, 51] in dogs. However, it seems unlikely that Lactobacillus play a role in the pathogenesis of CR in dogs since they are uncommonly depicted as an opportunistic pathogen [52], and were not reported to be elevated in dogs with CR in the study by Tress and others [10] or in humans with chronic rhinosinusitis.

Results of the present study showed that systemic antimicrobials do not seem to significantly influence the NM in dogs with fungal rhinitis. In human beings with chronic rhinosinusitis, contradicting results have been published with variable effect on the diversity, evenness and bacterial burden [41, 44, 53–55]. Another study in dogs with nasal neoplasia also showed that pretreatment with antibiotics did not significantly alter the NM [10]. The lack of effect could be due to a small concentration of drug reaching the nasal mucosa or a high resilience [44] of the NM to short-term antibiotic treatments and makes the use systemic antimicrobial questionable in canine chronic nasal diseases.

The influence of age and bodyweight on the NM is unclear in dogs, based on previous studies this influence seems weak or absent [9, 10, 12]. Facial conformation however has been associated with significant changes of the NM in healthy dogs [12]. These changes were mostly present in dogs of brachycephalic breeds compared to other breed types, but minor variations were also observed between dolichocephalic and terrier breeds. Antibiotic pretreatment has also been reported to influence NM at varying degrees in humans and dogs [10, 41, 53–55] and the possible influence of antifungal treatment is unknown. For these reasons we decided to take age, bodyweight, breed type (meso-/dolichocephalic or terrier breed) and treatment status (antibiotic and antifungal) into account along with disease status (FR, CR or control group) for the RDA, as we believe these individual factors were the most likely to influence the variance in microbiota community composition. Sex and living environment (rural versus

industrial regions) were considered unlikely to influence the NM [9, 10, 12].

The present study is essentially descriptive. We did not measure local microenvironmental parameters such as intranasal pH, humidity or temperature. Neither did we determine viral or fungal populations and the host immune response, preventing interpretation of the NM in light of these parameters.

Another limitation concerns the size of the group, essentially the dogs with chronic idiopathic rhinitis, which is moreover a heterogeneous disease, of unclear and possibly variable etiology.

A long-term follow-up was not performed in dogs with fungal rhinitis to evaluate the evolution of the NM in the presence or absence of relapse or recurrence of the disease.

And finally, the present study was not designed to assess the effect of antimicrobial treatment: the molecules and duration of treatment were not standardized and the number of dogs in the treated group was small. This could possibly explain why we failed to show statistical differences between treated and non-treated groups.

In conclusion, in dogs with chronic nasal diseases such as FR and CR, major alterations are present compared to healthy dogs while more subtle but significant differences might distinguish both diseases. Most dogs with fungal rhinitis probably did not recover their core microbiota at cure. The NM in dogs with fungal rhinitis at cure was unpredictable and a longer follow-up is needed to draw a conclusion. The present study lays the first groundwork to the realization and comprehension of the complex interactions between the nasal microbiota and nasal *Aspergillus fumigatus* infection in dogs. Further studies are warranted to discover if modulation of the nasal microbiota might be an interesting perspective for the treatment of this disease.

Methods

Study sample

Client-owned dogs with a diagnosis of fungal rhinitis (FR group) or chronic idiopathic rhinitis (CR group) were prospectively recruited. The CR group was mainly included in order to differentiate changes in NM due to the presence of inflammation from changes in NM specifically associated with fungal rhinitis.

A control group of healthy dolichocephalic dogs, age and breed matched with the FR group, was also recruited. Part of the dogs with fungal rhinitis that were treated and cured were examined at checkup (cured FR group).

Diagnosis of fungal rhinitis was based on the presence of compatible clinical signs and per-endoscopic identification of fungal plaques with turbinate destruction. Additional diagnostic procedures consisted of computed tomography of the head, histopathology and fungal culture or polymerase chain reaction (PCR). All dogs were treated with endoscopic debridement of the fungal plaques followed by a 15-min enilconazole infusion protocol [56]. Control rhinoscopy was performed 3 to 6 weeks after treatment. Cure was based on resolution of clinical signs and absence of fungal plaques. As a non-negligible amount of the dogs included in the FR group were treated with antimicrobial and/or antifungal treatment at the time of sampling, the potential effect of these treatments on the NM was also investigated.

Diagnosis of chronic idiopathic rhinitis was based on compatible clinical signs, endoscopic and/or histopathologic lesions. Other nasal diseases such as fungal rhinitis, neoplasia, oronasal defect or foreign body were excluded based on different examinations including endoscopy, computed tomography of the head, histopathology, culture or PCR results from nasal samples.

All healthy dogs were exempt of clinical signs and had a normal clinical examination and blood work.

In all dogs, questions were asked concerning ongoing local or systemic medical treatment. Except for dogs in the control group, the presence of antimicrobial or anti-inflammatory treatment before or at the time of sampling was not an exclusion criterion.

Sample collection

This study was approved by the animal ethical committee (N° 16–1854, 27/10/2016) and all samples were obtained with the consent of the owners.

For sample collection, dogs were premedicated with a combination of butorphanol (Butomidor®, Richter Pharma) and medetomidine (Medetor®, CP-Pharma) intravenously. Propofol (Propovet®, Zoetis) on demand was used for induction. Under general anesthesia, to prevent sample contamination, a sterile speculum was inserted into the nare to allow the passage of a sterile swab (Copan®, FLOQSwabs™, 553C, Brescia, Italy) in the distal third of the nasal cavity. Sample collection was performed either by EV, FB or CC. In case of unilateral fungal rhinitis, the affected nasal cavity was sampled. In diseased dogs, sample collection was performed before rhinoscopy. The nasal mucosa was brushed using three careful circular movements before withdrawal of the swab through the speculum. The tip of the saw was cut and stored in a sterile cryotube and banked at -80 °C until further analyses.

DNA extraction and high throughput sequencing

Based on the manufacturer's instructions, total bacterial DNA was extracted from the nasal swabs with the DNEasy Blood and Tissue kit (QIAGEN Benelux BV; Antwerp, Belgium). Spectrophotometry (NanoDrop

ND-1000, Isogen, De Meern, The Netherlands) was used for total DNA concentration measurement and purity evaluation.

After DNA extraction from samples, quantification of the bacterial load was performed with a quantitative real-time PCR targeting the V2-V3 region of the 16S rRNA gene with the following primers: forward (5'-ACT CCTACGGGAGGCAGCAG-3') and reverse (5'-ATT ACCGCGGCTGCTGG-3') as previously described [57]. The standard curve was based upon tenfold dilution of a quantified PCR product. This PCR product was purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Leiden, The Netherlands), quantified with PicoGreen targeting double-stranded DNA (Promega).

For bacterial identification, bacterial 16S rRNA gene amplicons were generated via amplification of the V1-V3 hypervariable regions of the 16S rRNA gene using the following primers: forward (5'-GAGAGTTTGATY MTGGCTCAG-3') and reverse (5'-ACCGCGGCTGCT GGCAC-3') and Illumina overhand adapters. The DNA was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. A final quantification, performed by quantitative PCR, of each sample in the library was performed using the KAPA SYBR" FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run.

Amplicon profiling analysis

Alignment and clustering were done with MOTHUR software package (v1.41.0) with an OTU clustering distance of 0.03 and based on the SILVA database (V1.32) of full-length 16S rRNA gene sequences. Vsearch algorithm was used for chimera detection [58]. After the chimera removal, reads corresponding to chloroplastic and mitochondrial 16S rRNA genes and reads whose taxonomic assignation fall outside the bacterial kingdom are removed during the cleaning process. From 16,220,278 raw reads, we obtained 14,714,808 reads after cleaning (length and sequence quality). Finally, we retained 6000 reads (median 5999 reads per sample) to adjust for uneven sequencing depth across samples. All biosample raw reads were deposited at the National Center for Biotechnology Information (NCBI) and are available under de Bioproject ID PRJNA841569.

Alpha- and beta diversity

Subsample data sets including bacterial richness, evenness and α-diversity were obtained with MOTHUR at species level using the Chao1 index, Simpson index-based measure and the inverse Simpson's index respectively. Beta-diversity at species level was assessed with MOTHUR using a dissimilarity matrix of Bray–Curtis. Non-metric multidimensional scaling plots for visual assessment were performed based on a Bray–Curtis dissimilarity matrix at species level with Rstudio (R v1.2.5033 package vegan v2.5–6 and ggplot2 v3.3.0) to represent the β-diversity between groups (FR versus CR versus control group, diagnosis versus cure within the FR group, treated versus non-treated dogs within the FR group).

Statistical analysis

Redundancy analysis

A RDA on values at genus level was performed to evaluate the relationships between the NM and the different potential explanatory variables (age, bodyweight, breed type, disease status and, among the FR group, antimicrobial or antifungal treatment status) that could influence/shape it. Forward selection was conducted to select significant variables using the "ordiR2step" function (with adjusted R2 coefficient) from the vegan package [59].

Relative abundances of taxa

A Kruskal–Wallis test with Benjamini-Hotchberg FDR correction followed by Tukey's multiple comparison test in STAMP (v2.1.3) were used to identify differences in relative abundance at phylum, family, genus and species level (FR versus CR versus control group and treated versus non-treated dogs within the FR group).

Alpha- and beta diversity

Bacterial richness, evenness, α-diversity, good's.coverage index and bacterial load were compared between the three groups (FR versus CR versus control group and treated versus non-treated dogs within the FR group) using a Kruskal–Wallis test and Dunn post-hoc test with Bonferroni correction or a Wilcoxon rank test for paired samples (diagnosis versus cure within the FR group). These analyses were performed using XLstat (2020.5.1, Addinsoft, Paris, France). Differences were considered significant for a p-value < 0.05.

Beta-diversity was estimated with AMOVA (analysis of molecular variance; 10,000 iterations) and beta-dispersion was assessed with HOMOVA (analysis of molecular variance homogeneity; 10,000 iterations).

Linear discriminant analysis (LDA) effect size (LEfSe) score

LEfSe was performed to detect differences in bacterial composition between groups (FR versus CR versus control group) at phylum, family, genus and species level with MOTHUR (significant for an LDA score > 3.0 [60].

Abbreviations

NM	Nasal microbiota
FR	Fungal rhinosinusitis
CR	Chronic idiopathic rhinitis
OTU	Operational taxonomic unit
rRNA	Ribosomal ribonucleic acid
DNA	Deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RDA	Redundancy analysis
LEFSe	Linear discriminant analysis effect size
NMDS	Non-metric multidimensional scaling
AMOVA	Analysis of molecular variance
HOMOVA	Homogeneity of molecular variance
LDA	Linear discriminant analysis

Acknowledgements
The authors would like to thank Albert Belinda, Phan Kim-Thu and Romijn Sylvain for their help in samples collection and storage.

Authors' contributions
EV and CC conceived and designed the study. EV, AF, FB, BT, GD and CC participated in data acquisition for the study. EV, AF and BT analyzed the data. EV, AF and BT performed the statistical analyses. EV and CC interpreted the results. EV and CC wrote the manuscript and all the authors read and approved the final version of the manuscript.

Funding
The study was conducted with personal funding.

Availability of data and materials
All sample raw reads associated with this study have been deposited at the National Center for Biotechnology Information (NCBI) under the accession number PRJNA841569.

Declarations

Ethics approval and consent to participate
Samples from healthy domestic dogs were collected according to a protocol approved by the Ethical Committee of the University of Liège (protocol #1854) and with the informed consent of the owners. All methods were carried out and reported in accordance with relevant guidelines and regulations (including ARRIVE guidelines).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 28 May 2022 Accepted: 17 March 2023
Published online: 15 April 2023

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Discussion – Perspectives - Conclusion

Discussion

The **first objective** of this work was to compare the results of paired nasal samples analyzed by conventional bacterial culture and by 16S rRNA gene amplicon sequencing. Primary nasal bacterial infections are uncommon in dogs ; however, secondary bacterial colonization can occur in the context of underlying infectious, inflammatory, or neoplastic nasal diseases (Windsor and Johnson, 2006; Peeters and Clercx, 2007). In clinical practice, dogs referred for rhinoscopic evaluation with acute or chronic nasal discharge are frequently receiving antimicrobial therapy, either systemic or local, sometimes empirically, and other times based on the results of a nasal swab culture (Henry et al., 2026). Nevertheless, the clinical relevance and diagnostic value of this test, as well as the appropriateness of antibiotic use in such cases, remain questionable. Several practical questions arise when managing these patients, particularly regarding the utility of nasal culture to guide treatment. Study 1 was designed to address these issues and to assess whether conventional culture methods reliably reflect the nasal bacterial communities identified by high-throughput sequencing.

- *Are clinical suspicions of secondary bacterial rhinitis reliable?* In most cases, such infections are presumed based on the clinical history and the appearance or nature of the nasal discharge, typically mucopurulent, especially when occurring in a patient with a pre-existing chronic nasal condition. For instance, a sudden worsening of clinical signs in a dog with chronic idiopathic rhinitis, accompanied by purulent secretions, is often interpreted as indicative of a bacterial overgrowth or dysbiosis. However, mucopurulent nasal discharge is not specific to bacterial infections and may simply reflect a flare-up of the underlying disease (Lobetti, 2014). In reality, distinguishing between true secondary bacterial infection and non-infectious exacerbation remains challenging. In study 1, two dogs were clinically suspected to have bacterial infection, yet their nasal microbial profiles obtained through 16S rRNA gene sequencing were not consistent with this hypothesis. In all other dogs, the microbial profile was more consistent with a dysbiotic pattern. These findings suggest that misclassification of dogs as bacterially infected may occur in clinical practice, potentially leading to inappropriate use of antimicrobial therapy.

- *How reliable are nasal swab culture results compared with 16S rRNA gene sequencing in dogs with chronic nasal disease?* The advent of DNA sequencing has improved our understanding of the composition and dynamics of bacterial communities, both commensal and opportunistic (Rodrigues Hoffmann et al., 2014; Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017).

Nevertheless, such advanced techniques remain largely inaccessible for routine diagnostic purposes. It is therefore important to assess the degree of correlation between results obtained from conventional nasal swab cultures and those from sequencing. Findings from study 1 revealed good agreement (among the most abundant sequenced taxa, all taxa with a relative abundance >10%, the one taxon with the highest relative abundance was detected by culture) between the two methods in approximately one-third of cases, moderate agreement (among the most abundant sequenced taxa, the one taxon with the highest relative abundance was not detected by culture, but other lower abundance taxa were detected by culture) in another third, and poor agreement (none of the most abundant sequenced taxa were identified by culture while at least one of them was expected to grow on the selected media) in the remaining third. Consequently, it is challenging to accurately characterize the nasal microbiota based solely on culture results, limiting the extrapolation of meaningful microbiological insights from culture alone.

- Can nasal swab culture be reliably used to guide antimicrobial therapy in dogs with suspected secondary bacterial rhinitis? In chronic nasal conditions such as chronic idiopathic rhinitis or nasal neoplasia, where complete resolution of the underlying disease is often not achievable, antimicrobial therapy may be warranted in selected cases (Windsor and Johnson, 2006; Kaczmar et al., 2018). However, results from study 1 indicate that culture findings alone may be insufficient to guide optimal antibiotic choice because the identification, through sequencing, of dominant or clinically relevant bacterial species that require specific growth conditions is not uncommon. Additionally, a positive culture result, even when characterized by pure and abundant growth, does not necessarily indicate that the isolated colony represents a dominant or major taxon within the nasal microbiota. In a recent study comparing nasal culture results from different sampling sites (Niedenführ et al., 2024), culture results were site-specific. Consequently, different antimicrobial treatments may have be selected depending on the site of culture, which is why they concluded that nasal bacterial cultures should not be recommended as a routine diagnostic measure. When antimicrobial therapy is needed, a pragmatic strategy might involve initiating topical therapy directed against the bacterial genera most frequently associated with bacterial rhinitis, such as *Enterobacteriaceae* and *Staphylococcus* (thiamphenicol topical solution, ciprofloxacin drops, tobramycin drops, …). The role of saline irrigation also needs to be considered; by physically clearing biofilms, excess mucus, and debris, it may reduce opportunistic species and promote a restoration of the commensal microbiota. In the absence of clinical improvement within 72 hours, additional diagnostic procedures should be considered, including aerobic and anaerobic cultures as well as *Mycoplasma* PCR, to better characterize the infectious process. Where available, DNA

sequencing could provide further refinement in identifying the bacterial populations involved and inform targeted therapeutic decisions.

- What is the role of systemic antimicrobial therapy in the management of secondary bacterial rhinitis in dogs ? At present, there is a lack of studies specifically evaluating the impact of oral antibiotic therapy on the nasal bacterial community, making it challenging to determine the true efficacy of systemic antimicrobial interventions. In study 1, the bacterial load, intrinsic diversity values, beta-diversity and beta-dispersion did not significantly differ between dogs treated with antimicrobials, ongoing or recent, compared to untreated dogs. However, that study has not been specifically designed to answer this question. In human beings, the available data suggest that systemic antibiotics exert at most a modest and variable influence on the sinonasal bacterial community and do not confer clear clinical benefit in CRS (Head et al., 2016, Lux et al., 2020, Siu et al., 2021). Topical antimicrobial strategies have gained increasing attention in recent years (Lim et al., 2008, Ramakrishnan et al., 2018, Miyake and Bleier, 2019), which raises the possibility that topical therapy could represent a preferable alternative in veterinary medicine as well. Importantly, the overuse of antibiotics carries the risk of fostering antimicrobial resistance, highlighting the need for prudent and evidence-based prescribing. Further research directly comparing the efficacy of systemic and topical antimicrobial approaches in canine patients with secondary bacterial rhinitis is warranted to establish optimal treatment guidelines. Importantly, clinicians should also bear in mind that secondary bacterial rhinitis represents a consequence rather than the primary cause of the underlying nasal disease. Consequently, antimicrobial therapy alone is unlikely to provide sustained clinical improvement unless the underlying disorder is identified and appropriately managed.

The **second objective** of this work was to expand current knowledge of the healthy canine nasal microbiota, with a particular focus on dogs predisposed to fungal rhinosinusitis, and to evaluate the influence of intrinsic and extrinsic factors on its composition. At the outset of this study, information on the nasal microbiota of healthy dogs was scarce (Rodrigues Hoffmann et al., 2014; Ericsson et al., 2016; Isaiah et al., 2017; Tress et al., 2017), despite the fact that a precise understanding of the baseline microbial community is essential for detecting disease-associated alterations. Defining the core microbiota allows the identification of microbial species or taxa consistently shared among individuals, which are likely to play a key role in maintaining the stability and functionality of the nasal microbial ecosystem. Equally important was the evaluation of potential intrinsic and extrinsic confounding factors, such as age, sex, skull conformation,

environment, that could influence the nasal microbiota independently of disease status. The identification of these confounding factors is crucial for identifying the variables that must be considered and controlled in future microbiota studies.

The results from healthy non-brachycephalic dogs in study 2 and study 3 demonstrated that the core nasal microbiota is predominantly composed of Proteobacteria, representing more than 70% of relative abundance (RA). Within this phylum, the genus *Moraxella* was consistently the most abundant. However, not all healthy dogs exhibited a *Moraxella*-dominated profile (defined as >50% RA). In a smaller subset of individuals, the nasal microbiota was dominated by other genera, such as *Suttonella*, *Pseudomonas*, or *Lactobacillus*. These patterns were observed in both study 2 and study 3, as well as in previous research (Tress et al., 2017; Isaiah et al., 2017). Additionally, some healthy dogs displayed a more heterogeneous composition, with no single taxon exceeding 50% RA, a finding also reported in earlier studies (Tress et al., 2017). Together, these findings indicate a general dominance of *Moraxella* in the healthy canine nasal microbiota. However, they also underscore the existence of considerable inter-individual variability, represented by multiple distinct community profiles that are all associated with health.

The extent to which microbial profiles, or components thereof, remain stable within an individual dog over its lifetime (intra-individual variability) remains unknown. It can be hypothesized, however, that certain intrinsic and extrinsic factors may influence both resident and transient microbial populations, as has been described in humans (Kumpitsch et al., 2019). Such factors may include host-related elements, particularly the local immune system, as well as inhaled air containing particulate matter and the physical or chemical characteristics of the surrounding atmosphere. This consideration is particularly relevant in dogs, whose pronounced sniffing behavior increases the interaction between the nasal cavity and the external environment.

In study 2, neither living environment (rural vs. urban) nor sex was found to significantly influence the nasal microbiota. Nevertheless, based on previous studies, this absence of a detectable effect does not necessarily contradict the hypothesis that environmental factors may influence microbial composition, but rather suggests that such effects may depend on the scale and magnitude of environmental differences. Indeed, all dogs in study 2 lived in Belgium, a relatively small country with limited environmental variation. Consequently, the contrast between “rural” and “urban” environments may not have been sufficient to generate detectable differences in nasal microbial communities. In constrast, one previous study (Isaiah et al., 2017) reported differences in microbial composition between dogs residing in Alabama and California, two regions with markedly

different climates and separated by considerable distance. Similarly, Ericsson et al. (2016) described nasal microbiota profiles dominated either by *Mycoplasmataceae* or *Moraxellaceae* in almost equal proportions within a cohort of research animals. In contrast, *Mycoplasmataceae*-dominated profiles were not identified in healthy client-owned dogs in study 2 and study 3. The specific genetic background, housing conditions, and controlled environment of the research dogs included in the study by Ericsson et al. (2016) may therefore have contributed to shaping their nasal microbiota differently from that of client-owned dogs living under more heterogeneous household conditions in Belgium.

Age-related influences on the nasal microbiota have been demonstrated in human beings (Bomar et al., 2018) and suggested in cats (Dorn et al., 2017). In contrast, in both study 2 and study 3, age was not significantly associated with differences in the nasal microbiota of dogs. This lack of association may reflect species-specific differences in lifespan and developmental stages compared with humans. Notably, very young dogs were not included in either study, leaving open the possibility that substantial microbial changes occur during the first year of life in this species.

Interestingly, in study 2, brachycephalic dogs exhibited marked differences in nasal microbiota composition compared with non-brachycephalic dogs. These differences are hypothesized to result from selection pressures exerted by other taxa (e.g., those colonizing the nares or introduced via oropharyngeal reflux) and/or from local microenvironmental variations linked to differences in airflow distribution (Hostnik et al., 2017). Given that brachycephalic breeds are not predisposed to chronic nasal diseases, they were excluded from study 3. Nevertheless, these findings should be considered when designing and interpreting future studies on the canine nasal microbiota. The unique microbial composition identified in brachycephalic dogs, characterized by reduced bacterial diversity and a distinct community structure compared to mesocephalic and dolichocephalic breeds, may contribute to their apparent resistance to chronic nasal diseases. Anatomical differences, including shortened nasal cavities, altered airflow dynamics, and increased oropharyngeal reflux, likely create a microenvironment less favorable to colonization by opportunistic nasal pathogens such as *Aspergillus fumigatus*. This raises the intriguing possibility that some of the protective features of the brachycephalic nasal microbiota could be harnessed therapeutically. Future research exploring whether microbiota transplantation from brachycephalic dogs to breeds predisposed to chronic nasal diseases could modulate nasal microbial communities and improve disease resistance may represent an innovative approach.

As expected, the composition of the canine nasal microbiota differs markedly from that reported in humans, likely reflecting substantial interspecies differences in anatomy, lifestyle, and environmental exposures. However, notable similarities can be observed with the nasal microbiota of outdoor cats, such as the high relative abundance of *Moraxella* (Dorn et al., 2017), a finding consistent with the closer anatomical and lifestyle characteristics shared between dogs and free-ranging felines.

The **third objective** of this work was to investigate whether a distinct dysbiosis of the nasal microbiota is associated with fungal rhinosinusitis, in comparison with healthy dogs and those with chronic idiopathic rhinitis. To our knowledge, study 3 provides the first characterization of the nasal microbiota in dogs with fungal rhinosinusitis using DNA sequencing. Dogs with chronic nasal diseases exhibited consistent alterations in their nasal microbiota compared to healthy controls, evidenced by increased species richness and α-diversity, as well as distinct clustering patterns on NMDS plots. We hypothesize that, in these diseases, local inflammation, infection, and/or turbinate destruction led to substantial microenvironmental changes within the nasal cavities. These alterations may disrupt normal anatomy, airflow patterns, and local immune defenses, thereby creating conditions that favor the overgrowth of certain resident bacterial strains or the establishment of opportunistic species.

Taken together, the findings from study 2 and study 3 indicate that both facial conformation, particularly brachycephalic morphology, and disease status are significant determinants of the canine nasal microbiota. These factors should be carefully considered in the design and interpretation of future research on the nasal microbiome in dogs.

The alterations of the nasal microbiota in dogs with chronic nasal diseases were characterized by a marked reduction in the relative abundance of Proteobacteria, predominantly driven by a decrease in *Moraxella*. A similar reduction in *Moraxella* has been reported in a study comparing dogs with chronic idiopathic rhinitis and nasal neoplasia to healthy controls using DNA sequencing (Tress et al., 2017). Collectively, these findings support the hypothesis that *Moraxella* may act as a keystone taxon, serving as a guardian or indicator of canine nasal health. One plausible mechanism is that, in chronic nasal disease, impaired mucosal defense mechanisms permit the colonization and proliferation of other bacterial taxa, which in turn outcompete *Moraxella* and disrupt its ability to persist within the nasal environment.

Although fungal rhinosinusitis and chronic idiopathic rhinitis were both associated with major disruptions of the resident nasal microbiota, our results also suggest that the microbial profiles of these two diseases are distinct. Each condition was associated with specific alterations. In dogs with fungal rhinosinusitis, taxa such as *Porphyromonas*, *Enterobacteriaceae*, and *Staphylococcus* were detected more frequently and at higher relative abundances. While these taxa were also present in healthy dogs, they occurred only in low abundance, suggesting that the local changes induced by fungal infection, such as inflammation and tissue damage, may create a favorable environment for their proliferation. Notably, in study 1, which compared culture-dependent and culture-independent analyses of nasal swabs, these same genera were also enriched in dogs with fungal rhinosinusitis, supporting the repeatability of these findings. In study 3, even after successful treatment of fungal rhinosinusitis, five of the nine dogs continued to exhibit high relative abundances of these taxa. This raises important questions regarding their persistence: do these organisms gradually return to baseline following resolution of infection, or might they contribute to the recurrence of clinical signs or disease relapse? Long-term follow-up studies will be essential to clarify their role in post-treatment nasal microbiota dynamics.

In dogs with chronic idiopathic rhinitis in study 3, an increase in *Pasteurella* was particularly marked. In study 1, *Pasteurella* spp. ranked as the first or second most abundant taxon in 5 of the 29 included dogs, all of which were affected by chronic idiopathic rhinitis. A similar enrichment of *Pasteurellaceae* has been previously reported in dogs with chronic idiopathic rhinitis and nasal neoplasia (Tress et al., 2017). In both study 3 and study 1, *Pasteurella multocida* and *Pasteurella canis* were among the detected species. These organisms are absent from the core nasal microbiota of healthy dogs, as defined by the results of study 2 and study 3. Their role in chronic idiopathic rhinitis remains unclear; however, it warrants further investigation. *Pasteurella multocida* possesses a variety of virulence factors and is recognized as the causative agent of atrophic rhinitis in swine (Horiguchi, 2012). *Pasteurella canis*, typically an inhabitant of the canine oral cavity, is considered an opportunistic pathogen in both humans and animals.

Future Perspectives

Future research directions

Several promising avenues warrant further exploration to advance our understanding and management of canine nasal diseases in relation to the microbiota.

Since the major limitation in respiratory microbiota research, both in veterinary and human medicine, lies in methodological heterogeneity. Variations in sampling depth, swab type, DNA extraction kits, targeted 16S rRNA hypervariable regions, sequencing platforms, and bioinformatic pipelines significantly influence microbial profiles (Klindworth et al., 2013; Ramakrishnan et al., 2016; Pollock et al., 2018). Therefore, harmonization of these methodological steps would greatly enhance the comparability of studies and facilitate more robust meta-analyses across research groups.

The temporal dynamics of the canine microbiota remain largely unexplored, and understanding the normal temporal variation is important for the development of treatments for suspected microbiome-related conditions. One study (Isaiah et al., 2017) reported a temporal variability in alpha-diversity in detection dogs sampled 7 weeks apart. The studies in the current work were mostly cross-sectional, apart from the 9 dogs followed-up after treatment for fungal rhinosinusitis in study 3. In humans, longitudinal studies have shown that the nasal microbiota of healthy individuals is characterized by a relatively stable core community, although each individual maintains a distinct microbial signature and transient fluctuations may occur over time (Wagner Mackenzie et al., 2019, Zhou et al., 2024).

In particular, antimicrobial treatments may represent a major ecological perturbation capable of altering community composition, and distinguishing treatment-induced shifts from normal temporal variability will require longitudinal investigations. As mentioned in the discussion, there is a lack of studies specifically evaluating the impact of oral and topical antibiotic therapy on the nasal bacterial community in dogs, making it challenging to determine the true efficacy and ecological consequences of these therapeutic interventions. In human medicine, different therapeutic strategies exist regarding antibiotic use in chronic rhinosinusitis. Some clinicians favor systemic antibiotics, for example prolonged courses of oral macrolides because of their anti-inflammatory properties (Wallwork et al., 2006), whereas others emphasize topical antimicrobial therapies, especially following endoscopic sinus surgery, to achieve high local drug concentrations while limiting systemic exposure (Jervis-Bardy et al., 2012). These differing approaches highlight the ongoing debate regarding the role of antibiotics in sinonasal disease and underline the need to better understand their ecological impact on the nasal microbiota.

A critical research objective will be to elucidate the relationship between nasal dysbiosis, whether characterized by a loss of microbial diversity or the overrepresentation of specific taxa, and the pathogenesis of conditions such as fungal rhinosinusitis and lymphoplasmacytic rhinitis. A

major unresolved question is whether the dysbiosis observed in these conditions represents a primary event that facilitates pathogen establishment or inflammation, or instead a secondary consequence of disease-related changes in the nasal environment, such as mucosal inflammation, altered mucus composition, or tissue damage. In the case of fungal rhinosinusitis, fungal colonization may eshape the bacterial community by altering nutrient availability, oxygen gradients, or host immune responses within the nasal cavity. Conversely, pre-existing bacterial dysbiosis could theoretically create ecological conditions that facilitate fungal colonization or persistence. Longitudinal studies tracking bacterial community dynamics alongside clinical progression and therapeutic interventions could provide insight into causal or consequential microbial shifts in these diseases. These studies are however associated with several challenges. Repeated sampling requires sedation or anesthesia, which can limit feasibility and owner compliance over time. In addition, the relatively low prevalence of some nasal diseases and variability in clinical management can complicate recruitment and cohort standardization. Methodological issues such as the low biomass of nasal samples, potential contamination, variability in sampling sites, and multiple environmental or therapeutic confounding factors may also influence microbiota composition and complicate the interpretation of microbial changes and their relationship with disease progression.

Moving beyond compositional analyses, functional investigations using shotgun metagenomic sequencing and metatranscriptomics are needed to better characterize the metabolic potential and biological activity of nasal microbial communities. While 16S rRNA sequencing provides valuable information on bacterial community structure, it does not allow the identification of functional genes or active metabolic pathways. Shotgun metagenomics can provide insights into the genetic repertoire of the microbial community, including pathways involved in biofilm formation, antimicrobial resistance, nutrient acquisition, and host–microbe interactions. Metatranscriptomic approaches could further reveal which microbial genes are actively expressed within the nasal environment, thereby offering a more dynamic view of microbial activity and its interaction with the host mucosa.

Particular attention should be given to microbial metabolites that may influence mucosal immunity and inflammation. In the upper respiratory tract, certain commensal bacteria are known to produce metabolites such as free fatty acids, and other bioactive molecules that can modulate epithelial barrier function, regulate cytokine production, and inhibit colonization by pathogenic microorganisms (Bomar et al., 2016; Man et al., 2017). Therapeutically, these advances could pave the way for innovative, targeted interventions aimed at restoring a balanced and protective nasal

microbial ecosystem. Rather than focusing solely on pathogen eradication, future therapeutic strategies may aim to modulate the microbial community structure and function to promote mucosal health. One potential approach involves the use of intranasal probiotics, which seek to introduce beneficial commensal bacteria capable of competing with pathogenic organisms, producing antimicrobial metabolites, or modulating local immune responses.

Future therapeutic strategies

Microbiota restoration strategies

As our understanding of the canine nasal microbiota continues to expand, interest has shifted toward therapeutic strategies that seek not only to eliminate pathogens but also to restore a balanced microbial ecosystem. Probiotic approaches aim to introduce beneficial commensal bacteria capable of competing with pathogenic organisms, producing antimicrobial compounds, or modulating host immune responses. In human studies, certain commensal taxa such as *Corynebacterium* and *Dolosigranulum species* have been associated with a healthy nasal microbial ecosystem and reduced colonization by respiratory pathogens, making them potential candidates for intranasal probiotic therapies (Martens et al., 2018). Although still largely experimental, early investigations suggest that targeted microbial supplementation could influence microbial community composition and potentially improve mucosal immune homeostasis.

Within this context, the genus *Moraxella*, which appears to represent a dominant and consistent component of the healthy canine nasal microbiota in several studies, may represent an interesting candidate for microbial restoration strategies. If future functional studies confirm that Moraxella species contribute to maintaining nasal microbial stability or inhibiting opportunistic pathogens, targeted approaches aimed at promoting or restoring this genus—either through probiotic administration or ecological modulation of the nasal environment—could potentially represent a novel therapeutic avenue in canine nasal disease.

Additional microbiota restoration strategies are also being explored. Nasal microbiota transplantation, conceptually similar to fecal microbiota transplantation used in gastrointestinal diseases, has been proposed as a method to restore healthy sinonasal microbial communities in cases of severe dysbiosis (Shekhar et al., 2025). Another emerging concept is the gut–airway axis, whereby the intestinal microbiota may influence respiratory immune responses through systemic immune signaling and microbial metabolites (Cavalcanti et al., 2023). Modulation of the gut

microbiota through diet, prebiotics, or probiotics could therefore indirectly influence respiratory health and susceptibility to airway inflammation.

Inspiration may also be drawn from human medicine, where the management of chronic rhinosinusitis increasingly incorporates microbiota-informed therapeutic strategies aimed at restoring microbial balance rather than solely eliminating pathogens. One possible intervention is nasal saline irrigation (Suh et al., 2011), which mechanically removes mucus, debris, and microbial biofilms while improving mucociliary clearance. By reducing microbial load and disrupting biofilm structures, irrigation may help rebalance the sinonasal microbial ecosystem and enhance the efficacy of topical treatments. In addition, topical antibiotic therapies have been explored in selected cases of chronic rhinosinusitis. Intranasal antibiotic irrigations, including mupirocin-based preparations, have been shown to significantly reduce sinonasal colonization by *Staphylococcus aureus* in patients with surgically recalcitrant chronic rhinosinusitis. However, the broader and longer-term ecological effects of such treatments on the overall sinonasal microbiota remain largely unknown (Jervis-Bardy et al., 2012).

Bacteriophage therapy

Another emerging therapeutic strategy is bacteriophage therapy, which uses viruses that specifically infect and lyse bacterial pathogens. Owing to their high host specificity, bacteriophages can selectively eliminate individual bacterial species while largely sparing commensal microbial communities, potentially reducing the ecological disruption and dysbiosis associated with broad-spectrum antibiotic therapy (Kortright et al., 2019). This targeted mode of action may be particularly relevant in the context of microbiota-based therapies. As our understanding of the canine nasal microbiota improves, specific bacterial taxa may eventually be identified as contributors to dysbiosis or chronic inflammation, allowing bacteriophages to selectively target these microorganisms while preserving the overall structure and function of the resident microbial ecosystem. In human medicine, topical phage therapy has shown encouraging results for the treatment of recalcitrant chronic rhinosinusitis, particularly against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Experimental studies have demonstrated effective antibacterial activity following local administration, while early clinical studies have shown topical delivery by nasal irrigation, sprays or gels to be feasible and well tolerated, although larger controlled clinical trials are still required to establish its clinical efficacy (Zhang et al., 2018; Uyttebroek et al., 2021). Although experience in veterinary medicine remains limited, interest in bacteriophage therapy is steadily

increasing as a potential alternative or adjunct to conventional antibiotics for the treatment of multidrug-resistant bacterial infections in companion animals, including canine otitis, pyoderma and wound infections (Loponte et al., 2021). To date, however, no studies have investigated its application in canine nasal disease, highlighting an interesting avenue for future research.

Postbiotic therapies

Postbiotic (metabolite-based) therapies represent another emerging avenue for microbiome modulation. Rather than introducing live microorganisms, these approaches aim to administer microbial-derived bioactive compounds, including short-chain fatty acids, peptides, enzymes and other metabolites, that reproduce the beneficial functional effects of commensal microbes. Such compounds have been shown to inhibit pathogen growth, enhance epithelial barrier integrity, regulate mucosal immune responses and reduce inflammation, thereby contributing to host protection. Compared with live probiotics, postbiotics may also offer practical advantages, including greater stability, easier standardization and improved safety, particularly in immunocompromised individuals, while avoiding the challenges associated with microbial colonization (Salminen et al., 2021). Although their application in respiratory diseases remains at an early stage, postbiotics represent a promising strategy for modulating host–microbiota interactions without introducing live microorganisms.

Although these microbiota-based approaches remain largely investigational, they illustrate how advances in microbiome research may shift future therapeutic strategies from broad pathogen eradication toward restoration and selective modulation of the resident microbial ecosystem. Translating these concepts to veterinary medicine may ultimately enable more targeted, individualized and ecologically sustainable management of chronic nasal diseases in dogs.

Conclusion

This work provides new insights into the composition, variability, and disease-associated alterations of the canine nasal microbiota, while critically evaluating the diagnostic value of conventional bacterial culture in the management of chronic nasal diseases. Through the combination of culture-based and DNA sequencing approaches, we demonstrated that conventional nasal swab cultures show limited concordance with sequencing results and may be insufficient to reliably guide antimicrobial therapy in suspected cases of secondary bacterial rhinitis.

These findings highlight the need for prudent, evidence-based antimicrobial use and support the exploration of topical rather than systemic treatments in selected cases.

By characterizing the healthy nasal microbiota in dogs, this thesis defines a core community largely dominated by *Moraxella*, while also revealing notable inter-individual variability and the influence of factors such as facial conformation and disease status. Brachycephalic breeds exhibit distinct microbial profiles, underscoring the importance of considering host morphology in study design and data interpretation.

In dogs with chronic nasal diseases, we identified a reproducible pattern of dysbiosis characterized by reduced *Moraxella* abundance and the proliferation of opportunistic taxa. Moreover, fungal rhinosinusitis and chronic idiopathic rhinitis, although sharing some alterations, display distinct microbial signatures—most notably the enrichment of *Porphyromonas*, *Enterobacteriaceae*, and *Staphylococcus* in the former, and *Pasteurella* in the latter. The persistence of some altered taxa even after clinical cure of fungal rhinosinusitis raises important questions about their role in disease recurrence.

Overall, this thesis emphasizes that both intrinsic factors (e.g., breed morphology) and disease processes significantly shape the canine nasal microbiota. These findings not only advance the fundamental understanding of nasal microbial ecology in dogs but also pave the way for microbiota-informed diagnostic and therapeutic strategies aimed at improving the management of chronic nasal diseases in veterinary medicine.

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