

## Comparative bacterial analysis of chronic and aggressive periodontitis in a sample population from Uruguay

Andrea Badanian, ORCID: 0000-0001-8397-6639, Grade 3 Professor, Microbiology Department, School of Dentistry, Universidad de la República<sup>1</sup>

Luis Bueno, ORCID: 0000-0002-7837-6492, Grade 5 Professor, Periodontics Department, School of Dentistry, Universidad de la República

Virginia Papone, ORCID: 0000-0003-4403-3376, Grade 5 Professor, Microbiology Department, School of Dentistry, Universidad de la República

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### Abstract

**OBJECTIVES:** To compare the prevalence of certain periodontal pathogens in aggressive and chronic periodontitis in 101 Uruguayan patients according to the valid classification at the time the studies were conducted.

**METHODS:** This analysis is based on studies conducted to detect periodontal pathogens in patients with chronic and aggressive periodontitis in Uruguay using conventional and molecular methods. Both studies analyzed the same microorganisms and used the same recovery and analysis techniques. *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevotella intermedia* and *Fusobacterium nucleatum* were studied.

**RESULTS:** A higher prevalence of *F. nucleatum*, *T. forsythia* and *P. gingivalis* was detected in chronic cases, whereas higher rates of *F. nucleatum* and *P. intermedia* were observed in aggressive ones. Within the last one, a higher proportion of *P. gingivalis* and *T. forsythia* was detected in the generalized cases and *A. actinomycetemcomitans* in the localized ones.

**CONCLUSIONS:** The patterns detected are in line with those included in the literature for our country.

**Keywords:** prevalence, microbiota, periodontitis.

### Introduction and background

At the time of conducting and completing this work, the valid classification was that arising from the *Workshop for a Classification of Periodontal Diseases and Conditions* organized by the *American Academy of Periodontology* (AAP) in 1999 <sup>(5-6)</sup>.

According to this classification, chronic periodontitis is the most common type and typically progresses slowly. In contrast, aggressive periodontitis can lead to loss of tooth-supporting tissue in a short period. Aggressive periodontitis included localized aggressive periodontitis (LAP) and generalized aggressive periodontitis (GAP) according to the extent of periodontal destruction <sup>(7)</sup>.

Recently, a new classification of periodontal diseases based on stages, extent/distribution and grades has been agreed, without distinguishing between aggressive and chronic periodontitis. Staging is dependent on the severity and complexity of disease management (extent of damaged or lost tissue), the degree of extent and distribution compared to localized or more generalized cases, and grades mainly as an indication of rate of progression <sup>(8)</sup>.

To correlate the clinical patterns of our studies with this new classification, regarding staging, we can say that our patients had a pocket depth  $\geq 5$  mm and a bone loss  $\geq 2$  mm. As for extent and distribution, there is a difference regarding the distinction between generalized chronic and aggressive cases, and localized aggressive cases.

Finally, in grade there is a difference regarding the slower rate of progression of chronic cases in contrast to aggressive cases.

In periodontal disease, numerous studies based on bacterial specificity as responsible for the disease allowed us to focus on and establish the role of some microorganisms such as *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, *P. intermedia* and *F. nucleatum* in periodontal disease <sup>(9)</sup>.

According to different studies, the microorganisms usually detected in chronic periodontitis belong to the red complex, such as *P. gingivalis* <sup>(3,9-10)</sup>.

In turn, the development of aggressive periodontitis has been linked to *A. actinomycetemcomitans* and *P. gingivalis*, the former being particularly important in localized cases and the latter in generalized cases <sup>(3,11-12)</sup>. However, links have been found with another group of periodontopathogens such as *T. forsythia*, *P. intermedia* and *F. nucleatum* <sup>(3,9)</sup>.

In any case, these microorganisms do not act in isolation, but interact with each other, and this is how they determine the effects of disease <sup>(9)</sup>.

Socransky et al. demonstrated that microorganisms in the subgingival plaque combine with each other creating bacterial complexes, and categorized them according to colors: green, yellow, blue, purple, orange, and red. These colors indicate the correlation of these complexes with the different degrees of severity of periodontal disease and its progression

<sup>(11)</sup>. The microorganisms that prevail in periodontal pathologies are the ones in Socransky's red and orange complexes and also *Aggregatibacter actinomycetemcomitans* <sup>(3,7,12-14)</sup>.

The main differences between health and disease are based on the predominance of red and orange *clusters*. The red complex that appears later in biofilm development is formed by periodontopathogenic bacteria, namely: *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia* <sup>(11,14)</sup>.

On the other hand, *A. actinomycetemcomitans* plays a role in aggressive periodontitis, particularly in LAP, and it may also be important in the onset of the disease and then replaced by other obligate anaerobes as the disease progresses <sup>(12)</sup>.

Up to the time of conducting these studies in patients in Uruguay, we knew that the relevance of the microorganisms studied in periodontal cases in the country was the same, but there were no local studies.

With the advent of molecular techniques, it was possible to determine the reality in our country without the risks of loss of viability implied when recovering samples in the laboratory using conventional techniques applied to periodontal microorganisms, mostly obligate anaerobes.

The microorganisms analyzed in this comparison were two members of the red complex, *P. gingivalis* and *T. forsythia* and two of the orange complex *P. intermedia* and *F. nucleatum*. It was interesting to include the latter in our studies given their ability to form bridges, which is relevant to *biofilm* formation <sup>(14)</sup>.

We also studied *A. actinomycetemcomitans*, which has historically been linked to periodontitis, particularly LAP <sup>(3,12)</sup>.

This study seeks to conduct a comparative analysis of the prevalence patterns of these five periodontal pathogens among the periodontitis cases studied.

## Materials and methods

Samples were obtained from 101 selected patients with chronic and aggressive periodontitis from the Clinic of Periodontics of the School of Dentistry, Universidad de la República. Fifty-one patients with chronic periodontitis and 50 patients with aggressive periodontitis were studied; they had received no prior periodontal treatment and had signed the informed consent. Patients with diabetes, arthritis, ulcerative colitis, HIV, cancer and cardiovascular disease, pregnant women and those who had been treated with antibiotics and/or anti-inflammatories three months before the study were excluded. The Ethics Committee of the School of Dentistry, Universidad de la República, approved the design of both studies following MERCOSUR regulations and the Helsinki Declaration on research involving human subjects.

Supragingival plaque was taken to obtain the samples, using sterile gauze to avoid bleeding. Medium sterile paper points were placed deeply into the pocket and left for 15 seconds to collect the samples. They were then placed in 1.5 ml of RTF (*Reduced Transport Fluid*)<sup>(15)</sup>. Each sample included eight medium sterile paper points (Nº 25) from four sites selected in each quadrant.

The samples were subjected to both traditional bacteriological techniques and molecular genetic techniques (PCR) at the Microbiology Department, School of Dentistry, Universidad de la República.

Traditional bacteriological diagnosis was made for the facultative Gram-negative bacillus *A. actinomycetemcomitans* and for *P. gingivalis* and *P. intermedia* (both pigmented strict anaerobic Gram-negative bacilli). We also conducted PCR identification for the microorganisms above and for *T. forsythia* and *F. nucleatum*. Samples were processed, stirred vigorously for 45 to 60 seconds, and then serial dilutions in RTF were prepared.

TSVB was used to recover *A. actinomycetemcomitans*: trypticase soy, bacitracin (75 µg/ml), vancomycin (5.0 µg/ml), 10 ml of horse serum (10%) through 1:10 dilution in RTF and incubation in a candle jar (to create capnophilic conditions) for 7 days at 37°C. *Brucella Agar* was used to recover the pigmented anaerobes: menadione (200 µg/ml) and hemin (5 mg/ml) and laked blood in 1:10, 1:100 and 1:1000 dilutions in RTF, streaking 100 µl of the last two dilutions and incubating in an anaerobic jar for 14 days at 37°C using the *Oxoid*® anaerobic indicator.

The original sample and 100 µl samples of the original sample were stored at -30°C for PCR processing.

The following strains were used as positive controls: *Aggregatibacter actinomycetemcomitans* (ATCC 29522), *Porphyromonas gingivalis* (BAA-308), *Prevotella intermedia* (ATCC 25611), *Tannerella forsythia* (ATCC 43037) and *Fusobacterium nucleatum* (ATCC 25586).

Identification of *A. actinomycetemcomitans* culture was performed by colony morphology, Gram staining, positive catalase test and negative MUG (4-Methylumbelliferyl-β-D-galactopyranoside) to study lactose fermentation, and also with *bioMérieux*® API kit in case of doubt.

Pigmented anaerobes were identified through pigment production, fluorescence to differentiate *P. gingivalis* from *P. intermedia* and *bioMérieux*® API kit in case of doubt <sup>(1-2)</sup>.

The genome of each control strain was extracted using the *Bead*™ Genomic DNA Kit (as described by manufacturer *Zymo Research*®). DNA concentration (ng/µl) was quantified with *NanoDrop*® 2000.

Microbial genome databases were used to search for and select 16S rRNA sequences from the wild strain as targets in the design of species-specific and/or serotype-specific

oligonucleotides. Additionally, we used bibliographic information from studies with validated oligonucleotides for the species <sup>(1-2)</sup>.

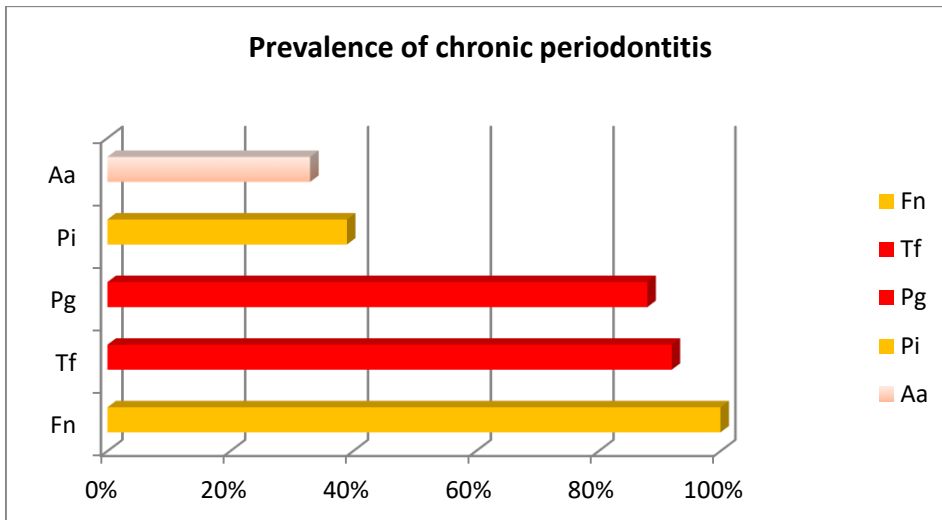
### **Statistical analysis**

Absolute frequencies in percentages were used. The populations were compared using the Student's T-Test with a significance level of 0.05.

### **Results**

As mentioned above, *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were analyzed applying both traditional bacteriological and molecular techniques. We found a strong correlation in the results obtained through both techniques: a correlation equal to or higher than 85% (95% for *A. actinomycetemcomitans*, 93% for *P. intermedia* and 85% for *P. gingivalis*). When we found differences in the results obtained through the two techniques, the molecular technique result was considered valid and presented in this work.

In chronic periodontitis, the most prevalent microorganism was *F. nucleatum* from the orange complex, occurring in 100% of patients. Additionally, most cases (approximately 90%) presented *T. forshyta* and *P. gingivalis* from the red complex. *P. intermedia*, from the orange complex, was recovered in 39% of patients, while *A. actinomycetemcomitans* was recovered in patients with chronic periodontitis but its prevalence was noticeably lower (33%) (Chart 1).



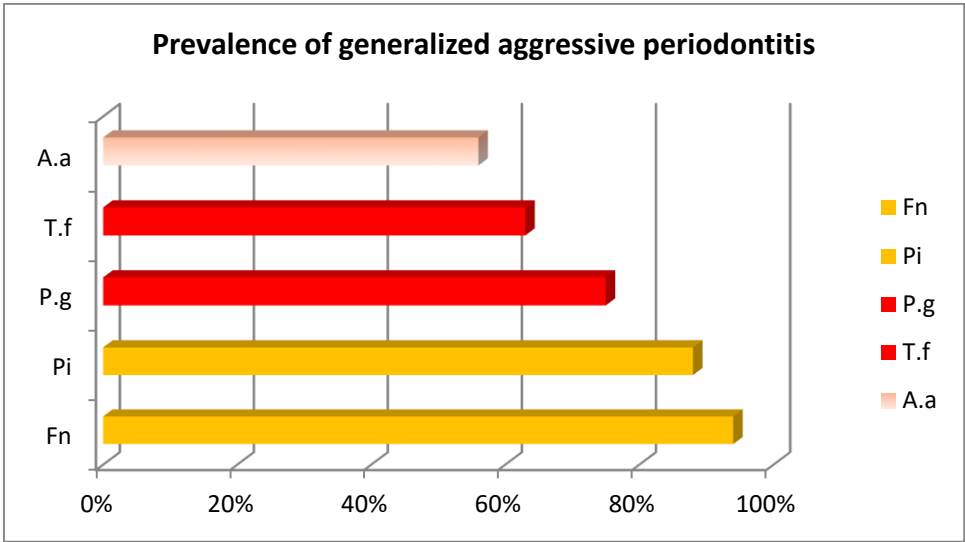
**Chart 1 Prevalence of chronic periodontitis**  
**Fn=Fusobacterium nucleatum 100%, Tf = Tannerella forsythia 92%, Pg = Porphyromonas gingivalis 88%, Pi= Prevotella intermedia 39%, Aa = Aggregatibacter actinomycetemcomitans 33%**

In both aggressive types of periodontitis, *F. nucleatum*, from the orange complex, was also important as it was the most prevalent microorganism in the generalized cases and in chronic periodontitis (Chart 2).

*Prevotella intermedia*, the other orange complex microorganism studied, was more prevalent than in the chronic cases, in particular in the localized cases, where it was the prevailing microorganism (Chart 3).

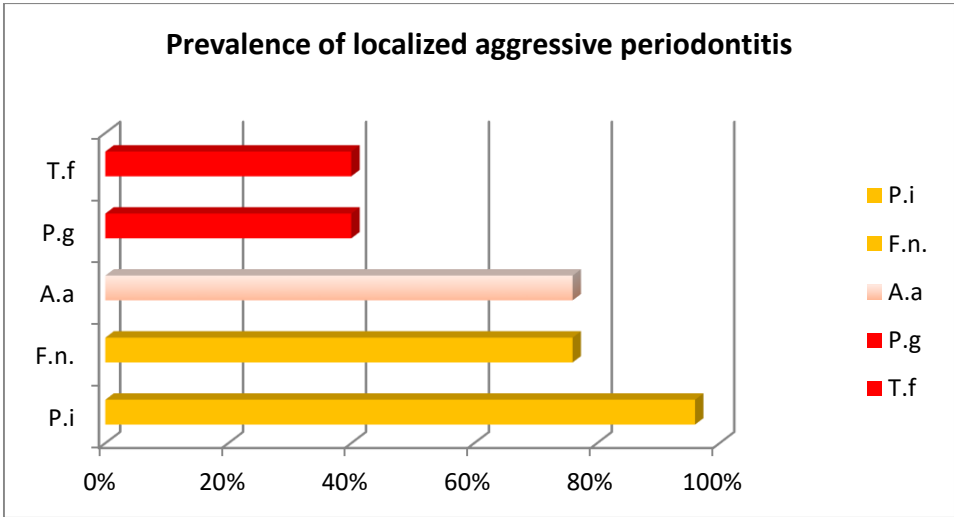
Furthermore, in aggressive cases, there was a higher prevalence of *A. actinomycetemcomitans* and a lower prevalence of the red complex microorganisms studied (*P. gingivalis* and *T. forsythia*) in relation to chronic cases. In aggressive cases, there are different results when distinguishing between generalized and localized cases. While the microorganisms from the red complex were more relevant in the generalized cases, *A. actinomycetemcomitans* prevailed in the localized cases, although in both cases there was higher prevalence than in chronic cases (Charts 2 and 3).





**Chart 2** Prevalence of generalized aggressive periodontitis

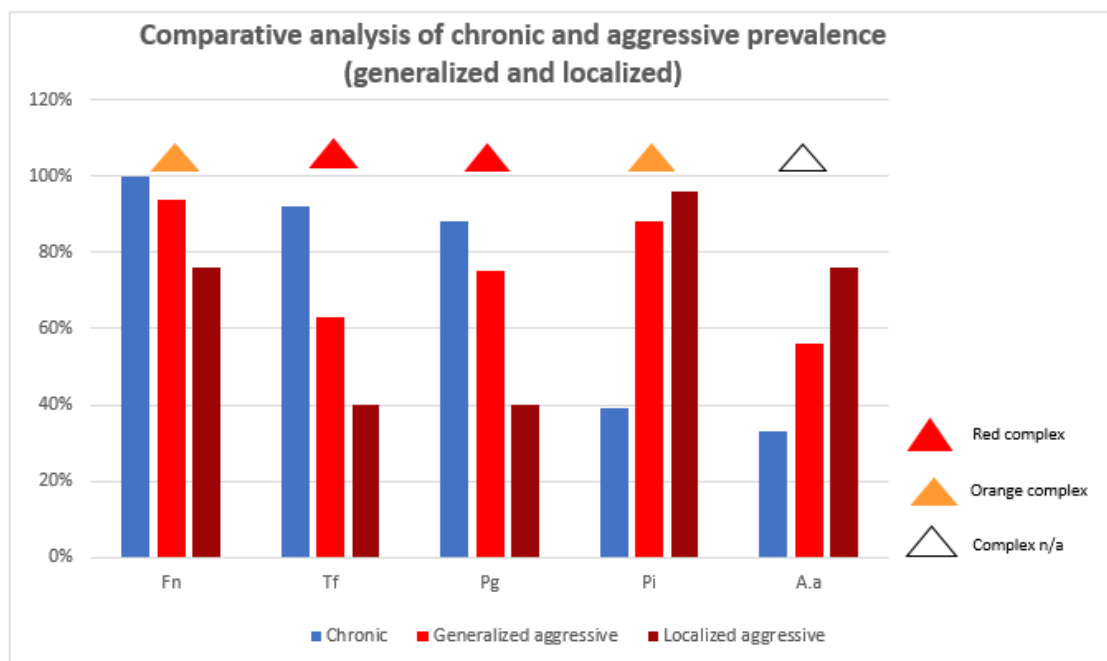
***Fn*=Fusobacterium nucleatum 94%, *Pi*= Prevotella intermedia 88%, *Pg* = Porphyromonas gingivalis 75%, *Tf* = Tannerella forsythia 63% *Aa* = Aggregatibacter actinomycetemcomitans 56%**



**Chart 3** Prevalence of localized aggressive periodontitis

***Pi*= Prevotella intermedia 96%, *Fn* = Fusobacterium nucleatum 76%, *Aa* = Aggregatibacter actinomycetemcomitans 76%, *Pg* = Porphyromonas gingivalis 40%, *Tf* = Tannerella forsythia 40%**

The following chart and table aim to facilitate the comparative analysis of the prevalence of each bacterial species studied in the different cases. (Chart 4 and Table 1)



**Chart 4 Comparative analysis of chronic and aggressive prevalence (generalized and localized)**

*Fn = Fusobacterium nucleatum, Tf = Tannerella forsythia, Pg = Porphyromonas gingivalis, Pi= Prevotella intermedia, Aa = Aggregatibacter actinomycetemcomitans*

**Table 1 Comparative analysis of prevalence of chronic periodontitis, GAP and LAP**

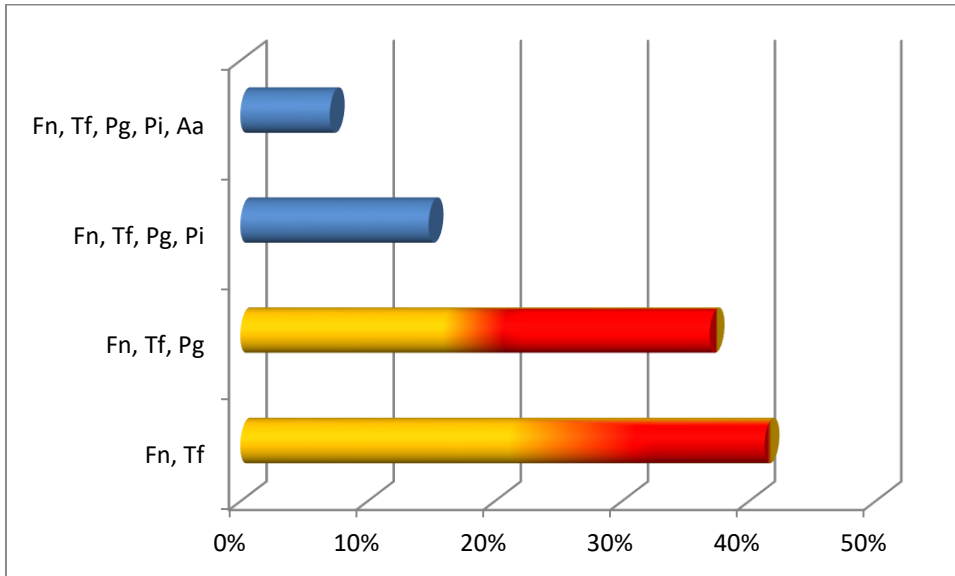
*Fn= Fusobacterium nucleatum, Tf= Tannerella forsythia, Pg= Porphyromonas gingivalis, Pi= Prevotella intermedia, Aa = Aggregatibacter actinomycetemcomitans*

	Fn	Tf	Pg	Pi	Aa
Chronic	100%	92%	88%	39%	33%
GAP	94%	63%	75%	88%	56%
LAP	76%	40%	40%	96%	76%

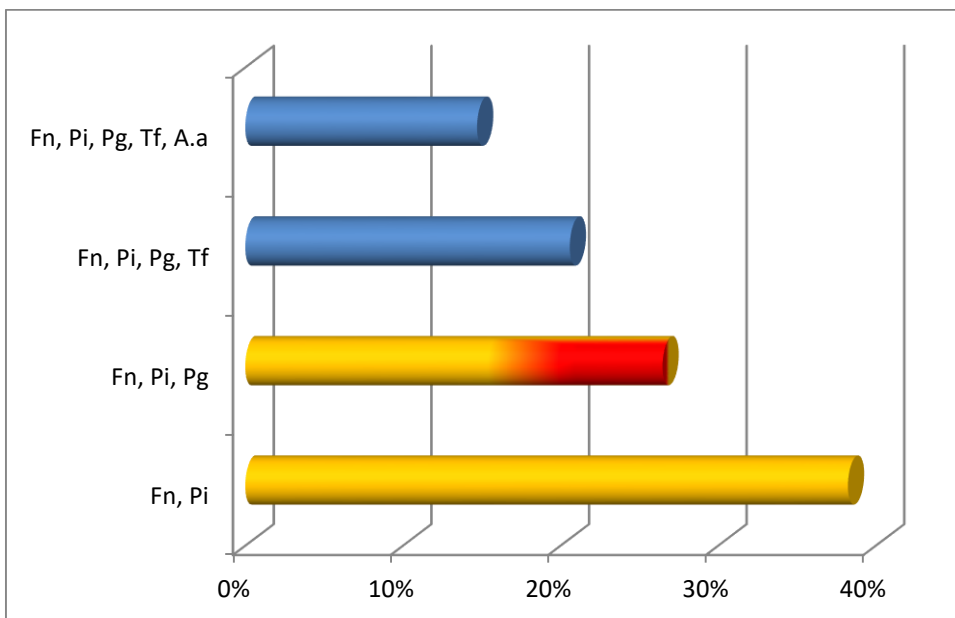
When considering the co-detection of species, where microorganisms are probably forming *clusters* or associations, we observed the following patterns for chronic periodontitis (Chart 5), generalized aggressive periodontitis (Chart 6) and localized aggressive periodontitis (Chart 7). As can be seen, the most common co-detection in the chronic cases was *F. nucleatum* with *T. forsythia*, followed by the association of *F. nucleatum*, *T. forsythia* and *P. gingivalis* (both red complex microorganisms).

Additionally, the most common co-detection in the two aggressive types was *F. nucleatum* and *P. intermedia*. In generalized aggressive cases, the second main

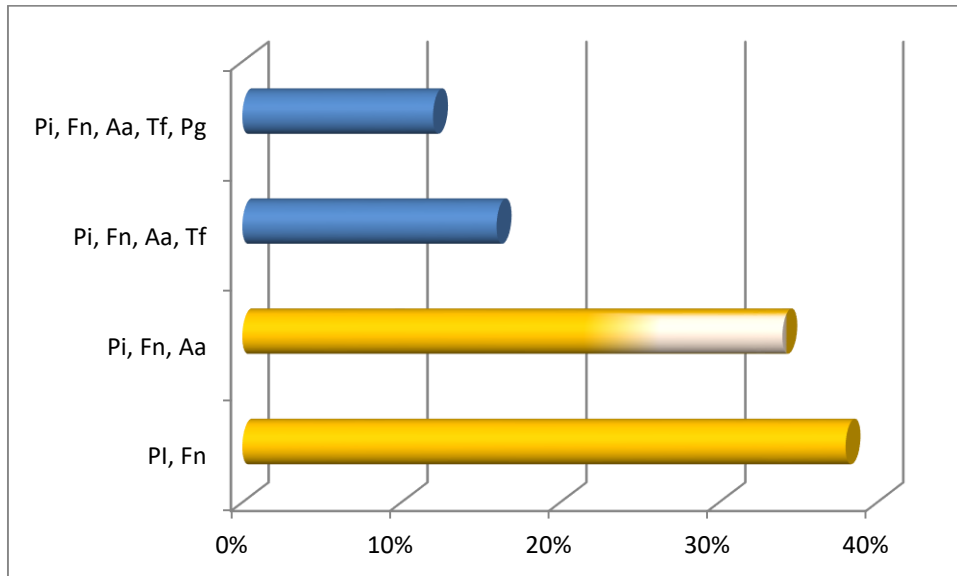
co-detection found was *F. nucleatum*, *P. intermedia* with *P. gingivalis*, while in localized aggressive cases it was *F. nucleatum*, *P. intermedia* (both from the orange complex) with *A. actinomycetemcomitans*.



**Chart 5. Co-detections in chronic periodontitis**  
**Fn=*Fusobacterium nucleatum*, Tf=*Tannerella forsythia***  
**Pg=*Porphyromonas gingivalis* Pi=*Prevotella intermedia*,**  
**Aa=*Aggregatibacter actinomycetemcomitans* Fn + Tf = 41%, Fn + Tf**  
**+ Pg = 37%, Fn + Tf+ Pg+ Pi = 15%, Fn + Tf+ Pg+ Pi + Aa = 7%**



**Chart 6. Co-detections in generalized aggressive periodontitis**  
**Pi=*Prevotella intermedia* Fn=*Fusobacterium nucleatum*,**  
**Pg=*Porphyromonas gingivalis* Tf=*Tannerella forsythia*,**  
**Aa=*Aggregatibacter actinomycetemcomitans*, Fn + Pi = 38%, Fn + Pi**  
**+ Pg = 26%, Fn + Pi+ Pg+ Tf = 21%, Fn + Pi + Pg+ Tf + Aa = 15%**



**Chart 7. Co-detections in localized aggressive periodontitis**  
**Pi=Prevotella intermedia Fn=Fusobacterium nucleatum,**  
**Aa=Aggregatibacter actinomycetemcomitans Tf=Tannerella forsythia**  
**Pg=Porphyromonas gingivalis, Pi +Fn = 38%, Pi + Fn + Aa = 34%, Pi**  
**+Fn + Aa+ Tf = 16%, Pi + Fn + Aa+ Tf +Pg = 12%**

The microbiota analyzed in the GAP cases was more similar to that of chronic periodontitis cases (greater representation of the red complex) than to that of LAP cases. Regarding *A. actinomycetemcomitans* and *P. intermedia*, it was more prevalent in both aggressive types than in chronic cases.

## Discussion

Dental plaque is considered a *biofilm*. Microorganisms are not structured in a random order, which means that some species are more frequently associated with certain species than others <sup>(9,11)</sup>.

Some species show a limited number of interactions with other species and others act like bridges, like *F. nucleatum*, which adheres to a vast number of species <sup>(16)</sup>.

In the Uruguayan patients studied, the most prevalent microorganisms in chronic periodontitis were *F. nucleatum* from the orange complex, and *T. forsythia* and *P. gingivalis* from Socransky's red complex.

It is not surprising for the last two microorganisms to be present in such high proportions in patients with chronic periodontitis, as they are microorganisms closely associated with the etiopathogenesis of the disease <sup>(3)</sup>.

Furthermore, in the aggressive cases, these red complex microorganisms appeared in a lower rate than in chronic cases, particularly in localized cases. This pattern of higher prevalence of the red complex in the generalized versus the localized cases is consistent with studies conducted in other regions <sup>(13)</sup>.

The high levels of *F. nucleatum* detected in both chronic and aggressive cases can be explained by its relevance in bacterial adhesion as it expresses multiple adhesins that allow late colonizers to bind. In addition, *F. nucleatum* may tolerate higher oxygen concentrations than *P. gingivalis*, which facilitates the generation of oxygen-reduction conditions. This promotes the growth and development of oxygen and other obligate anaerobes <sup>(16)</sup>. *F. nucleatum* can also raise pH through ammonia generation, which neutralizes the acid produced by the fermenting microorganisms, thus creating a more favorable environment for *P. gingivalis* and other acid-sensitive organisms <sup>(16)</sup>.

*P. intermedia*, from the orange complex, had a higher prevalence in aggressive cases than in chronic cases. The fact that it appears at lower rates in chronic cases in our patients is similar to what other studies report, where subjects with high rates of *P. gingivalis* had little or no presence of *P. intermedia* <sup>(3)</sup>.

Our results, where *P. intermedia* was the most prevalent microorganism in aggressive cases, are comparable to those reported in other studies conducted in Germany and Africa <sup>(17-18)</sup>.

In contrast, some studies in Korea and Australia report a greater presence of *P. intermedia* in chronic and generalized aggressive forms than localized aggressive ones <sup>(19-20)</sup>.

We must remember that orange complex species such as *P. intermedia* and *F. nucleatum* allow for the colonization of red complex microorganisms <sup>(3)</sup>, so the high numbers recovered for *F. nucleatum* in the three types of periodontitis and from both species in aggressive periodontitis (GAP and LAP) are revealing.

Regarding *A. actinomycetemcomitans*, there was a higher prevalence in aggressive cases versus chronic cases in our patients (and with greater emphasis on localized ones), which is compatible with the importance of this microorganism in aggressive cases, in particular in localized cases <sup>(3)</sup>.

## Conclusions

The studies conducted for the microorganisms analyzed and the comparison of the different periodontitis cases show that in Uruguay there is microbiota which is similar to that found in other regions <sup>(3,21)</sup>. In aggressive cases, *A. actinomycetemcomitans* appeared in higher rates than in the generalized cases, which is compatible with the results of studies conducted in other countries <sup>(3,5)</sup>.

Red-complex microorganisms *T. forsythia* and *P. gingivalis*, were particularly prominent in chronic processes, and even more in generalized aggressive cases than in localized ones.

Furthermore, *F. nucleatum* stood out in all cases, but particularly in chronic and generalized aggressive cases, where its role in the bacterial community is probably key. Something similar happened with *P. intermedia* from the orange complex, which prevailed in aggressive cases, particularly in localized cases.

We found a greater similarity in the microbiota studied between generalized aggressive cases and chronic cases than between localized aggressive cases and chronic cases. Additionally, aggressive cases have a higher rate of *A. actinomycetemcomitans* and *P. intermedia* compared to chronic cases, though they are more prevalent in LAP than in generalized cases.

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