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To cite this version:

M. Rober, M. Quirynen, A.D. Haffajee, E. Schepers, W. Teughels. Intra-oral microbial profiles of beagle dogs assessed by checkerboard DNA-DNA hybridization using human probes. Veterinary Microbiology, 2007, 127 (1-2), pp.79. 10.1016/j.vetmic.2007.08.007 . hal-00532305

HAL Id: hal-00532305

https://hal.science/hal-00532305v1

Submitted on 4 Nov 2010

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PII: S0378-1135(07)00395-1
DOI: doi:10.1016/j.vetmic.2007.08.007
Reference: VETMIC 3786

To appear in: *VETMIC*

Received date: 8-5-2007
Revised date: 1-8-2007
Accepted date: 6-8-2007

Please cite this article as: Rober, M., Quirynen, M., Haffajee, A.D., Schepers, E., Teughels, W., Intra-oral microbial profiles of beagle dogs assessed by checkerboard DNA-DNA hybridization using human probes, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.08.007

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Intra-oral microbial profiles of beagle dogs assessed by checkerboard DNA-DNA hybridization using human probes

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Abstract

Some investigators suggest a similarity between the oral microbiota of dogs and humans. The *in vivo* assessment of ecologic relationships among bacterial species and between bacterial species and their habitat is difficult to carry out. Consequently, this aspect is often neglected in animal oral microbiological studies. This study aimed to examine the proportions of 40 bacterial species in samples from 5 intra-oral habitats in beagle dogs using checkerboard DNA-DNA hybridization. Microbial samples were taken from subgingival and supra gingival plaque, the tongue, tonsils and cheek mucosa in 7 beagle dogs. Samples were individually evaluated for their content of 40 bacterial species and the percentage of total DNA probe count was determined for each species, at each habitat. All tested species could be detected in all sampled habitats but each habitat had a distinct community structure. The microbiotas colonizing the hard surfaces in the oral cavity were quite different from the microbiotas colonizing the soft tissues. Bacterial species that are in humans considered to be periodontopathogens, are present in high proportions. This study underlines the importance of the habitat and the host on the local microbial profile.

Keywords: Supra- and subgingival plaque, Soft tissue microbiota, Dog, Periodontitis, Oral microbiology, Ecology

1. Introduction

Some investigators suggest that there is a similarity between the oral microbiota of dogs and humans (Syed et al., 1980; Cutler and Ghaffar, 1997). In dogs, as in humans, the most common oral diseases originate from changes in the oral microbial ecology and often lead to suffering and tooth loss (Takada and Hirasawa, 2000; Hirasawa et al., 2000). Oral bacteria may even spread to other sites in the body and can induce histopathological changes in kidney, myocardium, and liver (DeBowes et al., 1996). The oral cavity is considered to be a microbiological entity (Quirynen et al., 2001) in which hundreds of different bacterial species, viruses and yeasts are living together. Consequently, the oral microbial composition is complex and the *in vivo* assessment of ecologic relationships among bacterial species and between bacterial species and their nonbacterial habitat has been difficult to carry out. This relates to the laborious microbiological techniques needed to assess a wide range of bacterial species in a large number of samples taken from clinically or microbiologically distinct

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habitats (for review see Socransky and Haffajee, 2005). For humans, this technical limitation has been overcome in large part by the use of more rapid techniques for microbial assessment such as checkerboard DNA-DNA hybridization (Socransky et al., 1994).

Although the checkerboard DNA-DNA hybridization technique has provided important insights in the oral microbial ecology over the past decade (for review see Socransky and Haffajee, 2005), the microbial ecology in the canine oral cavity still remains in its infancy. Such basic information is necessary for the improvement of canine oral health care, for the microbiological validation of periodontal therapy (Weinberg and Bral, 1999), and for providing a basis for uncovering the universal characteristics of the pathogenic and protective species, by comparing species that fill similar habitats in different hosts (Elliott et al., 2005).

This study used checkerboard DNA-DNA hybridization and 40 DNA probes made from human oral bacterial species to determine the proportions of bacterial species in the oral cavity of beagle dogs. The present investigation aimed to determine the proportions of 40 bacterial species in supra- and subgingival dental plaque and on the oral soft tissue surfaces (tongue, cheek, tonsil).

2. Materials and methods

2.1. Subject population

Seven male beagle dogs with an average age of 2.98 (+/- 0.26) years were enrolled in this study. They were housed at the animal care facility of the Catholic University of Leuven according to American Veterinary Medical Association guidelines and were fed commercial dry and wet food. The ethical committee for animal experimentation of the Catholic University approved the protocol, and a veterinarian (MR) monitored the animals during the entire course of the study. The dogs were in a good systemic condition but had naturally occurring moderate periodontitis. The average probing pocket depth was 2.7 ± 0.1 (standard error of mean) mm with localized pockets ≥ 4 mm. 40.7 ± 8.1 % of the pockets bled upon probing. The dogs never had received dental homecare or antimicrobial therapy.

2.2. Sample collection

Samples were collected under sedation using medetomidine hydrochloride (Domitor®, Pfizer Inc., Belgium) and ketamine (Anesketin®, Eurovet Animal health, The Netherlands). Buprenorfine

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hydrochloride (Temgesic®, Schering-Plough, Belgium) was administrated additionally as a painkiller. Every effort was made to avoid cross-contamination during the sampling process. After gentle air-drying and isolation with cotton rolls, supragingival plaque samples were taken from tooth 109, 209, 309, and 409 using sterile Gracey curettes. After removal of the remaining supragingival plaque, subgingival plaque samples were taken from the same teeth by inserting 8 paper points in the gingival sulcus for 20 seconds. After drying the cheek mucosa, tongue, and tonsils, soft tissue samples were taken by gently stroking each site for 10 seconds with a cotton swap. Samples of supragingival plaque and biofilms from the oral soft tissues could have been contaminated with saliva. However, the results from human studies have indicated that this potential contamination has little effect since the microbial profiles of the different oral sites as well as the biofilms that form on the teeth are quite distinct and also different from the microbial profile of saliva (Mager et al., 2003). The potential of contamination during the sample taking procedure is a recognized “hazard” of sample taking although literally hundreds of published papers on the oral microbiota of humans have used similar sample taking procedures.

All samples were placed into individual tubes containing 150 µl of TE buffer to which 150 µl of 0.5M NaOH was added. The samples from all dogs were individually analysed for the presence of 40 bacterial species using the checkerboard DNA-DNA hybridisation technique. The 40 bacterial species studied are listed in figure 1.

2.3. Microbiological assessment

The checkerboard DNA-DNA hybridisation technique was previously described (Socransky et al., 1994). In brief, the samples were lysed and the DNA placed in lanes on a nylon membrane using a Minislot device. After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 with the lanes of DNA at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes to 40 bacterial species were hybridised in individual lanes of the Miniblotter. After hybridisation, the membranes were washed at high stringency and the DNA probes were detected using an antibody to digoxigenin conjugated with alkaline phosphatase and chemifluorescence detection. Signals were detected using AttoPhos substrate and were read using a Storm Fluorimager, a computer linked instrument that read the intensity of the fluorescent signals resulting from the probe-target hybridisation. Two lanes in each run contained standards at concentrations of the 10^5

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and 10^6 cells of each species. Signals evaluated using the Storm Fluorimager were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect the signal was recorded as zero.

2.4. Data analysis

The total DNA probe count for each species was computed for each sampled habitat in each dog. The proportion (percentage of the DNA probe count) that each species comprised of the total DNA probe count was also calculated. The significance of differences among the proportions of the 40 test species were sought using the Kruskal Wallis test, adjusted for multiple comparisons (Socransky et al., 1991). Cluster analysis was performed using the proportions of the 40 species at the different sample locations in the 7 dogs. Similarities were computed using the minimum similarity coefficient (Socransky et al., 1982) and sorted using an average unweighted linkage sort (Sneath and Sokal, 1973).

3. Results

It should be stressed once more that the data represent the results of hybridizing DNA from canine oral bacterial species with DNA probes constructed using DNA from human oral isolates.

3.1. Comparison of microbial profiles in supra- and subgingival plaque and of the cheek, tongue, and tonsil habitats.

Figure 1 presents the mean percentage of the DNA probe count of samples from the five different intra-oral habitats in the seven dogs. The microbial profiles differed markedly, with 34 of 40 species differing significantly among sample locations. In particular, proportions of *Aggregatibacter actinomycetemcomitans*-like species were significantly higher on the tonsil than on the other sample locations. When compared to the other habitats, the subgingival habitat shows significantly higher proportions of human *Prevotella intermedia*, *Streptococcus constellatus, Campylobacter rectus* and *Campylobacter showae*-like species whereas the proportion of bacteria that hybridized to the human *Actinomyces naeslundii* genospecies 2 probe was significantly lower. Similarly, lower proportions for *Parvimonas micra, Fusobacterium nucleatum ss. polymorphum* and *Streptococcus intermedius*-like species were detected in the supra gingival plaque. Proportions of *Eikenella corrodens*-like species

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were significantly higher on the soft tissues when compared to supra- and subgingival plaque while *Treponema denticola*like species proportions were significantly higher in supra- and subgingival plaque. *Porphyromonas gingivalis*-like species were one of the three most prevalent bacterial species in all sample locations without major site specificity. The two other most prevalent bacterial species showed some tissue-type specificity: *E. corrodens*-like species colonized preferably soft tissues and *T. denticola*-like species, hard tissues. Location specificity was observed for *Leptotrichia buccalis*-like species on the tongue, *A. actinomycetemcomitans*-like species on the tonsil, *Fusobacterium nucleatum ss. vincentii*-like species on the cheek, *Veillonella parvula*-like species in the supragingival and *Fusobacterium periodonticum*-like species in the subgingival plaque.

Cluster analysis was performed to seek similarities in microbial profiles among the five habitats (Fig. 2). Two clusters were formed with more than 80% similarity. One cluster comprised supra- and subgingival plaque (*i.e.* hard tissue habitats) while a second cluster was made up of the soft tissue habitats (*i.e.* tonsil, cheek, tongue).

3.2. Comparison of hard tissue and soft tissue samples.

Because of the results of the cluster analysis shown in figure 2, the data for the hard and soft tissues were averaged and compared. The microbial profiles for the soft and hard tissue habitats were markedly different with 19 of 40 species differing significantly between sample locations. The species that differentiated the two cluster groups are shown in figure 3. *P. gingivalis, T. denticola, Tannerella forsythia, S. constellatus, C. rectus* and *C. showae*-like species were in significantly higher proportions on hard tissue habitats, whereas *E. corrodens, A. actinomycetemcomitans, Fusobacterium nucleatum ss. nucleatum, P. micra, Prevotella melaninogenica, Prevotella nigrescens, A. naeslundii genospecies 2, Campylobacter gracilis, Streptococcus gordonii, Streptococcus oralis, Streptococcus intermedius, Selenomonas noxia* and *Actinomyces israelii*-like species were in significantly higher proportions in soft tissue habitats.

3.3. Comparison of hard tissue samples.

As shown in Fig. 4a, there is a statistically significant difference in the prevalence of 26 species between hard tissue locations (*i.e.* supra- and subgingival plaque). Of these 26 species, 20 species were present in higher proportions in the subgingival plaque. Supra gingival plaque contained

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however higher proportions of *P. gingivalis, F. periodonticum, F. nucleatum ss. vincentii, A. actinomycetemcomitans, Prevotella acnes* and *A. naeslundii* genospecies 2-like species.

3.4. Comparison of soft tissue samples.

In general, the proportions of the 40 different bacterial species are less different between the soft tissue habitats than between hard tissue habitats. As shown in Fig. 4b, there is a statistically significant difference in the prevalence of only 11 species on soft tissue habitats (*i.e.* cheek and tongue mucosa and tonsil). In particular, when compared to the other soft tissue locations, statistically significant different proportions can be detected on the cheek for *N. mucosa, F. nucleatum ss. vincentii* and *E. corrodens*-like species and on the tonsils for *A. actinomycetemcomitans* and *S. anginosus*-like species.

4. Discussion

Examination of complex mixtures of micro-organisms has been hampered in the past by at least two factors. The first is the tradition of focusing on a small number of species thought to be pathogenic. The second is the absence of reliable, rapid identification techniques to evaluate large numbers of bacterial species in large numbers of samples (Socransky and Haffajee, 2005). Historically, the supra- and subgingival plaque microbiota of dogs has been analyzed by culture techniques (Harvey et al., 1995). Recently, molecular techniques using 16S rRNA gene sequencing were used to study the canine oral microbiota, but were based on a bacterial culturing procedure (Elliott et al., 2005; Hardham et al., 2005). This is a time-consuming, labor intensive, and expensive undertaking that limited the number of samples which were examined. Additionally, culture techniques can underestimate the numbers and species depicted in a sample if the optimal culturing conditions are not met. For example, the recognized human periodontal pathogen *T. forsythia* was not detected by culture until the unusual growth requirements of this organism were determined. Checkerboard DNA-DNA hybridization has shown to overcome these problems.

Additionally, none of these studies (Harvey et al., 1995; Elliott et al., 2005; Hardham et al., 2005) analyzed the soft tissue microbiota and Elliott et al. (2005) did not differentiate between supra- and subgingival plaque in their analysis. The current study, in accordance with other human studies, clearly showed that there were microbiological differences between the sub- and supragingival

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microbiota. In accordance with the current study, Elliott et al. (2005) showed major differences in the microbiota between plaque samples and a pooled saliva sample. However, in this latter publication, the pooled saliva sample was frozen before culture, in contrast to the plaque samples; and it is likely that this influenced the relative proportions of bacterial species.

In the current study, as a proportion of the 40 species examined, 5% of the plaque bacteria belonged to the genera *Actinomyces*. This is slightly less than the proportion previously reported in dogs (Dent and Marsh, 1981; Elliott et al., 2005) and substantially less than proportions found in human plaque samples using the same checkerboard DNA-DNA hybridization (Ximenez-Fyvie et al., 2000). Our data showed that, similar to humans, *A. odontolyticus*-like species were one of the dominant species on the tongue of dogs (Hallberg et al., 1998; Mager et al., 2003). Additionally, these were also one of the dominant species in plaque and on soft tissue surfaces of the dog, a finding in contrast with the human data derived from oral samples (Mager et al., 2003). Similar to the human studies, *A. naeslundii* genospecies 2-like species were overall the dominant species in the dogs, its extreme low proportion in dog subgingival plaque tends to highlight the importance of the host on bacterial colonization patterns.

The proportions of streptococci were, as previously shown, slightly lower than those typically found in human plaque (Wunder et al., 1976; Dent and Marsh, 1981; Elliott et al., 2005). However, they were not so different to support the suggested hypothesis of streptococci fulfilling a different role in dog plaque than in human plaque (Wunder et al., 1976; Elliott et al., 2005). However, it should be pointed out that these latter 2 authors used culture dependent techniques to estimate the number of streptococci compared with the checkerboard DNA-DNA hybridization technique employed in the current study. Nonetheless, it has been shown that the recovery of streptococci could be increased by using an improved Mitis Salivarius agar medium (Takada et al., 2006). In accordance with the human microbiota, the dominant streptococcal species in our study was *S. mitis* (Frandsen et al., 1991; Mager et al., 2003). *S. sanguinis*-like species were a less dominant streptococcal species except in subgingival plaque. The low proportion of this species may explain why Syed and coworkers (1980; 1981) could not detect this species in dogs by bacterial culturing and is in accordance with previous work (Wunder et al., 1976; Takada et al., 2006).

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Although high proportions of *Fusobacterium*-like species could be detected from all sites sampled, the proportions were clearly lower than the 20-40% reported by Syed and coworkers (1980; 1981) but higher than for the *F. nucleatum*-like species reported by Elliott and coworkers (2005).

In contrast to the observations of Elliott and coworkers (2005), bacteria that hybridized to the human periodontopathogens probes, *P. gingivalis*, *T. forsythia*, *T. denticola,* also known as the "red complex", and *A. actinomycetemcomitans* were detected in high proportions in the current study. In addition, all dogs appeared to harbor these species. However, these findings are consistent with previous reports using different microbial identification techniques such as 16S rRNA gene sequences (Hardham et al., 2005), bacterial culturing (Syed et al., 1980; Dahlen et al., 1982; Allaker et al., 1997) or DNA hybridization (Norris and Love, 1999; Kohal et al., 2004). Interestingly, the proportions for "red complex" bacteria were much higher than the 7% (subgingival) and 2.8% (supragingival) which were reported for humans (Ximenez-Fyvie et al., 2000). The present study confirms that the soft tissues of dogs harbor relatively high numbers of *P. gingivalis*, *T. forsythensis*, *T. denticola,* and *A. actinomycetemcomitans*-like species. These surfaces could therefore act as a reservoir for recolonization of the hard tissues after debridement.

There were limitations to the current study. The 40 bacterial species that were examined were those thought to be important in human dental plaque and their relative proportions were calculated based upon the total load of these 40 species. Other species that may be present in even larger proportions than those examined in the current study, could have been overlooked.

Additionally, The checkerboard DNA-DNA hybridization technique uses whole genomic probes to identify the bacterial species. Whole genomic probes are constructed using the entire genome of a bacterial species as the target and thus can be quite sensitive. One of the criticisms of these probes is that the use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species (Socransky and Haffajee, 2005). Therefore, it is possible that the identification of the species in the dog, using the checkerboard technique and probes developed from human isolates, was not correct at species level. To stress this, we used the terminology "-like species". Similar species in different hosts (*i.e.* humans and dogs) can differ by almost 7% in the 16S rRNA gene (Ochman and Wilson, 1987). In addition to 16S rRNA gene differences, genetic differences will also be present. For example, human *P. gingivalis* isolates are catalase-negative whereas canine *P. gingivalis* isolates are catalase-positive (Harvey et al., 1995;

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Isogai et al., 1999). This recognition was the basis for reclassifying canine *P. gingivalis* to *Porphyromonas gulae* (Fournier et al., 2001). Therefore, the *P. gingivalis*-like species detected by the checkerboard DNA-DNA hybridization technique were most likely *P. gulae*. On the other hand, because the checkerboard DNA-DNA hybridization technique uses whole genomic DNA probes it allows the detection of closely related species, permitting its use for the examination of various biofilms in different hosts. Other concerns have been that the whole genomic DNA probes might not detect all strains of a given species and that the probes would have a low sensitivity in terms of the numbers of cells that they detect (Socransky and Haffajee, 2005). Investigations at The Forsyth Institute, however, using whole genomic DNA probes have indicated that many of the concerns regarding their use are unjustified or can be overcome (Socransky et al., 2004). Moreover, the sensitivity of the DNA probes is adjusted to detect 10^4 human cells and potential cross-reactivity was previously tested. In all, 93.5% of potential cross-reactions to 80 cultivable human species tested exhibited signals <5% of that detected for the homologous probe signal. The use of competitive hybridization and probes prepared by subtraction hybridization and polymerase chain reaction minimizes cross-reactions for closely related taxa. Nevertheless, in future studies of the oral bacterial ecology of dogs and the association of biofilms on different oral surfaces, specific probes made from specific canine species might be preferred in the checkerboard format to provide better clarification of the dog oral microbiota.

5. Conclusions

Within the limits that the current study only used 7 dogs which were co-localized and received the same diet, it was found that all of the test species could be detected on all sampled surfaces in the Beagle dogs. Further, it was demonstrated that each surface had a distinct community structure, underscoring the importance of the local habitat and the host on the local microbiota. The microbiotas colonizing the hard surfaces in the oral cavity were somewhat similar but quite different from the microbiotas colonizing the soft tissues. Further, bacterial species that hybridized to probes of bacterial species that are considered to be human periodontopathogens were present in high proportions in the dogs. These findings suggests that the beagle dog model may be useful for examining mechanisms associated with the initiation and progression of periodontal infections as well as therapeutic interventions that may have relevance in the human.

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6. Acknowledgements

This study was supported by the NIDCR (Bethesda, USA), grant DE015360 (Principal Investigator Marc Quirynen), by the National Fund for Scientific Research (Brussels, Belgium), grant G0240.04 and by the Catholic University Leuven (Leuven, Belgium), grant OT03/52. W. Teughels was supported by the K.U.Leuven Research fund.

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8. Figure captions

Figure 1. Mean percentage DNA probe count (+/- SEM) from samples from the 5 intraoral locations in the 7 dogs. Species are ordered according to their mean proportions in supra gingival plaque. Significant differences among sample locations were determined using Kruskal Wallis test and adjusted for multiple comparisons (Socransky et al., 1991). The letters behind bars reflect a significant difference with one of the 5 different sample locations (a: supra gingival plaque; b: subgingival plaque; c: tongue; d: tonsil; e: cheek).

Figure 2. % similarity (minimum similarity coefficient). Dendrogram of a cluster analysis of the mean species proportions from the 5 sample locations. A minimum similarity coefficient was employed and an average unweighted linkage sort. Two clusters were formed at > 75% similarity.

Figure 3. Mean percentage DNA probe count (+/- SEM) from samples from the soft tissue and hard tissue locations in the 7 dogs. The data of all soft tissue habitats (tongue, tonsil cheek) and hard tissue habitats (supra and sub gingival plaque) were averaged. Species are ordered according to their mean proportions on hard tissue locations. Significant differences among sample locations were determined using Kruskal Wallis test and adjusted for multiple comparisons (Socransky et al., 1991). The grey shading represent significant differences between both habitats.

Figure 4.a. Mean percentage DNA probe count (+/- SEM) from samples from the hard tissue locations in the 7 dogs. Species are ordered according to their mean proportions in supra gingival plaque. Significant differences among sample locations were using Kruskal Wallis test and adjusted for multiple comparisons (Socransky et al., 1991). The grey shading represent significant differences between both habitats.

Figure 4.b. Mean percentage DNA probe count (+/- SEM) from samples from the soft tissue locations in the 7 dogs. Species are ordered according to their mean proportions on the tongue. Significant differences among sample locations were determined using Kruskal Wallis

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test and adjusted for multiple comparisons (Socransky et al., 1991). The letters behind bars reflect a significant difference with one of the 5 different sample locations (a: tongue; b: tonsil; c: cheek).

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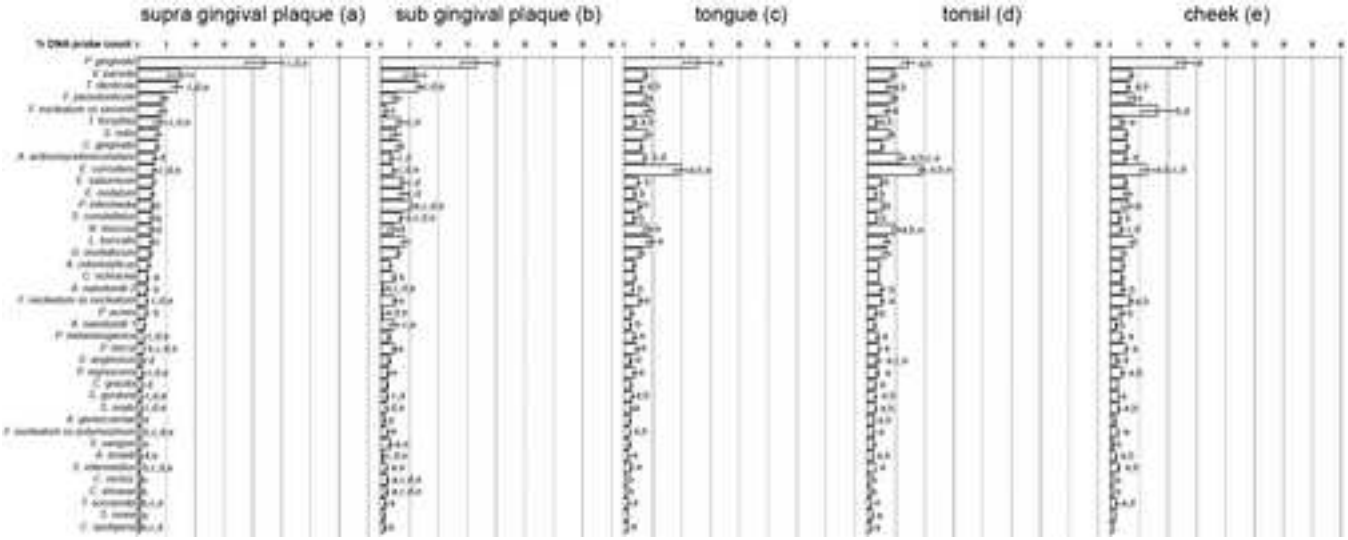

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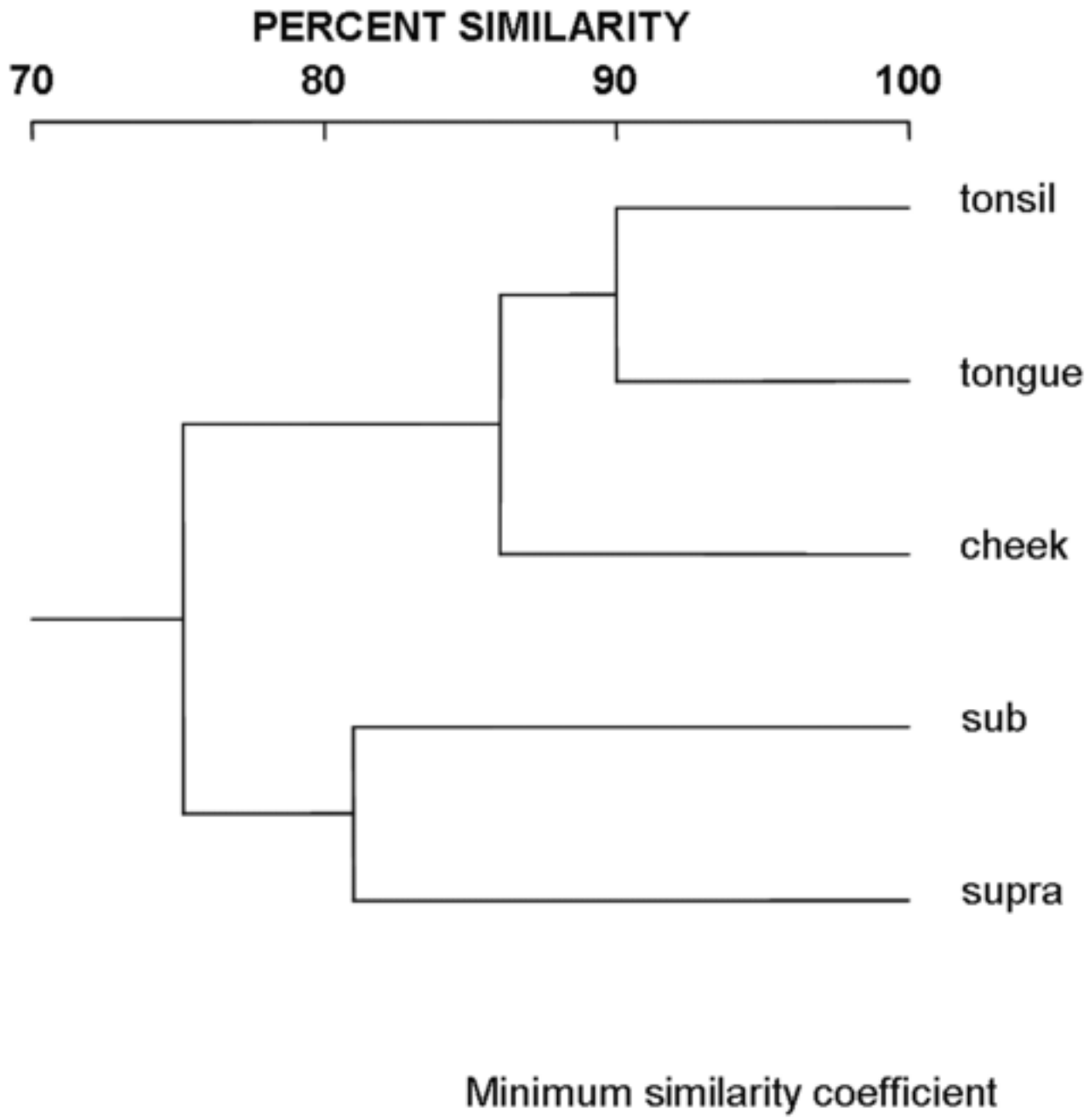

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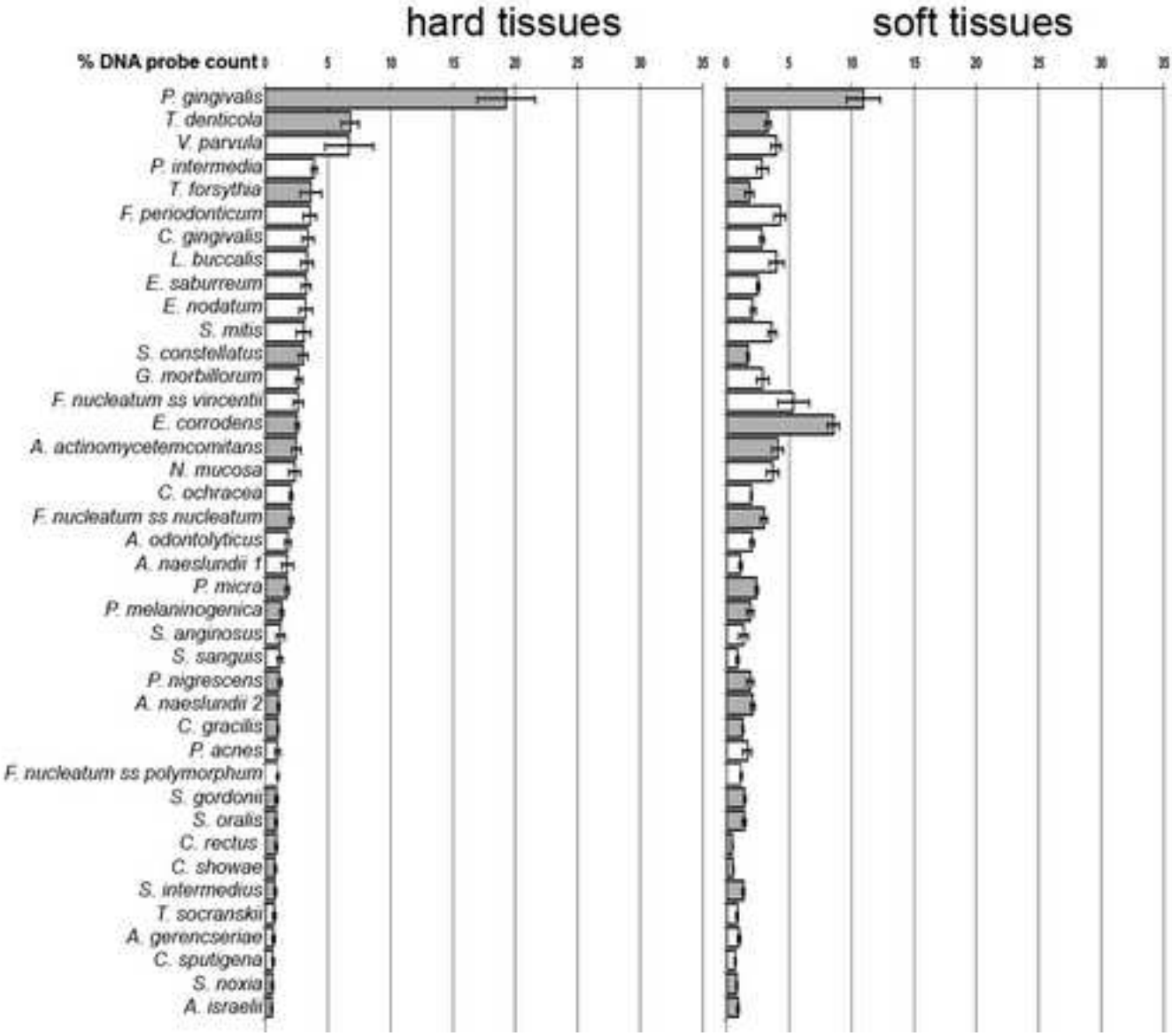

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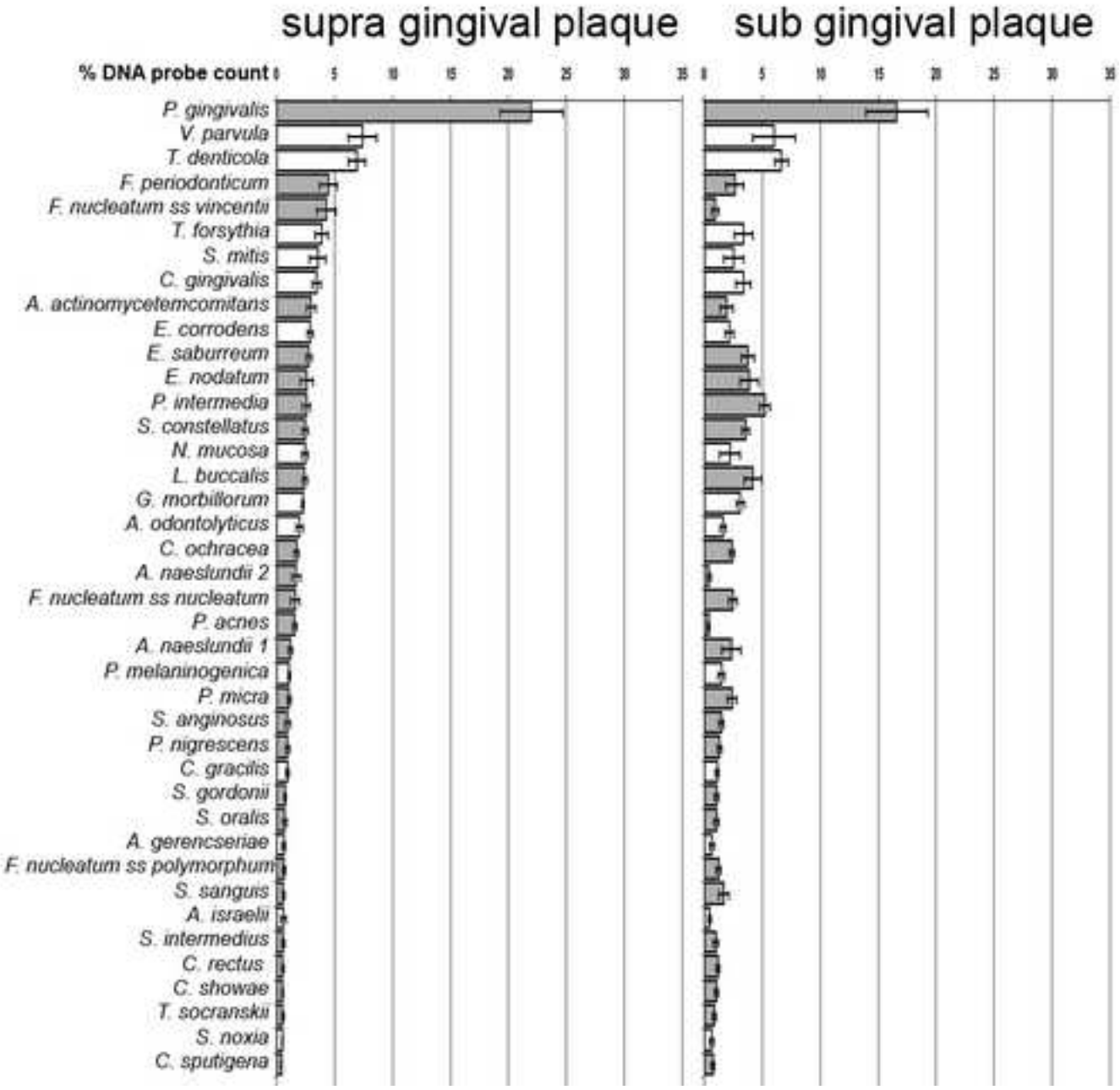
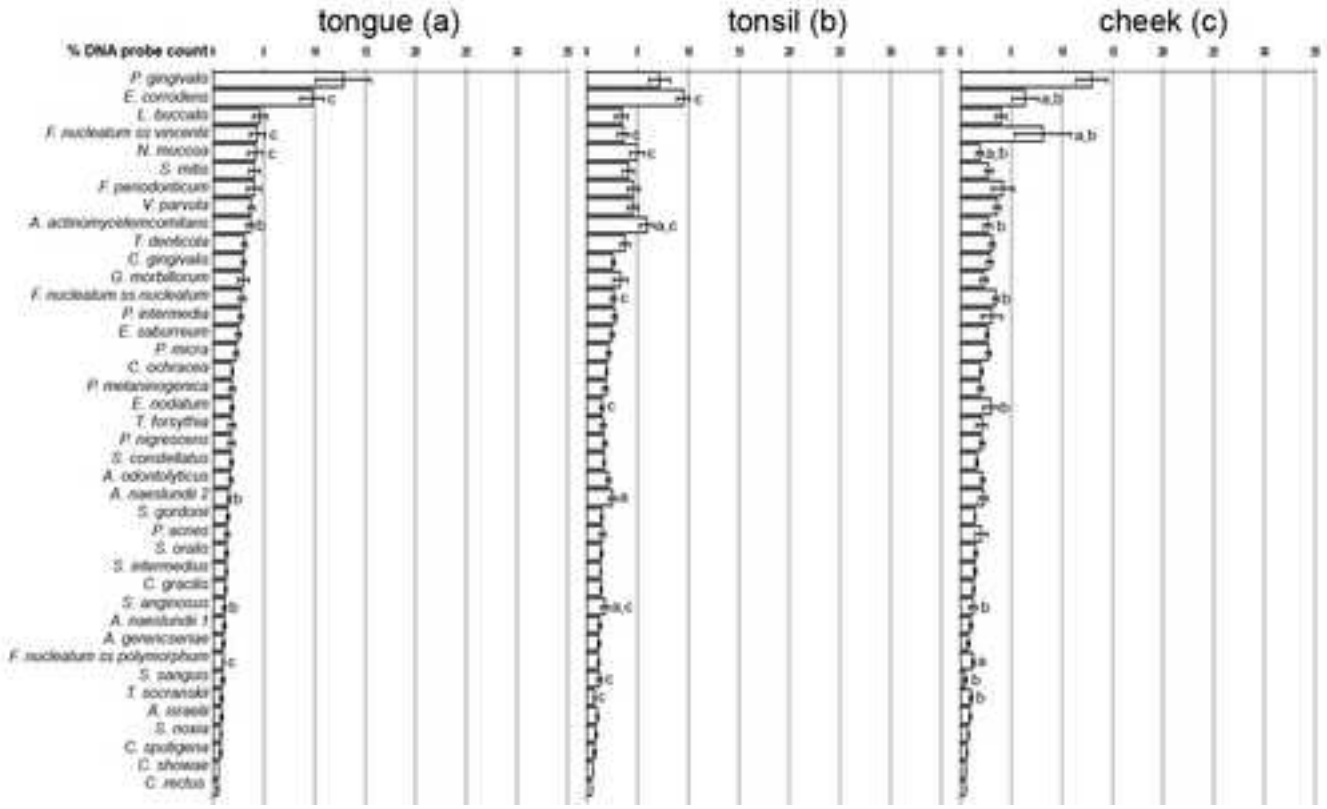
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