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Subgingival microbial profiles as diagnostic markers of destructive periodontal diseases: A clinical epidemiology study

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Abstract

Aims. To describe the subgingival microbial profiles of the major putative periodontal pathogens and investigate their role as diagnostic markers for destructive periodontal diseases in an untreated and isolated population. ***Materials and methods.*** The source population consisted of all subjects aged ≥ 12 years in an isolated Brazilian population. An interview and a full-mouth clinical examination were conducted and subgingival plaque samples were obtained from four sites per subject. PCR analyses were used to identify the following micro-organisms: *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia, Prevotella intermedia* and *Campylobacter rectus.* ***Results.*** Among the 214 clinically examined subjects (81% response), 170 of the 195 dentate subjects provided plaque samples. Two subgingival microbial profiles were identified: absence of all micro-organisms but *Campylobacter rectus* or co-occurrence of *Tannerella forsythia* and *Porphyromonas gingivalis.* Using a combination of microbiological and interview information, the smallest overall misclassification in the diagnosis of extensive clinical attachment loss ≥ 5 mm was 8.8% (4.7% of non-cases and 22% of cases), but this was not different from the 7.6% (2.3% non-cases and 24.4% cases) obtained using clinical and interview information ($p = 0.292$). ***Conclusion.*** Specific microbial profiles could be identified in this isolated population. They did not result in significant superior diagnostic accuracy when compared to traditional clinical markers.

Key Words: *epidemiology, biofilm, microbiology, periodontal diseases, specificity, sensitivity*

Introduction

It is generally accepted that destructive periodontal diseases are caused by a consortia of micro-organisms that interact in a highly structured and spatially organized biofilm [1], displaying properties that are more than the sum of its constituent members [2].

The possible relationship of bacterial consortia in the subgingival biofilm was first described by Socransky et al. [3] in 1998. Although this is the reference study for understanding the microbial complexes in the subgingival plaque in relation to periodontal diseases, it is based on site-specific information on probing depth (PD) and bleeding on probing (BoP), mostly from periodontal patients seeking dental treatment. Therefore, it is of extreme importance that further research evidence with subject-centered analyses is conducted in the field to investigate micro-organism co-occurrence in the subgingival biofilm of specific populations [4].

In clinical periodontology, it was promoted as common practice to sample subgingival plaque from periodontal patients to identify alleged periodontal pathogens using routine laboratory techniques such as culture, DNA–DNA hybridization or PCR [5]. This screening has been implicated as a way to improve periodontal diagnosis [3,6] and thereby also the therapeutic outcome in periodontitis subjects. In a more distant perspective improved outcomes might be achieved through the identification [7] or

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vaccination [8] of sites or subjects that present a high risk of periodontal destruction. As clinical periodontal diagnostic tools are crude (a blunt metal probe with millimeter increments) [9], it is indeed possible that specific laboratorial microbiological profile tests could complement the periodontal clinical examination [10,11] or provide a more accurate and precise indicator of ongoing destructive periodontal disease [12]. However, there is additional need for research in the role of microbial testing for the diagnosis of periodontal disease or health states. Hence, a recent systematic review concluded that there is no strong evidence supporting the benefit of microbial testing, partly because of heterogeneity between studies and party due to a lack of high quality studies, as the evidence-base chiefly consists of case reports or case series without controls [13].

Epidemiological studies of isolated populations permit the investigation of the periodontal microbial profile from an undisturbed perspective, since the absence of supra- and subgingival therapeutic interventions and the limited tradition for oral hygiene procedures provide an opportunity to assess the established subgingival microbiota, rather than a microflora characterized by a transient colonization pattern [12,14]. Using a clinical epidemiological approach, the present study aims to describe the patterns of occurrence of five of the major periodontal pathogens and to investigate their role as adjunct diagnostic markers for periodontal diseases in an untreated and isolated population from Brazil.

Materials and methods

Study population

The study population was defined as all subjects living in the micro-area of Cajaíba, which consists of six beaches located along the south-eastern coastline of Brazil between the states of Rio de Janeiro and Sao Paulo. Cajaíba is effectively isolated from the landside by the impenetrable Juatinga ecologic reserve and its impassible mountain range [15]. Entry to the area may be made from the seaside, but, owing to the presence of the Cajaíba and Juatinga headlands, the distances to towns outside the area are substantial, just as economic constraints serve to maintain the population in isolation. Consanguinity is frequent among the inhabitants, most of whom live from subsistence fishing. Neither dental nor medical care has been offered to this population, preventive programs are non-existent and access to dental services is very limited. No tradition for oral hygiene practices is established among the inhabitants. The present study was made possible when the Non-Governmental Organization (NGO) named 'Sorriso Marinho' ('Marine Smile') made their way to the area with a view to provide emergency dental assistance in the form of tooth extractions and temporary fillings.

Since no updated information was available regarding the demographics of this population a census was performed prior to the study, which identified 264 subjects aged 12–82 years [16]. The study protocol and the informed consent form were reviewed and approved by the Ethics Committee of the University of Sao Paulo, Sao Paulo, Brazil. More detailed information about the study population and ethical considerations may be found elsewhere [16,17].

Data collection

Interview data. All 264 eligible subjects were visited at home during October 2005 and November 2006 and were interviewed by a trained dental assistant based on a structured written questionnaire. The interview questions were tested prior to the survey and the reproducibility of the final questionnaire was assessed [16]. The data collected through the interview included information on age (years), gender, occupation, income, literacy (yes/no), length of education (years), experience of relief-of-pain treatment (yes/no), frequency of tooth cleaning and smoking habits. The questions pertaining to smoking habits included duration of smoking habit for both current and former smokers (years); as well as the type and number of tobacco-containing items smoked on a daily basis for both current and former smokers. The tobacco-containing items used in the study population included commercial cigarettes or hand-rolled cigarettes (or corn straw hand-rolled cigarettes) and, in a few cases, cannabis and pipes. Subjects were considered smokers if they had smoked at least 100 cigarettes or the equivalent thereof during lifetime [18]. A few of the elderly subjects were unaware of their exact age, which was therefore estimated. Screening for diabetes was also carried out by measurements of the casual plasma glucose concentrations [16], but as only two dentate persons were screen-positives, this variable was not considered any further.

Clinical data. The clinical examinations were carried out by a single trained and calibrated periodontal specialist (PC) assisted by a recorder. All clinical examinations were performed under field conditions in the households of the participants using natural daylight and a headlamp as a source of illumination.

Probing depth (PD) and gingival recession (GR) were measured at six sites per tooth in all permanent teeth present, excluding third molars, using a mouth mirror (Mirror # 5, Hu-Friedy, Chicago, IL) and a manual periodontal probe (PCPUNC15, Hu-Friedy, Chicago, IL). The measurements were rounded to the lowest whole millimeter. The clinical attachment level (CAL) was calculated as the sum of the PD and GR values for each site. Two sites per tooth (midbuccal

and midlingual) were assessed for the presence of visible plaque (yes/no) and supragingival calculus (yes/no). Excessive amounts of supragingival calculus compromising the assessment of the periodontal conditions were removed by periodontal curettes (Gracey Curettes 5/6, 11/12, 13/14 Hu-Friedy, Chicago, IL) before probing. Measurement reproducibility of CAL and PD has previously been described [16,17]. Intra-examiner reproducibility was assessed by double recordings in 13 subjects (~ 6% of the study population), repeated 7 days after the first clinical examination. The intra-class correlation coefficient at the site level ranged between 0.87–0.90 for PD and 0.93–0.95 for CAL measurements and at the subject level (mean value) between 0.98–0.99 for both parameters.

Subgingival plaque sampling and laboratory procedures

The collection of the subgingival plaque samples was performed after the clinical examination by the same examiner. Four sites per subject were sampled: In subjects with PD ≤ 4 mm, four randomly chosen sites were sampled, whereas in subjects with at least one site with PD ≥ 4 mm we sampled the site with the highest PD per quadrant. The four subgingival samples were subsequently pooled to provide one sample per subject.

After careful removal of supragingival deposits with sterile periodontal curettes and isolation of the area with sterile cotton rolls, sterile paper points were inserted to the depth of the sulcus/pocket. The paper points were removed after 20 s and immediately placed in a microtube containing reduced Ringer's solution (1 ml) (Oxoid Ltd, Basingstoke, Hampshire, UK). The samples were kept at −20°C for 3–4 days until transportation to the laboratory of molecular biology, Taubaté University, Taubaté, Brazil, where they were stored at −80°C until processing.

The bacterial cells in the microtube were dispersed using a vortex mixer at maximal setting for 1 min. After this, the bacterial suspensions were thawed and centrifuged at 12 000 rpm for 3 min and the genomic DNA was extracted using PureLink™ Genomic DNA Purification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The presence of *Agregactibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Porphyromonas gingivalis* (*P. gingivallis*), *Tannerella forsythia* (*T. forsythia*), *Prevotella intermedia* (*P. intermedia*) and *Campylobacter rectus* (*C. rectus*) was established by a polymerase chain reaction (PCR) using the specific primers shown in Table I, in a Mastercycler Gradient thermocycler (Eppendorfs, Westbury, NY) under standard conditions [19].

The PCR products were separated on a 1.5% agarose gel (Invitrogen) by electrophoresis performed at 5 V/cm in Tris-acetate-EDTA buffer. The DNA bands on the gel were stained with 0.5% μg/ml ethidium bromide (Amersham, Arlington Heights, IL) and photographed under 300 nm UV light illumination. Positive and negative controls for all five species were included (Figures 1A–E) in order to check the primer specificity and eliminate the risk of an eventual DNA contamination. The positive controls were obtained by the amplification of standard micro-organisms (provided by the Osvaldo Cruz Foundation, FioCruz, RJ, Brasil) in the gels. The negative controls were employed using all the reagents needed for the amplification with the addition of ultrapure water instead of the microbial sample.

Data analyses

The co-occurrence of the micro-organisms was assessed by generating a pattern variable describing the simultaneous presence or absence of the five species. In principle 2^5 different patterns were possible. Using decision tree software (SmartDraw for Windows), the observed microbial patterns were illustrated by entering species according to increasing overall frequency of detection.

Table I. Primers used in the present study.

Target species	Probe sequence (5′-3′)	Product size
A. actinomycetemcomitans	5′-ATGCCAACTTGACGTTAAAT-3′ 5′-AAACCCATCTCTGAGTTCTTCTTC-3′	557 bp
P. intermédia	5′-TTTGTTGGGGAGTAAAGCGGG-3′ 5′-TCAACATCTCTGTATCCTGCGT-3′	575 bp
P. gingivalis	5′-AGGCAGCTTGCCATACTGCG-3′ 5′-ACTGTTAGCAACTACCGATGT-3′	404 bp
T. forshytia	5′-GCGTATGTAACCTGCCCGCA-3′ 5′-TGCTTCAGTGTCAGTTATACCT-3′	641 bp
C. rectus	5′-TTTCGGAGCGTAAACTCCTTTTC-3′ 5′-TTTCTGCAAGCAGACACTCTT-3′	598 bp
Universal	5′-GATTAGATACCCTGGTAGTCCAC-3′ 5′-CCCGGGAACGTATTCACCG-3′	602 bp

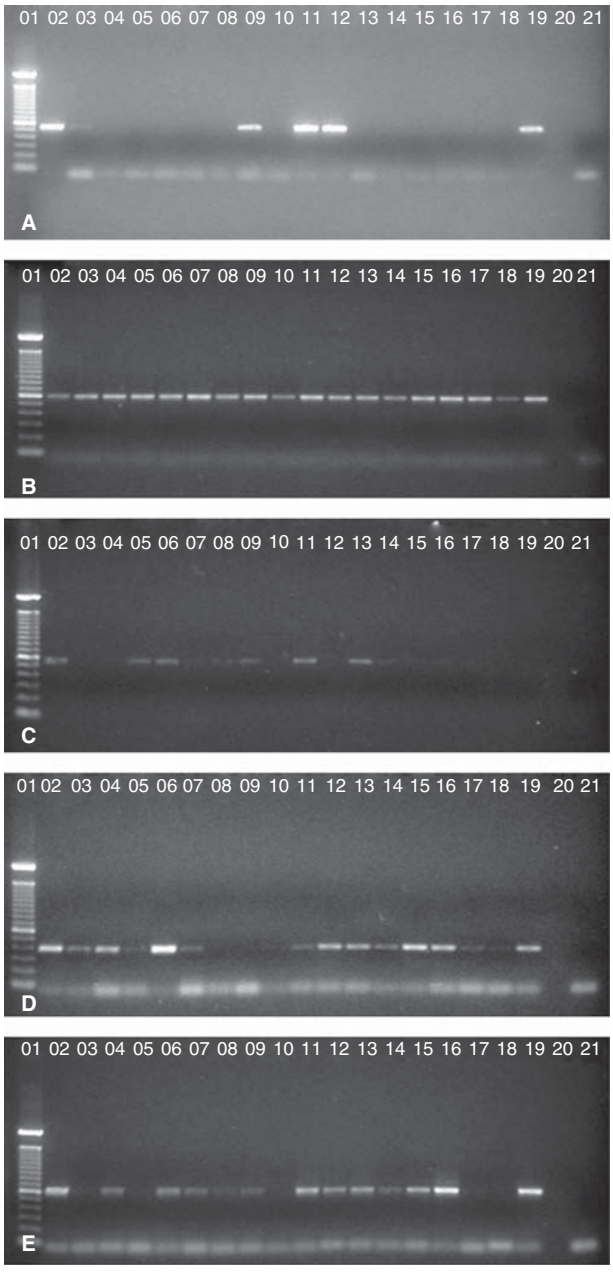

Figure 1. Agarose gels indicating the product of the PCR primers of: (A) *A.actinomycetemcomitans*, (B) *C. rectus*, (C) *P. intermedia*, (D) *P. gingivalis* and (E) *T. forsythia*. 01 – Ladder 100 bp, 02 – Positive control, 3–19 – Plaque samples, 21 – Negative control.

To assess the diagnostic accuracy of the dominant specific microbial profiles identified for the diagnosis of selected periodontal conditions, the overall misclassification percentage and the sensitivity and specificity values were calculated using Stata (StataCorp, College Station, TX). The sensitivity is a measure of the ability of the presence of a profile to correctly identify subjects with disease (outcome), whereas the specificity is a measure of the absence of the profile to identify absence of disease. Due to the lack of a widely accepted reference standard for the defining criteria for periodontitis [8], the following six periodontal case definitions were considered to represent clinical surrogates [20] for destructive periodontal diseases: Prevalent (at least one site with the condition) and extensive CAL ≥ 5 mm, ≥ 7 mm or PD ≥ 5 mm, respectively; where extensive was defined as having at least 30% of the sites affected.

In order to assess the value of the detection of the micro-organisms as potential diagnostic markers of the above periodontal conditions, a series of decision tree analyses were performed using the DTREG software (Phillip H. Sherrod, TN). In decision tree analysis, the decision tree is constructed by a binary split of the outcome (periodontal case status) variable into two 'child nodes'. The same process, called recursive partitioning, is then used to split the child nodes, and the resulting decision tree may be used to predict the value of the outcome variable. For each split, two decisions are made by the software: which predictor variable to use for the split and which set of values of the predictor variable go into the left, respectively the right, child node that results from a split. In principle, the software is able to build a tree so large that each subject ends up in their own terminal node. Such is not very informative and two options exist for stopping the recursive partitioning process. One option is to specify the minimum size of a node to split, while another option is to specify the maximum number of levels in the tree. For the purpose of the present analyses up to four levels were used in the decision tree analyses. Based on the decision trees we calculated the overall misclassification percentages, as well as the percentage of cases and non-cases—according to the case-definitions outlined above – that were misclassified by the decision tree algorithm.

In Scenario 1, the best predictors of periodontal case status (defined above) were sought among the five microbial candidate predictor variables along with age (12–19/20–29/30–39/40–49/50 + years), gender and smoking (never/current or former). In Scenario 2, the microbial variables were replaced by the clinical variables 'proportion of sites with supragingival calculus (<20%/20–50%/>50%)' and 'proportion of sites with visible plaque (<75%/≥75%)'. Figure 2 shows an example of the graphical output of decision tree analysis using Scenario 1 in the prediction of subjects with prevalent CAL ≥ 5 mm allowing for three levels in the tree.

Statistical tests were carried out using Student's *t*-test and Chi square tests as appropriate using a significance level of 5% ($\alpha = 0.05$).

Results

A total of 264 subjects aged 12–82 years were eligible for examination in this survey. Of the 214 subjects who consented (81.1% response) and received an interview and a clinical examination [16], 195 were

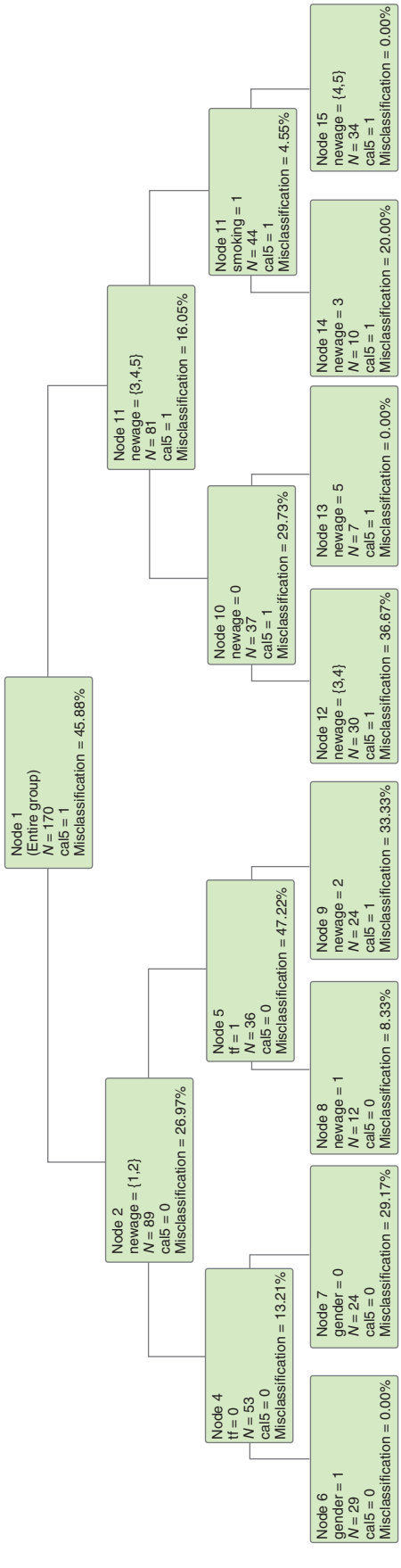

Figure 2. Example of a complex decision tree in DTREG. It describes a tree with a maximum of three levels allowed at predicting the outcomes prevalent CAL ≥ 5 mm, from the variables described for Scenario 1.

dentate and a total of 170 pooled subgingival plaque samples were obtained from them. No significant differences were found among those providing ($n = 170$) or not subgingival plaque samples ($n = 25$) (Table II).

PCR amplification was successful for all 170 subgingival samples. In seven subjects (4%) none of the five target species were detected. *C. rectus* was found in 154 subjects (91%) and in view of its ubiquity it was not considered in further co-occurrence analyses.

Among the combinations of the remaining four species, two main microbial profiles were identified. In Profile 1, none of the four micro-organisms were present ($n = 31$) and in Profile 2, both *T. forsythia* and *P. gingivalis* were present ($n = 77$) (Figure 3). Profile 1 showed very low sensitivity values, indicating that all four microbial species were rarely absent when disease was present and moderately high specificity values, resulting in a misclassification percentage ranging between 21.2% (extensive PD ≥ 5 mm) and 65.3% (prevalent CAL ≥ 5 mm). Profile 2 showed varying sensitivity values for the identification of the various periodontal states, ranging from 0–100%, and low-to-moderate specificity values (ranging from 54.4–76.9%). The overall misclassification percentage ranged from 28.8–43.5%, being highest for the two rare periodontal outcomes (extensive CAL ≥ 7 mm and extensive PD ≥ 5 mm) (Table III).

Table IV describes the results of the decision tree analyses of the two sets of predictors, Scenario 1 with microbiological information or Scenario 2 with clinical information, in the correct classification of subjects with prevalent or extensive CAL ≥ 5 mm. In Scenario 1, no improvement in the classification of subjects with extensive CAL ≥ 5 mm were seen beyond a two-level classification tree, which was based on the predictors age and *T. forsythia*; whereas a four-level classification tree provided the best prediction of prevalent CAL ≥ 5 mm. Correct classification of cases was better obtained using the Scenario 1 predictors ($p < 0.01$), whereas correct classification of non-cases was better achieved using the Scenario 2 predictors ($p < 0.01$). However, the overall misclassification percentages were rather similar across the two scenarios.

Figure 2 shows the actual decision tree corresponding to the 3-level decision tree generated in the framework of Scenario 1 (Table III). The tree shows that the absence of *T. forsythia* among subjects aged < 30 years was strongly associated with the absence of CAL ≥ 5 mm (0% misclassification); whereas subjects aged 30 + years who had a smoking history were quite likely to have CAL ≥ 5 mm (0% misclassification).

No classification trees could be built for the periodontal outcomes extensive CAL ≥ 7 mm and extensive PD ≥ 5 mm, owing to very few subjects in these categories. The only micro-organism which could be used to predict prevalent CAL ≥ 7 mm was *T. forsythia*. Again, no significant reduction ($p = 0.421$) in the

overall misclassification percentage was achieved when using the four-level decision tree as the best classification of subjects with CAL ≥ 7 mm for Scenario 1 predictors (9.4% using age, *T. forsythia*, smoking story and gender) compared to Scenario 2 predictors (10.0% using information about age, smoking story, gender, subgingival calculus and visible plaque), although the combination of age and *T. forsythia* from Scenario 1 predictors were statistically significant better in correctly classifying cases with prevalent CAL ≥ 7 mm (misclassification 27.7%) than were the corresponding Scenario 2 predictors comprising age, smoking history and percentage of sites with supragingival calculus (36.2% misclassification) ($p = 0.02$).

Table V shows the results of the decision tree analyses for subjects with prevalent PD ≥ 5 mm. Among the microbiological predictors, *T. forsythia* was again the most important, followed by *P. intermedia* and *C. rectus*. The inclusion of microbiological predictors resulted in a substantially better classification of cases ($p \leq 0.01$) but a substantially worse identification of non-cases ($p < 0.01$) than did the use of predictors from Scenario 2. The overall misclassification did not differ significantly whether predictors were chosen from Scenario 1 or 2 ($p \geq 0.08$).

Discussion

In this study we have explored the patterns of co-occurrence of five putative periodonto pathogens in an entire population characterized by limited tradition for oral hygiene procedures and no access to periodontal treatment and studied their utility as markers of periodontal destruction.

Our analyses showed two main profiles among the five micro-organisms studied: One profile was characterized by the absence of all micro-organisms except the nearly omnipresent *C. rectus*. In the other profile, *T. forsythia* and *P. gingivalis* were co-occurring. The latter profile corroborates the characteristics of the red complex previously described [3]. Some possible mechanisms underlying the co-occurrence of these species include the higher binding properties (co-aggregation or co-adhesion) of *P. gingivalis* to the primary colonizers in the subgingival biofilm [21,22], whereby *P. gingivalis* may be regarded a secondary colonizer [23].

The utility of the two identified microbial profiles as sole diagnostic markers of destructive periodontal disease was limited. Even though the profile involving the absence of *P. g*, *T. f*, *P. i* and *A. a* showed relatively high specificity values for extensive periodontal destruction (Table II), it was also clear that the misclassification of subjects was considerable. Hence, the discrimination using the two microbial profiles as the sole diagnostic markers was associated with misclassification percentages ranging from 30–80%, which effectively prevents their use for individual level diagnostic purposes. This is perhaps not surprising since the occurrence of both prevalent and extensive destructive periodontal disease, as defined here, are known to increase with increasing age, pointing to higher age as a key determinant. The need to consider

Table II. Demographic, biological, behavioral and clinical parameters among the dentate subjects who provided ($n = 170$) or didn't provide subgingival microbial samples ($n = 25$).

Parameter	Levels	With subgingival samples ($n = 170$)	Without subgingival samples ($n = 25$)	p-value
Age group, % 30 + years		47.7	52.0	0.68
Gender, % male		54.7	48.0	0.53
Illiteracy, %		30.6	24.0	0.50
Current or former smokers		36.5%	48.0%	0.27
Diabetes mellitus		1.2%	0%	0.59
Ever urgency treated subjects		60.0%	68.0%	0.44
Mean (SD) number of teeth present		20.9 (7.2)	19.6 (8.9)	0.41
% sites Supragingival Calculus	20–50%	34.1%	36.0%	0.85
	>50%	29.4%	36.0%	0.50
Prevalent CAL	≥5 mm	54.1%	48.0%	0.57
	≥7 mm	27.7%	36.0%	0.39
Extensive* CAL	≥5 mm	11.5%	21.2%	0.17
	≥7 mm	4.9%	7.0%	0.66
Prevalent PD ≥ 5 mm		36.5%	36.0%	0.96
Extensive* PD ≥ 5 mm		2.7%	3.9%	0.74

*≥30% sites with the condition.

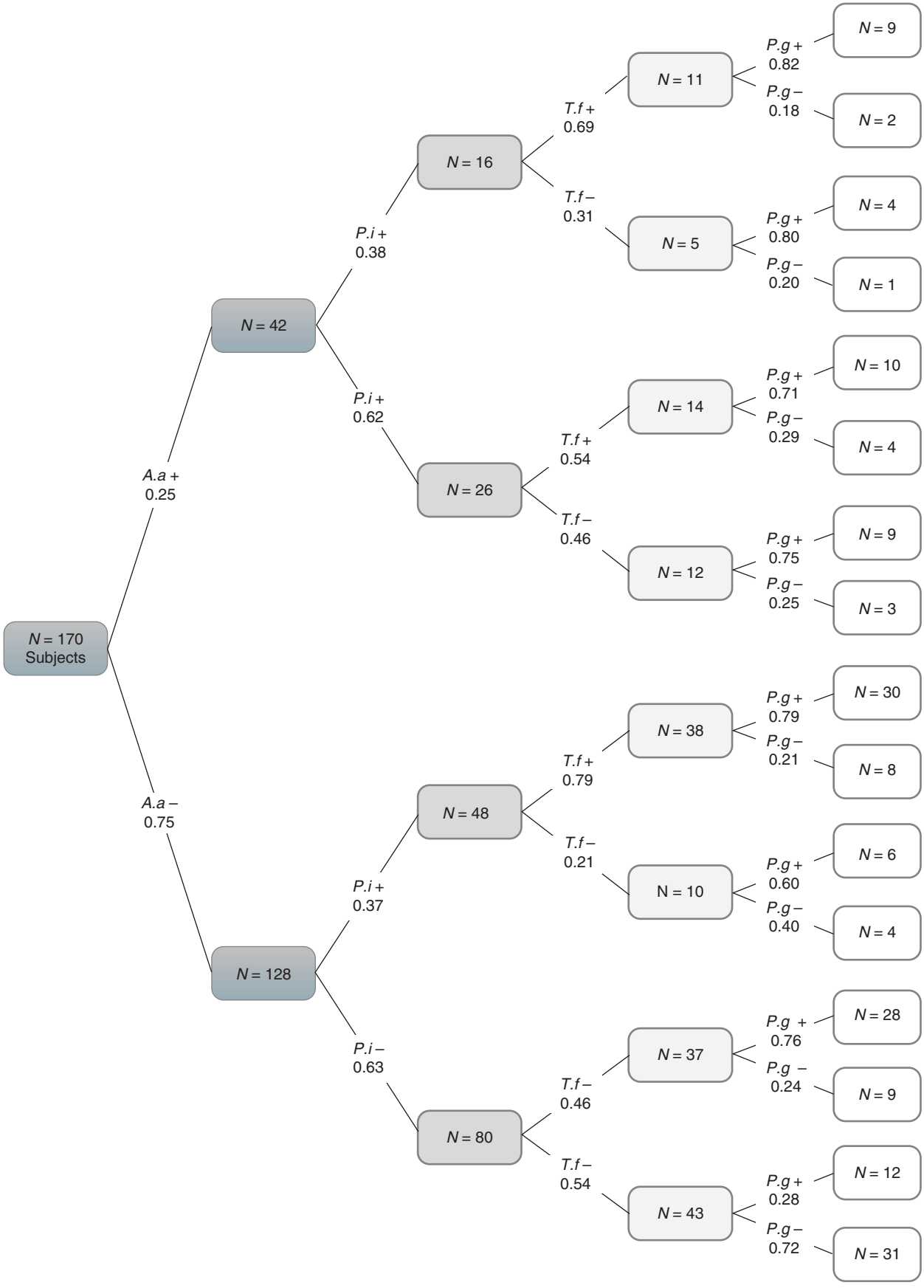

Figure 3. Decision tree analyses for the entire study sampled population.

Table III. Overall misclassification percentage, sensitivity and specificity of the two microbial profiles in distinguishing subjects according to periodontal disease status. Prevalent refers to the presence of one or more sites with the outcome whereas extensive means that at least 30% of the sites are affected.

Periodontal diagnosis		Microbial Profile 1 (absence of *T.f*, *P.g*, *P.i*, and *A.a*)			Microbial Profile 2 (presence of both *T.f* and *P.g*)		
		Misclassification	Sensitivity	Specificity	Misclassification	Sensitivity	Specificity
CAL ≥ 5 mm	Prevalent	65.3%	6.5%	67.9%	30.0%	64.1%	76.9%
	Extensive	41.2%	0%	81.5%	31.8%	50.0%	54.8%
CAL ≥ 7 mm	Prevalent	44.7%	2.1%	75.6%	27.1%	83.0%	69.1%
	Extensive	31.8%	0%	81.7%	35.3%	0%	54.4%
PD ≥ 5 mm	Prevalent	52.3%	3.2%	73.1%	28.8%	72.6%	70.4%
	Extensive	21.2%	0%	81.7%	43.5%	100%	55%

age was confirmed in the two sets of analyses where either microbiological or additional clinical information was considered in addition to the demographic and behavioral variables, as age was found to be the first predictor in the decision tree analysis of the periodontal status variables based on CAL recordings (Table IV). The effect of age on the prevalence and extent of PD was less strong, as manifested by age entering as a predictor only at level two in the decision tree analyses.

As regarding the analyses using either microbiological or clinical oral hygiene-related information in addition to demographic and behavioral information, they showed an interesting contrast, most clearly discernible in the analyses based on destructive periodontal disease defined by PD ≥ 5 mm and to a lesser extent also by CAL ≥ 5 mm. The use of the microbial information (Scenario 1) led to less misclassification of the cases, whereas use of the oral hygiene-related clinical information (Scenario 2) led to less misclassification of the non-cases (Table V). Unfortunately, the data material was too small to allow us to clarify if similar results would emerge when considering all possible predictors in a unifying analysis.

A number of clinical [3,19,24,25] and epidemiological studies [12,26,27] have related the presence of subgingival microbial species to either healthy or diseased/progressing periodontal sites. However, a paucity of them [3] has investigated how combinations of species, microbial consortia, associate with different states of periodontal health or disease. Our observation that the microbial profile characterized by the absence of all micro-organisms but *C. rectus* was rarely identified among periodontal cases, whether defined by prevalent or extensive CAL ≥ 5 mm, CAL ≥ 7 mm or PD ≥ 5 mm, is in agreement with studies of continued periodontal health during maintenance therapy [28]. Profile 2, which was

Table IV. Demographic, behavioral and biological with microbiological (Scenario 1) or selected clinical information (Scenario 2) in the diagnosis of subjects with prevalent and extensive CAL ≥ 5 mm. Prevalent refers to the presence of one or more sites with the outcome, whereas extensive means that at least 30% of the sites are affected. All candidate predictors are described in decreasing order of importance.

			Misclassification %					Misclassification %		
N tree levels	Scenario 1	CAL ≥ 5 mm	Total	Non cases	Cases	Scenario 2	CAL ≥ 5 mm	Total	Non cases	Cases
1	Age★	Prevalent	21.8	16.7	26.1	Age★	Prevalent	21.8	16.7	26.1
		Extensive	10.0	8.5	14.6		Extensive	10.0	8.5	14.6
2	Age★, *T.f*, SM	Prevalent	21.8	16.7†	26.1	Age, VP, SC	Prevalent	20.0	9.0	29.3
	Age★, *T.f*	Extensive	8.8	4.7	22.0	Age, SC	Extensive	8.8	3.9	24.4
3	Age★, *T.f*, SM, G	Prevalent	17.1	26.9†	8.7†	Age, SC, VP	Prevalent	18.2	15.4	20.7
	Age★, *T.f*, SM, *C.r*	Extensive	8.8	4.7	22.0	Age, SC, SM, VP, G	Extensive	7.6	4.6	17.0
4	Age★, *T.f*, SM, G, *A.a*, *C.r*, *P.g*	Prevalent	16.5	26.9†	7.6†	Age, SC, VP, SM, G	Prevalent	17.6	10.3	23.9
	Age★, *T.f*, SM, *C.r*, G	Extensive	8.8	4.7	22.0	Age, SC, SM, VP, G	Extensive	7.6	2.3	24.4

★Age was here categorized as 12–19, 20–29 and 30 + years.
†$p \leq 0.01$ for test of the null hypothesis that the scenario 1 and scenario 2 misclassification percentages are the same.
SM, smoking history; G, gender; VP, visible plaque; SC, supragingival calculus.

Table V. Demographic, behavioral and biological with microbiological (Scenario 1) or selected clinical information (Scenario 2) in the diagnosis of subjects with prevalent PD ≥ 5 mm (presence of one or more sites with the outcome). All candidate predictors are described in decreasing overall order of importance.

			Misclassification %					Misclassification %		
N tree levels	Scenario 1	PD ≥ 5 mm	Total	Non cases	Cases	Scenario 2	PD ≥ 5 mm	Total	Non cases	Cases
1	*T.f*	Prevalent	31.2	41.7†	12.9†	SC	Prevalent	25.3	17.6	38.7
2	*T.f*, age⋆, *P.i*	Prevalent	25.3	31.5†	14.5†	SC, age	Prevalent	25.3	17.6	38.7
3	*T.f*, age⋆, SM, *P.i*	Prevalent	24.1	33.3†	8.1†	SC, age, VP, G	Prevalent	25.3	17.6	38.7
4	*T.f*, age⋆, SM, *P.i*, *C.r*	Prevalent	21.8	20.4	24.2†	SC, age, VP, SM, G	Prevalent	22.9	16.7	33.9

⋆Age was here categorized as 12–19, 20–29 and 30 + years.
†$p \leq 0.01$ for test of the null hypothesis that the scenario 1 and scenario 2 misclassification percentages are the same.
SM, smoking history; G, gender; VP, visible plaque; SC, supragingival calculus.

characterized by the co-occcurrence of *T. forsythia* and *P. gingivalis*, could correctly classify cases with CAL or PD with both moderate specificity and sensitivity. This is also in agreement with a recent clinical longitudinal study aiming to assess levels of periodonto pathogens as predictors of chronic periodontitis progression [7]. However, the results for extensive CAL ≥ 7 mm and PD ≥ 5 mm should be interpreted cautiously as there were rather few such cases.

The utility of microbiological testing for diagnosing destructive periodontal diseases remains controversial [6,25,29]. Hence, the usefulness of a microbiological test would be evaluated on the basis of its ability not only to affect the diagnosis and thereby also the treatment plan but also on an unequivocally better treatment outcome, or a tangible benefit to the patient in terms of the costs or the level of discomfort associated with the treatment [11,13]. Regarding a possible economic benefit to the patient it must be borne in mind that the cost of the commercially available microbial tests is such that their use is not yet justified. However, if microbial testing is employed, our results indicate that the most cost-effective approach would be to test for *T. forsythia* only. Hence, this periodontal micro-organism stood out as a better candidate among the five periodonto pathogens tested for the identification of subjects with prevalent and extensive CAL and PD. A plausible explanation for this finding may relate to the statistically significant higher counts for this micro-organism found in gingivitis and periodontitis patients [25] when assessed by PCR compared to culture and other microbiological procedures.

Most of the previous studies on the relationship between periodontal status variables and microbiological findings have been based on other microbiological assessment techniques than the PCR, including culture or checkerboard technique. Both the PCR and the checkerboard techniques have the advantage of not depending on viable bacterial cells. Even though the checkerboard technique has been available since 1994 [30], it has been employed by only a few research centers around the world. This technique allows both the simultaneous semi-quantitative analysis up to 40 microbial species and a considerable number of microbiological samples at once, thus representing a quick and economic option. It also complies with the widely held view that quantification of the bacterial load is necessary to distinguish states of periodontal disease and health [31]. However, the results of the present study indicate that the PCR technique was able to discriminate the periodontal status of subjects based on their microbiological findings. Disadvantages of the checkerboard technique are related to its general detection limit equivalent to 10^4 cells for some bacterial species such as the *A. actinomycetemcomitans*. This feature therefore implies a higher chance of false-negative observations. Furthermore, it is based on the use of whole genomic DNA probes, which increases the possibility of cross-reactions among micro-organisms, leading to reduced specificity. The PCR technique is widely used by different research centers around the world and is extremely sensitive and specific in its ability to detect micro-organisms. It is more costly and time consuming than the checkerboard technique. Therefore, studies employing this technique usually focus on microbial species of higher interest. As any laboratorial technique, its valid application requires technical and methodological rigor to avoid false positive or negative findings. In the present study, the specificity of the primers was tested by *blast* (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and, for each species, the number of cycles, primer annealing temperature and reagent concentrations were tested to standardize each reaction and reduce possible interpretation difficulties. In the present study, we chose the PCR technique for its ability to detect with high specificity and sensitivity rates all the bacterial species analyzed [11,32]. The PCR technique has also been recommended for epidemiological studies assessing oral diseases [27]. The limitations in the number of

micro-organisms that can be studied by PCR obviously mean that there is a high probability that other microbial profiles might have resulted, had we included more or different microbial species. Owing to this limitation, we restricted the microbiological analyses to include only micro-organisms widely recognized as periodontal pathogens, including *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* [33]. However, it should be borne in mind that to allow a proper statistical analysis of a larger number of micro-organisms it would have been necessary to have a larger number of microbiological samples, which was not possible owing to the limited number of adult subjects in this isolated population.

The strengths of the present investigation include the microbiological sample size, the epidemiological design involving the inclusion of the whole range of manifestations of periodontal destruction in a population and the periodontally 'undisturbed' nature of the study population. Limitations include the cross-sectional design, as prediction models are ideally based on cohort data. However, the decision tree analyses, which have recently been employed as a mathematical modeling to assist in medical diagnostic assessment [34,35], was introduced here as a possible new useful mean to statistically model the complex microbial data in relation to periodontal disease status and, in this context, the present data can be considered the learning data set, which should be followed by a subsequent validation of the learning model on different data sets.

As new techniques and knowledge emerge within the field of microbiology more is discovered regarding the microbiota associated with periodontal diseases. Although great advances have been achieved so far, the complex understanding of the microbial ecology of dental biofilm in the periodontal field appears to be just beginning [6].

In conclusion, specific microbial profiles could be identified in this isolated population. They did not provide significant superior diagnostic accuracy when added to traditional clinical markers.

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