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Rodent models for oral microbiome research: considerations and challenges- a mini review

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Rodent models have been commonly employed in oral microbiota research to investigate the relationship between bacteria and oral disease. Nevertheless, to apply the knowledge acquired from studies conducted on rodents to a human context, it is crucial to consider the significant spatial and temporal parallels and differences between the oral microbiota of mice and humans. Initially, we outline the comparative physiology and microbiology of the oral cavity of rodents and humans. Additionally, we highlight the strong correlation between the oral microbiome of rodents and genetic makeup, which is influenced by factors including vendor, husbandry practices, and environmental conditions. All of these factors potentially impact the replicability of studies on rodent microbiota and the resulting conclusions. Next, we direct our attention toward the diversity in the microbiome within mice models of disease and highlight the diversity that may potentially affect the characteristics of diseases and, in turn, alter the ability to replicate research findings and apply them to real-world situations. Furthermore, we explore the practicality of oral microbial models for complex oral microbial diseases in future investigations by examining the concept of gnotobiotic and germ-free mouse models. Finally, we stress the importance of investigating suitable techniques for characterizing and managing genetically modified organisms. Future research should consider these aspects to improve oral microbiome research's translational potential.

KEYWORDS

oral, microbiome, microbiota, mice: mouse: rodents: animals, *in vivo*

1 Introduction

The use of mice and rats as a model has been crucial in understanding the causes, development, and potential treatments for several human diseases. Obtaining consistent results from the model was challenging for a long time due to the presence of inconsistencies in nutrition, infection, age of animals, housing, and several other factors. Although numerous research cited in the literature have provided new insights into several

human diseases, it is common for them to be deficient in sufficient information, limiting their translational value. While many studies mentioned in the literature are clear, it is also not uncommon for them to lack enough information to make judgment easier (1).

The microbiome is the collective genetic material of all the microorganisms that inhabit on and inside human body, including bacteria, protozoans, fungi and viruses. Under unfavorable conditions, few microbes have been shown to become pathogenic and may result in dental caries, periodontitis, abscesses, endocarditis, and various oral potentially malignant disorders/invasive neoplasms. Microorganisms of any type may result in or trigger the pathogenic process; while some are established conditions, the role of others is questionable. For instance, candida (or candidiasis) had always been considered carcinogenic (2), but their definitive role has now been deemed contentious to the latest World Health Organization updates of head and neck tumors in 2022 (3). Previous studies have repeatedly indicated a spatial role of the oral microbiome in the tumorigenesis of oral cancer (4). The role of periodontal diseases has been positively correlated with an increased risk of oral potentially malignant disorders (OPMDs) (5) and oral squamous cell carcinoma (OSCC) (6), which led the research to hypothesize that the inflammatory microbiome of periodontitis may also play a plausible role in development and progression of oral cancer. The need for an ideal animal model is thus deemed necessary for the duplication of these diseases and for basic, translational research. Rodent models have been used to study oral microbiomes and diseases associated with microbial dysbiosis. The chief advantages of the application of rodents for experimental procedures include ease of breeding and handling, comparatively lower maintenance cost, availability, and an adequately sized oral cavity for easy inoculation (and sample collection). Although rodents and humans share numerous morphological, histological, and physiologic characteristics, there are significant disparities in oral microbiome. Therefore, it is not unexpected that there are substantial variations in the composition of the gut microbiota, both in terms of the types of microorganisms present and their relative abundance. Research has demonstrated significant variations in microbial composition among different breeds of mice (7). Given these findings, it is natural to question using mouse models for extrapolating the oral microbiome to humans. One of the possible explanations is that there is currently no superior option. Here, we review the physiology and oral microbiology of the rodents in detail. Further, we focus on the mice models tried and tested for research involving oral microbiome and highlight the recent advancements in science that could be utilized to improve the oral microbiome's translational potential *in vivo* research.

2 Physiology of the oral cavity of mice and rats

The anatomical morphology, physiology, and histological characteristics of the oral cavity of rodents, one of the most

typical animal models, resembles the human rima oris (8). Rodents (order Rodentia), the largest mammalian group with extreme diversification, comprises 40% of all the mammal species of kingdom Animalia and are subclassified into three major suborders based on the anatomical variations and functional differences in masseter muscles (8, 9). All the rat-like/mice-like rodents are categorized as *Myomorpha*. The other two suborders are *Caviomorpha/Hystrichomorpha* and *Sciuromorpha* (9).

In contrast to human dentition (2/2I, 1/1C, 2/2PM, 3/3M), who have well-formed crowns and roots of all teeth (32 permanent), all rodents species have elodont aradicular incisors, lack the canines and bears a long space between incisors and 'cheek-teeth'-premolars and molars (9). It should be noted that the rats and golden hamsters also lack the premolars, yielding a dental formula as 1/1I, 0/0 C, 0/0 P and 3/3M (total 16 teeth). However, odontogenesis is akin to human tooth development involving the same types of cells, making these a perfect model for analyzing odontogenic lesions. Figure 1 shows photomicrographs of developing rat tooth germs and adjacent oral structures where we can find close resemblances with human tooth germs.

Further, the differences lie in the jaws, arrangement of the masseter, and zygomatic arch (10, 11). In contrast to humans, where the jaw movements are restricted to complex arrangement and synchronization of muscles at temporomandibular joints (TMJ), the few species of rats show unossified cartilaginous tissue at mandibular symphysis area, allows specific movements between the mandibular bones. The symphysis in human mandibles is fused with no movements. Three distinct layers have been identified viz. superficial masseter, deep masseter and zygomatico mandibularis (9), contrariwise, only two sets of fibres are seen in human, the superficial and deep. The similar angulation fibers and function of these strong muscles of mastication allow easy recapitulation of temporomandibular joint movement and disorders in animal models.

Pertaining lining epithelium in rats, which serves as the prime oral structure for analysis of oral microbiota and carcinogenesis, shows many similarities with the oral mucosa of the human mouth. Externally delineated by lips, labial skin which is orthokeratinized stratified squamous cell type contains abundant hair, adnexa, and numerous vibrissae, structures lacking in human (Figure 2). Further, unlike us, a cleft is noted between upper incisors; thus, the labial frenulum is not noted. The lingual frenum is also not seen in rats (12). The similar stratification of the epithelia is noted; however, the entire oral cavity is variably ortho-keratinized. The human oral cavity demonstrates the transition from keratinized labial mucosa to non-keratinization in the buccal and floor of mouth region to para- to ortho-keratinization on attached gingival or palatal mucosa. The tongue is specialized with thousands of taste buds; in contrast, the majority of taste buds are seen on the soft palate in rats, with missing uvula (8). However whether similar physiology, odontogenesis, and palatogenesis between human and rodent oral cavity have an influence on the oral microbial colonisation is not known.

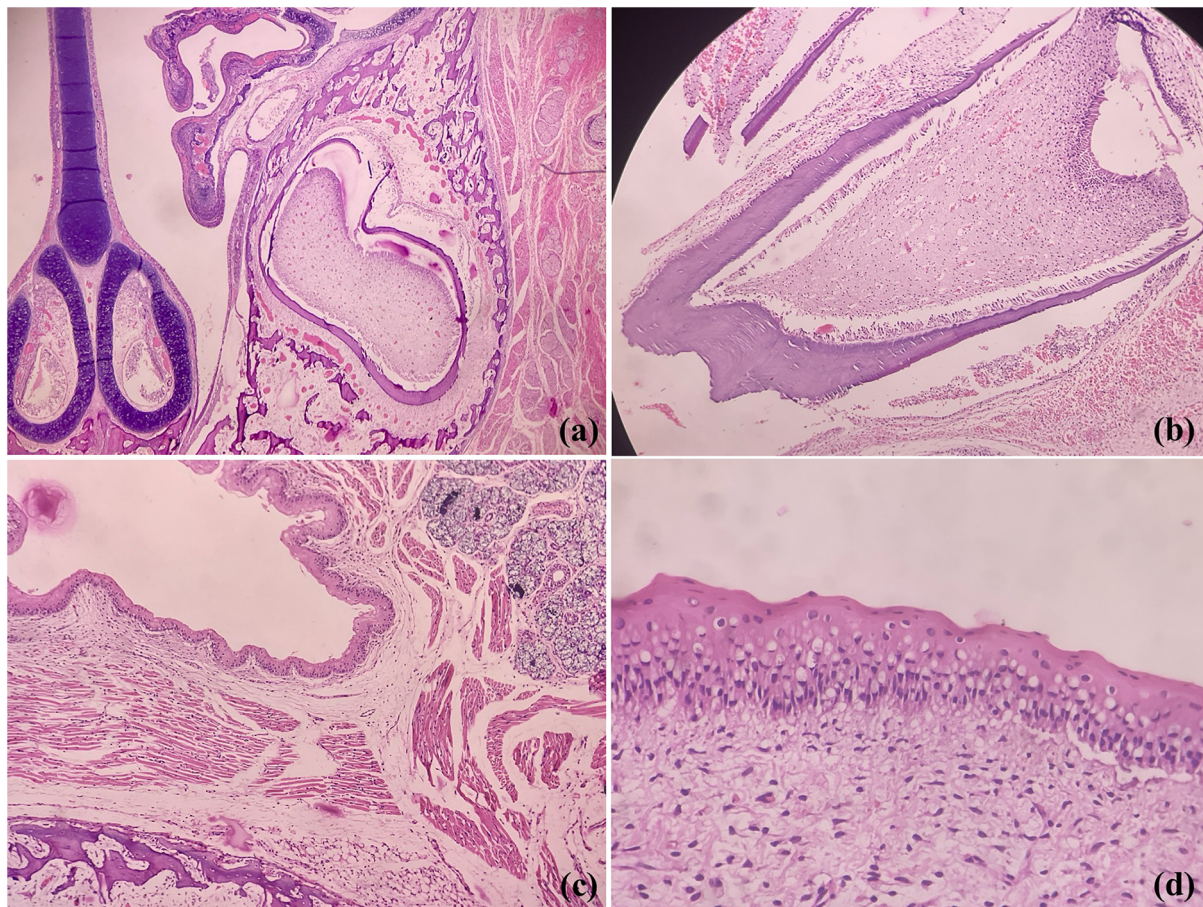


FIGURE 1

Photomicrographs of H&E stained sections showing coronal section of rodent face demonstrating developing tooth germs, maxillary sinus and nasal septum (a), developing molar (b), fetal epithelium, salivary gland tissue, bone and skeletal muscles (c) and oral epithelium (d) (Picture courtesy Dr Suganya Panneer Selvam, Oral Biologist, Saveetha Dental College and Hospitals).

3 Oral microbiome of rodents

The bacteria flora of the oral cavity of humans is dominated by the phyla Fusobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, and Spirochaetes, accounting for approximately 96% of the species detected (13). The normal human flora begins to develop postpartum; an infant is first exposed to microorganisms during birth (13). While the human gastrointestinal tract contributes a significant number of microbiota, the human oral cavity contains as high as 700 different species (13). The bulk of microbes in the oral cavity of rodents were reported to be attributed to the phyla *Firmicutes* and *Proteobacteria*, with most of them belonging to the class *Gammaproteobacteria* (14). The oral microbial communities in humans are more intricate than those in mice, with less than 50% of the oral microbiota in human samples comprising the top ten bacterial species/phylotypes in mice (15). A study revealed that only 27 types of mouth bacteria were common to both mice and humans (15). The earliest communities seen in the murine oral mucosa are similar to those found on the murine skin, indicating that the transmission of these communities likely occurs from the mother's skin during feeding (14). *Staphylococcus*

sp., *Streptococcus sp.*, *Lactobacillus sp.*, *Enterobacteriaceae sp.*, and *Enterococcus sp.* were shown to constitute the main species in the rodents (16).

4 Factors that influence oral microbiome composition of experimental rodents

4.1 Genetics of the mouse

In murine oral microbiome research, understanding the genetic background of various mouse strains is crucial for ensuring result accuracy and credibility. Studies indicate significant differences in the diversity and richness of the oral microbiomes among mouse strains, such as C57BL/6 or BALB/c (17, 18). These differences significantly affect research into the interactions between microbiota and host health, also challenging model selection and study design. Specifically, the notable differences in types and quantities of oral microbiota across mouse strains are crucial for autoimmune disease research. This underscores the importance of selecting the

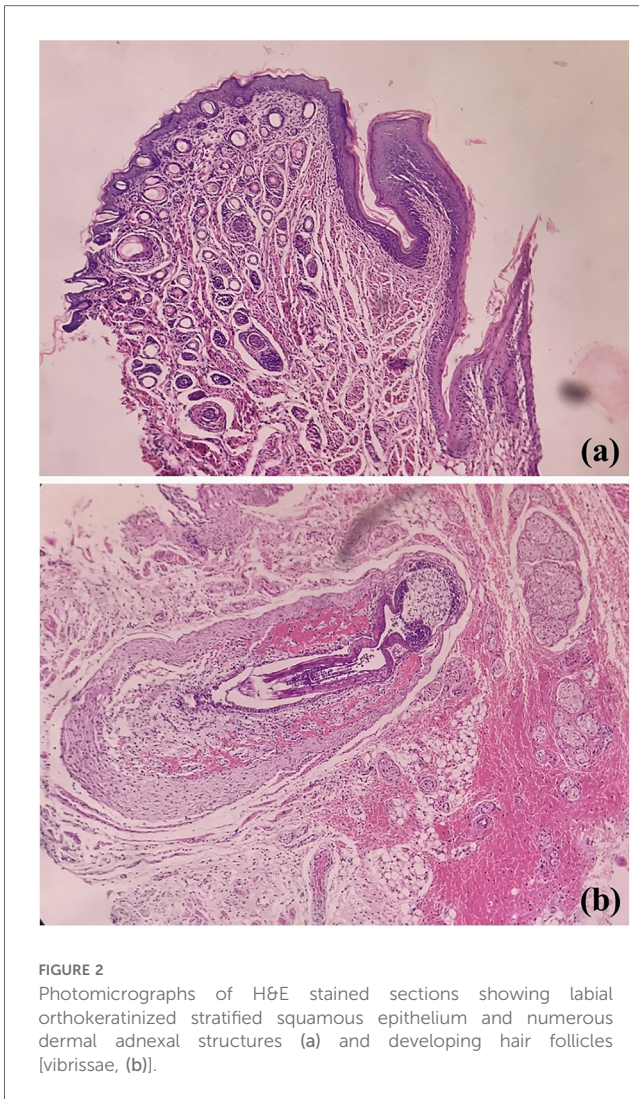


FIGURE 2
Photomicrographs of H&E stained sections showing labial orthokeratinized stratified squamous epithelium and numerous dermal adnexal structures (a) and developing hair follicles (vibrissae, (b)).

appropriate mouse strain for a deeper insight into the microbiome's role in specific disease models. Furthermore, research that identifies a direct link between specific genetic markers and the mouse oral microbiome composition enhances our understanding of microbiota-host interactions and offers a scientific basis for selecting suitable mouse models (19, 20). Researchers discovered that, despite identical genetic manipulations, different mouse strains show significant differences in oral microbiome composition when replicating the same pathological changes (21, 22). Further, it has been identified to have a more significant impact than the animal's gender in microbiome studies (23). Prior research has also demonstrated that mice obtained from various suppliers possess unique microbial communities in their oral, stomach, and fecal regions (24). These findings highlight the importance of accounting for the genetic background of mice in microbiome studies.

4.2 Diet

Research consistently shows that dietary changes significantly impact mice's oral microbiome composition and diversity.

Specifically, high-fat and sugar diets increase certain pathogenic bacteria in the oral cavity, whereas fiber-rich diets enhance microbial diversity, benefiting oral health (25). Studies using mouse models reveal that dietary changes affect the microbiome's composition and are closely linked to oral health status (26). For example, certain dietary habits can promote dental caries and periodontal disease development, closely associated with diet-induced changes in the oral microbiome. Adjusting mice's dietary composition allows researchers to explore the relationships between specific microbial communities and various physiological and pathological states. This approach improves our understanding of microbes' impact on oral and overall health and how dietary interventions can enhance health. Diet significantly impacts mouse models' oral microbiome composition; adjusting it is crucial for exploring the relationships between the oral microbiome and health states.

4.3 Housing during experiments

Laboratory environmental conditions, namely temperature, humidity, and housing density, significantly affect the oral microbiome composition in mice (27–29). These environmental factors can directly impact the oral microbiome's stability and indirectly alter microbial community balances by affecting mice's physiological states, immune responses, and dietary preferences (30). Extrinsic factors in the environment, such as the shedding of skin or dust particles from caretakers and scientists who handle the mice, as well as the pH of the water, water treatment and the type of food given, play a crucial role in shaping the microbial colonization in laboratory mice (24, 31, 32). The oral microbiome, in general, is crucial for various physiological and pathological processes and demonstrates high plasticity to environmental changes, highlighting its quick adaptability to surrounding shifts (33). Although existing literature highlights the significant impact of environmental conditions on mice's microbiome, further research is required to elucidate the specific mechanisms and effects on physiological and health statuses.

4.4 Stress during experiments

Physiological and psychological stress are key factors affecting the composition of the gut microbiome in mice, potentially exerting effects through changes in immune response mechanisms. Under stress conditions, mice exhibit significant changes in the structure of their microbial communities, including oral mucosa, which could further impact their health and susceptibility to diseases (34, 35). Studies have shown that stress affects the diversity and abundance of microbes, possibly by modulating the activity of the immune system, thereby altering the balance of the microbial community (34, 36, 37). Stress hormone cortisol has been directly implicated in modulating the gene expression profiles of the oral microbiome (38). This underscores the importance of stress management in experimental design, especially in research exploring the

relationship between oral microbiota and host health. Therefore, managing and measuring stress factors should be central to scientific research design to ensure the accuracy and comparability of results. Methodological considerations, including the assessment of stress levels, are critical for interpreting results and ensuring their reproducibility and generalizability.

4.5 Other related factors

Research shows that a mouse's immune system status, whether immunodeficient or hyperimmune, significantly influences its oral microbiome composition. Immunodeficient mice tend to harbor more pathogenic microbes in their oral cavity, whereas hyperimmune mice may suppress some beneficial microbes (39, 40). The use of antibiotics and other medications significantly impacts mice's oral microbiome. Specifically, antibiotics often reduce microbial diversity and lead to the emergence of antibiotic-resistant strains (41).

5 Currently used strains in oral microbiome research

In contemporary oral microbiome research, *in vivo* research employs several rodent strains to unravel the complex host-microbe interactions in infectious oral diseases. The choice of specific strains is guided by the unique characteristics of each strain, which offer unique advantages for specific aspects of these diseases, allowing researchers to examine genetic susceptibility, immune responses, and microbial dynamics.

5.1 Caries and apical periodontitis

Previously, Wistar, Sprague-Dawley, and Osborn strains were the most frequently used rat strains in caries research (42). Hsiao et al. used two rat models such as pathogen-free Sprague-Dawley (SD) rats as the Nutritional Microbial Bacterial Model and Sprague-Dawley adult rats as Pulp Disease Induction Models to observe the progress of dental caries and pulp disease (43). Some of the rodent models in caries microbiome research include NFS/N mice strain to determine the caries susceptibility (41), Sprague-Dawley rats for caries induction (42), and BALB/cA and C3H/HeN mice strains for the caries microorganism sensitivity (44). C57BL/6 mice was used to evaluate microorganism mediated pulpitis-induction (18, 44–45). Germ-free adult male C57BL/6 J mice was used recently disparity in metabolic activity and lactic acid generation between monozygotic twins with contrasting caries experiences and their oral microbiome (46). For investigations on microbe-induced apical periodontitis, the most commonly used models include C 57B/L6 mice and (BALB/c) mice (47–49). A recent study utilized apoE^{-/-} mice to establish a *P. gingivalis*-induced chronic apical periodontitis (CAP) model to understand the association of CAP with gut microbiota as well as atherosclerosis by 16S rRNA sequencing

and microbial metabolomics (50). Wistar rats were also used to study the effect of *F. nucleatum*-induced apical periodontitis on gut microbiota (51).

5.2 Periodontitis

By introducing multiple bacterial species into the model, researchers can mimic the intricate interactions and synergistic effects among different pathogens, the role of host immune responses in shaping the periodontal microbiota and disease progression, the interplay between the host immune system and the oral microbiome in driving periodontal tissue destruction, and assessing the efficacy of treatments in a more realistic disease context, thus providing insights into disease pathogenesis that may be missed in monomicrobial models. In this context, the periodontal inoculation model has been successfully used to evaluate mechanisms of periodontal inflammation, pathogenic differences between different periodontal bacteria, and alveolar bone loss (52, 53). C57BL/6, C57BL/6 J (WT, -TLR2(KO), -TLR4 (KO), -TLR2,&4KO) mice, CD1 Swiss mice, C57BL/6 J female mice are some of the strains, and their knockouts that have been used in experimental periodontitis with microbes (54). Klausen has reviewed the studies on periodontitis in rat models and concluded that germ-free Sprague-Dawley (SD) rats were the most commonly used rodent strains, followed by Lewis specific pathogen free (LEW-SPF) and Rowett SPF (ROW-SPF) (55). Major histocompatibility complex (MHC), cytokine, and adhesion molecule knockout mouse strains have also been used to evaluate the role of these mediators in *P. gingivalis*-mediated periodontal tissue destruction (56). The Baker mouse model, chemically induced mouse model, and murine incisor abscess model have been used to study the interaction of monomicrobial infections in apical periodontitis (57). In contrast, the murine back abscess model is used to study the host response to the polymicrobial mixed infection, including *P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum* (57). Owing to the genetic variances among the strains affecting the mutation of the immune components or in their adaptive immunity, these strains are differentially susceptible to experimental periodontal diseases, and several strains are quite resistant. BALB/c, AKR/J, DBA/2J and C3H/HeN mice are more susceptible than C57BL6 A/J 129/J, SJL/J and C3H/HeJ (58, 59). Recently, a research group successfully generated a chronic periodontitis model in mice by oral infection of the *P. gingivalis* P4 strain, which co-aggregated with a dominant mouse oral commensal bacterium (60). A recent study experiments on humans and mice model demonstrated that TH17 cells are necessary for the breakdown of periodontal tissue (40). The murine ligature-induced periodontitis (LIP) model is frequently employed to study the dysbiosis of the indigenous oral microbiome, which has coevolved with the murine host microbiome in periodontitis (61, 62). Arce et al. compared the microbial profiles from 16S rDNA gene sequencing datasets from nine different murine LIP models on C57/BL6 mice and demonstrated that though there were similar enrichment of certain microbes across different studies, the organization of the

microbial community was influenced by the induction of periodontitis and the specific study being conducted (61). They also illustrated the technical factors that influence the results of oral microbiome research in the periodontitis models, ranging from sample collection to bioinformatic pipeline, which has been emphasized previously (63).

5.3 Oral cancer

One of the earlier studies to demonstrate the role of oral microbes, precisely two periodontal pathogens in oral tumor progression, utilized male BALB/c mice models (64). Germ-free Swiss Webster male mice under gnotobiotic conditions were used to compare the tumorigenesis between OSCC-associated microbiomes after 4-nitroquinoline-1-oxide (4-NQO) induction (65). A recent study explored the impact of the microbiome and its metabolic pathways on regulating oral carcinogenesis using C57BL/6 mice and identified an increase in the biosynthesis of polyamines in saliva with oral cancer (66). Another study utilized BALB/c mice to explore the microbiome of OSCC in mice with experimental induced periodontitis where they identified *Porphyromonas* to be the most abundant genus (67). However, the impact of the variation in oral microbiome between the strains has not been taken into consideration in any of these studies. Given the significant differences between mice strains, Gnotobiotic or germ-free mice, Specific Pathogen-Free (SPF) Mice, Immunodeficient Mouse Models such as nonobese diabetic/severe combined immunodeficiency (NOD-SCID) or NOD SCID gamma (NSG) mice, Transgenic or Knockout Mice could be valuable for introducing specific microbial communities to study their effects on oral cancer development. Apart from rats and mice, male Golden Syrian hamsters (3–4 weeks) were also utilized to study the impact of smokeless tobacco on the oral microbiota and oral carcinogenesis (68).

The murine oral cavity is also ideal for the candida model and thus may serve perfectly for experimentation for clarification of the controversial role of candidal infection in OPMDs and OSCC. Various researchers have induced Candidiasis in germ-free mice (69, 70). In hyposalivation rat models (salivary glands were removed), it was demonstrated that disease progression was noted with more ALS transcripts (cell surface glycoprotein associated with adhesion) (69). Also, similar expression was noted in HIV-positive patients and experimental candidiasis in rats (70).

6 Gnotobiotic and germ-free rodents in oral microbiome research

In recent years, gnotobiotic animal models have emerged as a crucial tool for investigating intricate interactions between hosts and their microbiota. Gnotobiotic animal models consist of germ-free animals, devoid of any foreign organisms, and animals colonized with one or more specific microbes (71, 72). Germ-free mice are selectively bred in isolators that exclude entirely any contact with microorganisms, with the specific purpose of

ensuring their complete absence of detectable bacteria, viruses, and eukaryotic germs (73). Germ-free rodents have no microorganisms living in or on them, allowing researchers to precisely control an animal's oral microbiome through the direct inoculation of bacteria of interest, which facilitates the exploration of intricate host-microbe interactions within the oral cavity. These models offer valuable insights into the complexities of the oral microbial ecosystem and aid in the development of targeted therapeutic strategies for maintaining oral health. The idea of germ-free mouse colonies was first proposed by Louis Pasteur in 1885 and later established in the 1940s (74). Germ-free mice are used to investigate the complete absence of microorganisms or to create gnotobiotic animals that are only colonized by identified germs. Nevertheless, the production and upkeep of these mice necessitate specialized facilities, and the expenses, effort, and expertise needed to maintain them can render these models unattainable for several researchers (75). To ensure the absence of germs in mice, it is necessary to regularly check for contamination using various methods such as culture, microscopy, serology, gross morphology, and sequencing-based detection approaches (76). They also utilize PCR (both 16S and pathogen-specific), microscopy, and culturing techniques to test for bacteria. In addition, any distinct mouse strain to be examined in germ-free circumstances must be regenerated in these facilities, which restricts the number of diverse genotypes that may be feasibly studied (77). In addition, the upkeep of mice in isolators may render it unfeasible or arduous to carry out specific investigations, such as behavioral assessments or pathogen introductions. However, it is essential to differentiate this term from specified pathogen-free (SPF), as SPF animals are devoid of specific pathogens. Still, their internal microbiota is intricate and not clearly characterized (78). Gnotobiotic mouse models contribute significantly to our understanding of the oral microbiome's role in health and disease, enabling the investigation of dysbiosis, immune responses, and microbial contributions to conditions such as dental caries, periodontal diseases, and oral cancer.

7 Humanization strategies in germ-free mice for oral microbiome

Humanization strategies for oral microbiome research in germ-free mice involve introducing human oral microbial communities into mice raised in a germ-free environment. The resemblance of the transplanted oral microbiome in mice to that of the donor, i.e., the human microbiome, is dependent upon several factors like diet, socioeconomic background, ethnicity, exercise regime, etc. Differences in the donor and recipient anatomy and genetic, environmental, and immunological backgrounds are the key factors affecting this similarity. Human microbiome transplantation allows the oral samples from human donors to be transplanted into germ-free mice. Fecal Microbiota Transplantation (FMT), although primarily associated with the gut microbiome, has also been adapted for oral microbiome research (79). Human oral microbial communities extracted from

fecal samples are introduced into germ-free mice. He et al. have transplanted the microbiome of periodontitis patients into the mouse periodontium and observed a significant increase in osteoclasts, TNF- α , and IL-1 β in the latter (80). Human periodontitis microbiome has also been introduced in mouse by using subgingival plaque of periodontitis patients (81). However, several host factors could have a significant effect on microbial colonization (82). Another significant advantage would be the immunological response elicited by these humanized oral bacteria communities. Immunodeficient mice may be studied, but they will once more be unable to mimic the reaction that oral microbiological communities will have in a human setting. Despite limitations and challenges, humanized mice models hold promise in elucidating the pathogenesis and immunotherapeutic strategies in periodontitis. There have been notable findings from studies using humanized models. *A. actinomycetemcomitans*-specific IgG antibody responses, significant increment in the RANKL expression and alveolar bone resorption, as well as decreased OPG expression, was observed in the Aa-hu-PBL-NOD/SCID model (83). These models also shed light on the role of suppressor of cytokine signaling (SOCS) molecules in *A. actinomycetemcomitans*-induced osteoclastogenesis. By using a *P. gingivalis* infected HLA-DR1 humanized C57BL/6 mice model, the development of rheumatoid arthritis and its impact on bone density and systemic cytokine production were also analyzed (84). Recently, a team of researchers developed a human oral microbiota-associated (HOMA) mouse model by transplanting human saliva into germ-free mice and 85% of the genus-level taxa were identified to be similar to the donor (16).

8 Conclusion

Although the comparative physiology of rodents and humans emphasizes the significance of rodents as a model to study oral pathologies, the literature unequivocally demonstrates that genetics plays a significant role in oral microbiome variability among rodents. Diet and living conditions are also significant variables that must be considered, particularly when replicating human microbial diseases and comparing the findings of oral microbiome studies. Future research should prioritize replicating microbial studies with different rodent strains to demonstrate strong and reliable results regardless of diet, genotype, or environmental factors on the composition of the oral

microbiome. Exploring germ-free mice and then enhancing humanization strategies would be a way forward. Given the significant differences between the human oral microbiota and that of mice, it is essential to conduct thorough robustness checks before extrapolating the results to humans.

Author contributions

DG: Conceptualization, Data curation, Formal Analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. DP: Data curation, Writing – original draft, Writing – review & editing. ZL: Investigation, Methodology, Writing – original draft, Writing – review & editing. SP: Data curation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

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