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Fusobacterium nucleatum supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments

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The authors compared the differences in tolerance to oxygen of the anaerobic periodontopathic bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, and explored the possibility that *F. nucleatum* might be able to support the growth of *P. gingivalis* in aerated and CO_2-depleted environments. Both micro-organisms were grown as monocultures and in co-culture in the presence and absence of CO_2 and under different aerated conditions using a continuous culture system. At steady state, viable counts were performed and the activities of the enzymes superoxide dismutase and NADH oxidase/peroxidase were assayed in *P. gingivalis*. In co-culture, *F. nucleatum* was able to support the growth of *P. gingivalis* in aerated and CO_2-depleted environments in which *P. gingivalis*, as a monoculture, was not able to survive. *F. nucleatum* not only appeared to have a much higher tolerance to oxygen than *P. gingivalis*, but a significant increase in its numbers occurred under moderately oxygenated conditions. *F. nucleatum* might have an additional indirect role in dental plaque maturation, contributing to the reducing conditions necessary for the survival of *P. gingivalis* and possibly other anaerobes less tolerant to oxygen. Additionally, *F. nucleatum* is able to generate a capnophilic environment essential for the growth of *P. gingivalis*.

Keywords: oxidative stress, oral bacteria, anaerobes, interactions, continuous culture

INTRODUCTION

It is well known that elevated oxygen tensions within bacterial cells increase the enzymic and non-enzymic reduction of molecular oxygen to superoxide anions ($O_2^{\cdot -}$), which can form, by dismutation, H_2O_2 and O_2. H_2O_2, in turn, reacts with $O_2^{\cdot -}$ to form $OH^{\cdot}$ in the presence of iron complexes (Rosen & Klebanoff, 1979). These oxygen species are highly reactive and can cleave nucleic acids and oxidize essential proteins and lipids (Brawn & Fridovich, 1981; Harley *et al*., 1981). Strictly anaerobic micro-organisms do not possess the anti-oxidant systems needed to detoxify such reactive oxygen species. However, the susceptibility of anaerobes to oxygen varies even among closely related micro-organisms, and it has been suggested that it correlates with the levels of anti-oxidant enzymes present, superoxide dismutase (SOD) in particular (Park *et al*., 1992).

Abbreviations: SEM, scanning electron microscopy; SOD, superoxide dismutase.

As the oral cavity is an overtly aerobic environment, it is therefore likely that oral anaerobes encounter residual amounts of oxygen both in the early stages of dental plaque development and in established periodontal pockets (Marquis, 1995). Indeed, periodontal pockets have been reported to possess residual oxygen at one-tenth the level in air-saturated water (which is 0·021 μmol ml^{-1}) (Mettraux *et al*., 1984) and the average E_h (redox potential) in subgingival plaque appears to be only somewhat negative at about −50 mV (Kenny & Ash, 1969). Moreover, there is evidence in dental plaque of open channels that could deliver oxygen deep into the biofilm (Massol-Deya *et al*., 1994). Therefore, the survival of anaerobic bacteria in the mouth might be dependent on the specific tolerance to oxygen of each species and on microbial interactions within the community.

Porphyromonas gingivalis and *Fusobacterium nucleatum* belong to the group of strictly anaerobic bacteria associated with periodontal diseases (Ximenez-Fyvie *et al*., 2000). Due to its numerous putative virulence

factors, *P. gingivalis* is considered one of the major periodontopathic bacteria (Lamont & Jenkinson, 1998). *F. nucleatum* is also regarded as a key organism for dental plaque maturation due to its extensive co-aggregating capacity (Kolenbrander *et al*., 1989). Bradshaw *et al*. (1998) have suggested that *F. nucleatum* could be a 'bridge' or 'mediator' of co-aggregation between facultative and obligate anaerobic species and they proposed that this co-aggregation was the mechanism by which strict anaerobes, such as *P. gingivalis*, survived under aerobic conditions, due to the formation of microenvironments in which the facultative organisms mediated reducing conditions. However, our earlier studies (Diaz *et al*., 2000) showed that although *F. nucleatum* is an anaerobe, its capacity to adapt to and reduce an oxygenated environment is extremely high. Therefore, in the present study we explored the possibility that *F. nucleatum* might be able to protect *P. gingivalis* and other anaerobic micro-organisms less tolerant to oxygen. Accordingly, our aims were to determine whether *F. nucleatum* was able to support the growth of *P. gingivalis* in a continuous aerated co-culture, comparing this co-culture with the tolerance to oxygen of a monoculture of *P. gingivalis* grown under the same conditions. The levels of anti-oxidant enzymes in *P. gingivalis* were also assayed in order to compare them with our previously reported levels of the same enzymes in *F. nucleatum* (Diaz *et al*., 2000). As preliminary experiments showed that *F. nucleatum* does not require the external addition of CO_2 for growth in the chemostat, while CO_2 seems to be essential for *P. gingivalis*, we also explored the possibility that *F. nucleatum* could supply CO_2 for the growth of *P. gingivalis*.

METHODS

Micro-organisms and maintenance of the strains. *F. nucleatum* ATCC 10953 (the type strain) and *P. gingivalis* W50 (ATCC 53978) were used for all experiments and maintained, short-term, on anaerobic blood agar plates incubated at 37 °C in an atmosphere of $N_2/H_2/CO_2$ (90:5:5).

Growth conditions. For all experiments, if not otherwise indicated, the micro-organisms were grown under continuous culture conditions in BM medium (Shah *et al*., 1976) supplemented with 500 μg haemin l^{-1}. Growth in a 365 ml working-volume chemostat (BioFlo model C30, New Brunswick Scientific) was initiated by inoculating the growth chamber with a 24 h batch culture of the micro-organism(s) grown in the same medium under an atmosphere of $N_2/H_2/CO_2$ (90:5:5). After 24 h of batch-culture growth in the chemostat vessel, the medium reservoir pump was turned on and the medium flow adjusted to give a dilution rate of $D = 0{\cdot}069\ h^{-1}$ ($t_d = 10$ h). The temperature was controlled at 36 °C and the pH maintained at 7·4 by the automatic addition of 2 M KOH. The cultures were sparged with the appropriate gas mixture and the E_h was constantly monitored, as was dissolved oxygen. Culture purity was checked daily by Gram staining and cell viability was measured, at steady-state, by viable counts. Vortex mixing of co-culture samples, prior to viable counting, was carried out to disperse co-aggregated cells without affecting cell viability. Blood agar plates, with and without 50 μg kanamycin ml^{-1}, were used for viable counts of *P. gingivalis* and *F. nucleatum*, respectively. In the kanamycin-containing medium *P. gingivalis* was recovered with 100% efficiency. Under all conditions, steady state was achieved after about seven generations, as evidenced by sustained stability of the culture E_h and cell viability. Various culture parameters (see below) were then assayed daily for up to 9 d. Cultures grown under various conditions were also examined by scanning electron microscopy (SEM), as follows. For planktonic-phase analysis, approximately 1 ml of cell culture was removed from the chemostat and 5 μl was placed on a micro glass cover slip and allowed to dry. When formation of biofilms occurred over the chemostat inserts, the chemostat was disassembled and a small amount of the biofilm was mechanically removed with minimal disruption and placed directly onto cover slips. All samples were then placed in fixative solution containing 4% paraformaldehyde, 1·25% glutaraldehyde and 4% sucrose in PBS, followed by post-fixing in 1% OsO_4, and dehydration with increasingly concentrated ethanol solutions. Samples were analysed using a Philips XL30 field emission scanning electron microscope.

Initially, *P. gingivalis* was grown in a gaseous atmosphere of N_2/CO_2 (95:5) and then under the sequentially increased oxygenated conditions $N_2/CO_2/O_2$ (85:5:10) and $N_2/CO_2/O_2$ (75:5:20).

To evaluate the CO_2 requirement of the two organisms, each was grown axenically in a N_2/CO_2 (95:5) atmosphere and when steady state had been achieved, CO_2 was excluded from the gas mixture. To determine whether *P. gingivalis* could survive in a CO_2-depleted environment relying only on the amount of CO_2 produced by its own metabolism, a closed environment was used, rather than the chemostat, in which the gaseous atmosphere is continually replaced. For these experiments, *P. gingivalis* was grown in batch culture; two anaerobic jars were used for incubation at 37 °C, one gassed with N_2/CO_2 (95:5) and the other with N_2 alone. *P. gingivalis* cells growing at steady state in continuous culture under an atmosphere of N_2/CO_2 (95:5) were used as the initial inoculum. BM medium was used for all experiments and media were inoculated to produce an initial OD_{560} of 0·45, in a total volume of 50 ml. At late-exponential phase the OD_{560} was recorded, the pH was measured and the appropriate amount of culture, to produce an initial OD_{560} of 0·45, was transferred to fresh medium; these broths were incubated under the appropriate gaseous conditions.

The ability of *F. nucleatum* to support the growth of *P. gingivalis* under aerated conditions was determined by growing the bacteria in continuous co-culture under the same gaseous conditions as described above for the *P. gingivalis* continuous monoculture. In a parallel set of experiments CO_2 was excluded from the gas mixture.

Enzyme assays. Since *P. gingivalis* failed to grow under oxygen concentrations of 10% or more, the activity of anti-oxidant enzymes was assayed in cell-free extracts from the micro-organism grown under anaerobic conditions and under 3% and 6% O_2 in the incoming gas mixture. Extracts were prepared as described previously (Diaz *et al*., 2000). Protein content was determined using a Coomassie Plus Protein Assay Reagent Kit (Pierce) with bovine serum albumin as a standard. NADH oxidase and NADH peroxidase activities were assayed at 25 °C following the methods of Higuchi (1992). NADH oxidase was assayed by monitoring the oxidation of β-NADH in the reaction mixture (3 ml) at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7·0), 0·1 mM β-NADH and cell extract (0·125 mg protein). NADH peroxidase was assayed under anaerobic conditions, achieved

in a Thunberg-type cuvette, using the same reaction mixture as for NADH oxidase but with the addition of 0·3 mM H_2O_2. One unit of activity for both enzymes was defined as the amount of extract that catalysed the oxidation of 1 nmol NADH min^{-1}. SOD activity was measured at 550 nm by competitively inhibiting the reduction of cytochrome *c* at 25 °C, following the methods of McCord & Fridovich (1969). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7·8), 0·1 mM EDTA, 0·01 mM cytochrome *c*, 0·1 mM xanthine, sufficient volume of xanthine oxidase to produce a constant reduction of cytochrome *c* at a rate of 0·02 units of absorbance increase min^{-1} and cell extract (0·125 mg protein). One unit of SOD was defined as the amount of extract that decreased by 50 % the rate of reduction of cytochrome *c*.

Statistical analysis. Data were expressed as means ± standard deviations. Differences between means were analysed for statistical significance by Student's *t* test.

RESULTS

Under the aerated conditions initially tested (10 % and 20 % O_2), a monoculture of *P. gingivalis* declined according to washout kinetics. However, further experiments showed that *P. gingivalis* was able to maintain steady-state growth in environments containing lower oxygen levels (3 % and 6 %) and a statistically significant decrease in cell viability occurred as the oxygen concentration was increased. Anti-oxidant enzyme levels for *P. gingivalis* grown in these environments are shown in Table 1.

Also, as seen in Fig. 1, the exclusion of CO_2 from the gas mixture resulted in washout of a monoculture of *P. gingivalis* but not of *F. nucleatum*. It should be noted that $NaHCO_3$ added to the growth medium did not substitute for CO_2. Furthermore, batch culture experiments under a closed anaerobic atmosphere showed that the CO_2 produced by *P. gingivalis* metabolism was not sufficient to fulfil its growth requirements. As shown in Table 2, the micro-organism did not survive even a single transfer in a CO_2-depleted atmosphere.

In contrast to the low oxygen tolerance of a monoculture of *P. gingivalis*, the organism survived in co-culture with *F. nucleatum* in gaseous environments containing 10 % and 20 % O_2 (Table 3, Environment 1). Interestingly, a statistically significant increase in the populations of *F. nucleatum* was observed under moderate oxygen levels (10 %).

It can also be seen (Table 3, Environment 2) that *F. nucleatum* was able to satisfy the requirements for CO_2 of *P. gingivalis*, as the exclusion of CO_2 from the gas mixture did not have a marked effect on *P. gingivalis* population sizes. This contrasted with the inability of *P. gingivalis* to grow in monoculture without CO_2 (Fig. 1).

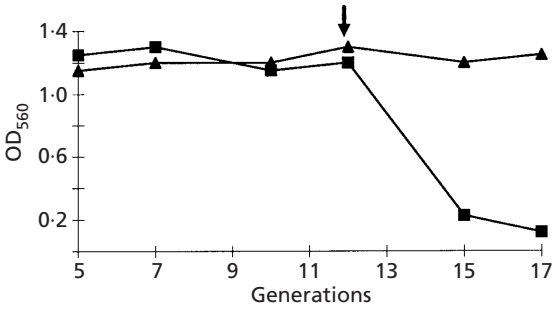

Fig. 1. Effect of CO_2 on the chemostat growth of *F. nucleatum* (▲) and *P. gingivalis* (■) as monocultures. The gas phase up until the 12th generation was N_2/CO_2 (95:5); CO_2 was then turned off (arrow).

Table 2. Effect of CO_2 on the batch culture growth of *P. gingivalis*

OD_{560} was measured at late-exponential phase. Initial OD_{560} for inoculum and transfers was 0·45. OD_{560} tr. represents the OD_{560} of transferred cultures at late-exponential phase, as explained in the text. The results are means ± SD of three separate experiments.

Atmosphere	OD_{560}	OD_{560} tr.
N_2:CO_2 (95:5)	1·15 ± 0·05 (pH = 7·5)	1·25 ± 0·04
N_2 (100)	1·05 ± 0·05 (pH = 7·4)	0·38 ± 0·06

Table 1. Effect of O_2 on the growth and specific activity of anti-oxidant enzymes of *P. gingivalis*

Gas condition†	E_h (mV)	Viable counts‡	NADH oxidase§	NADH peroxidase§	SOD§
N_2/CO_2 (95:5)	−487	9·79 ± 0·10	8·15 ± 0·72	9·04 ± 0·81	8·55 ± 0·62
$N_2/CO_2/O_2$ (92:5:3)	−420	9·36 ± 0·20*	12·77 ± 1·43*	11·12 ± 1·06**	8·45 ± 0·52
$N_2/CO_2/O_2$ (89:5:6)	−385	9·15 ± 0·10**	19·83 ± 1·18**	18·11 ± 1·82**	10·67 ± 0·57*

* $P < 0·05$ for results of anaerobic versus 3 % O_2 or 6 % O_2.

** $P < 0·001$ for results of anaerobic versus 3 % O_2 or 6 % O_2.

† Gas percentages indicate the incoming gas mixture. Dissolved O_2 was not detected in any of the above conditions.

‡ Mean ± SD of $\log_{10}$(c.f.u. ml^{-1}), $n = 6$.

§ Means ± SD of specific activity [units (mg protein)$^{-1}$], $n = 4$.

Table 3. Chemostat co-cultures of *P. gingivalis* and *F. nucleatum* grown under increasingly oxygenated conditions in CO_2-rich and CO_2-depleted environments

In Environment 1 ($n = 5$) CO_2 was included in the gas mixture. Anaerobic: N_2/CO_2 (95:5). 10% O_2: $N_2/CO_2/O_2$ (85:5:10). 20% O_2: $N_2/CO_2/O_2$ (75:5:20). In Environment 2 ($n = 9$) CO_2 was excluded from the gas mixture. Anaerobic: N_2 (100). 10% O_2: N_2/O_2 (90:10). 20% O_2: N_2/O_2 (80:20). Dissolved O_2 was not detected in any of the above conditions. Values represent viable counts expressed as means ± standard deviations of $\log_{10}$(c.f.u. ml^{-1}).

		Anaerobic	10% O_2	20% O_2
Environment 1	*F. nucleatum*	7·91 ± 0·05	8·52 ± 0·06†	7·76 ± 0·16
	P. gingivalis	9·05 ± 0·05	8·16 ± 0·08†	6·38 ± 0·68†
Environment 2	*F. nucleatum*	7·98 ± 0·18	8·52 ± 0·12†	7·36 ± 0·31*†
	P. gingivalis	8·00 ± 0·31*	8·10 ± 0·35	5·39 ± 0·79*†

* Results are significantly different to Environment 1 in the same gas phase ($P < 0·001$).

† Results are significantly different to anaerobic conditions in the same Environment ($P < 0·001$).

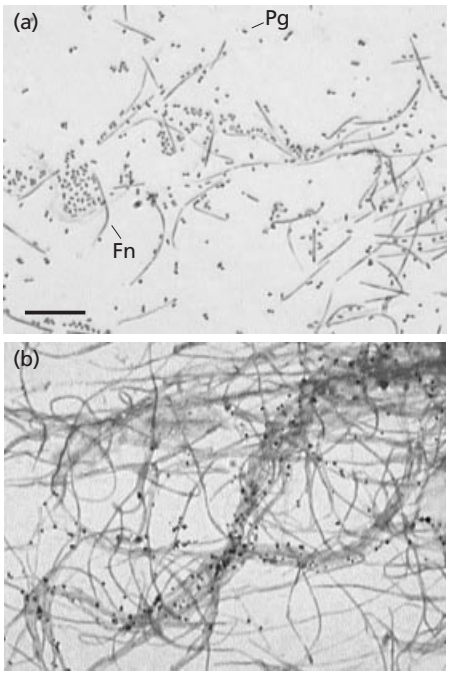

Fig. 2. Gram stains of the planktonic phase of a continuous co-culture of *P. gingivalis* (Pg) and *F. nucleatum* (Fn) grown under anaerobic conditions (a) and under 20% O_2 (b). Bar, 10 μm.

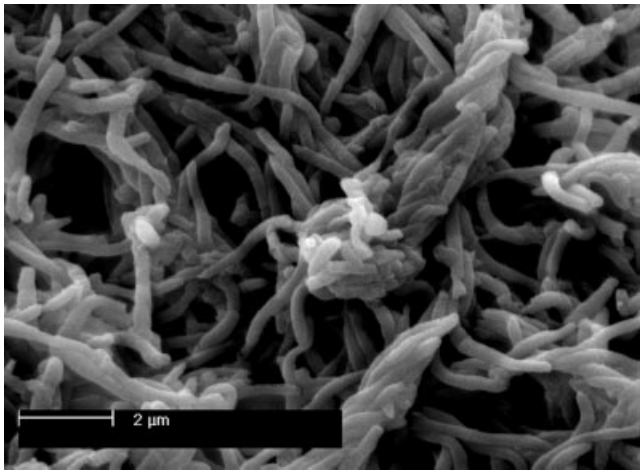

Fig. 3. Scanning electron micrograph of a biofilm formed by *F. nucleatum* and *P. gingivalis* grown as a continuous co-culture under 20% O_2. The rod-shaped *F. nucleatum* cells form an intricate network; *P. gingivalis* is difficult to distinguish but its presence was confirmed by viable counts.

The co-culture E_h was unaffected by the presence or absence of CO_2. Furthermore, the change from anaerobic conditions to 10% O_2 resulted in only a small change in E_h values, from about −500 mV to −430 mV. However, the culture E_h increased to about −150 mV under 20% oxygen and only under this condition were large planktonic aggregates and biofilms observed around the growth vessel inserts. Daily Gram stains and SEM analysis of the planktonic phase of the culture showed that *F. nucleatum* cells increased in length as the culture became more oxygen stressed; cells grown under 20% oxygen were at least five times longer than cells grown under anaerobic conditions (Fig. 2). SEM analysis of the biofilms formed under aeration showed that most obvious on the surface of these biofilms were *F. nucleatum* cells that appeared to form an intricate network (Fig. 3). However, viable counts performed on some of these biofilms revealed that *P. gingivalis* was present (data not shown). It should be noted that both co-aggregating and non-co-aggregating cells of both organisms were observed in the planktonic phase of the culture under all conditions, although the proportion of co-aggregating cells increased at higher oxygen concentrations (Fig. 2).

DISCUSSION

Of the 300 to 400 species isolated from the oral cavity, only a small group of micro-organisms, including *F. nucleatum* and *P. gingivalis*, is consistently associated with periodontitis (Socransky & Haffajee, 1994). These

two species, as is the case with other periodontopathogens, might colonize the supra- and subgingival plaques of periodontally healthy individuals for considerable periods of time prior to disease initiation, after which their levels increase (Ximenez-Fyvie *et al*., 2000). It is therefore important to determine how micro-organisms survive during the different stages of plaque development and to identify those factors regulating the ecological shifts that occur in the transition from health to disease.

Socransky *et. al*. (1998) demonstrated that bacteria in subgingival plaque exist as microbial complexes. Although *P. gingivalis* and *F. nucleatum* were identified as forming part of different complexes, a close association was shown to exist between the complexes to which each micro-organism belonged. In fact, the members of the *P. gingivalis* complex were rarely found in the absence of members of the *F. nucleatum* complex. Our data provide an explanation for this apparent dependence of *P. gingivalis* on *F. nucleatum*, suggesting that the latter might create the necessary reduced conditions and supply CO_2 for the survival of the former.

The present study clearly shows that, although *F. nucleatum* and *P. gingivalis* are anaerobes, there is a marked difference in their oxygen tolerance. Our previous findings showed that *F. nucleatum* is able to survive in chemostat cultures sparged with proportions of oxygen even higher than the oxygen content of air (Diaz *et al*., 2000), while the present findings show that *P. gingivalis* is not able to survive, in the same system, when the gas phase of the culture contains more than 6% oxygen. It should be noted that the ability to metabolize oxygen by a culture of *F. nucleatum* or *P. gingivalis* may depend upon the cell numbers (determined by nutrient availability) used in this system, but it reflects the potential of the organisms to survive oxygen tensions that might occur in the oral environment.

Interestingly, if the currently detected levels of the anti-oxidant enzymes in *P. gingivalis* are compared with our previous report for *F. nucleatum* (Diaz *et al*., 2000), the major differences occur in NADH oxidase activity. Under anaerobic conditions, *F. nucleatum* produced 190 units of activity (mg protein)$^{-1}$, while *P. gingivalis* activity was only 8·15 units (mg protein)$^{-1}$, a difference of more than 20-fold. In contrast, SOD activity in *F. nucleatum* was 10 times lower than in *P. gingivalis*. This indicates that, in these two micro-organisms, SOD activity does not correlate with tolerance to oxygen, which is at variance with previous suggestions for other species (Park *et al*., 1992). NADH oxidase/peroxidase usually exists as a double system, able to metabolize both molecular oxygen and H_2O_2 (Poole *et al*., 2000), while the role of SOD is the detoxification of $O_2^{\cdot-}$ with the formation of H_2O_2 (McCord & Fridovich, 1969). Therefore, high levels of SOD without correspondingly high levels of NADH oxidase/peroxidase activities could be toxic for anaerobic micro-organisms because of the generation of large amounts of H_2O_2 that can not be adequately detoxified, as seems to occur in *P. gingivalis*. The negative aspect of the NADH oxidase/peroxidase systems appears to be that as they are flavin-based enzymes, the formation of $O_2^{\cdot-}$ can be promoted (Imlay & Fridovich, 1991). Therefore, it seems that an efficient radical detoxification system in the absence of catalase, as is the case for *F. nucleatum* and *P. gingivalis*, depends on the delicate balance between these two anti-oxidant enzymes, although higher proportions of NADH oxidase/peroxidase seem more beneficial for *F. nucleatum*.

The present results clearly show that *F. nucleatum* is able to support the growth of *P. gingivalis* under aerated conditions in which the latter cannot survive as a monoculture. Indeed, the fact that the populations of *F. nucleatum* increase under oxygen stress indicates its capacity to survive in natural environments that might be partially oxygenated, in contrast to the low tolerance to oxygen of *P. gingivalis*. Thus, these results suggest that the capacity of *F. nucleatum* to protect *P. gingivalis* from oxidative damage might be one of the reasons why there seems to be a close *in vivo* association between these two micro-organisms (Socransky *et al*., 1998). Additionally, this study shows that the growth of *F. nucleatum* does not require a capnophilic environment, whereas CO_2 seems to be essential for *P. gingivalis*. The fact that *P. gingivalis* survived in co-culture with *F. nucleatum* without the addition of CO_2 indicates that the latter is able to satisfy the CO_2 requirement of the former. This might constitute another metabolic interaction that explains the close association between the pair. Other possible interactions between the two micro-organisms include the presence of proteolytic enzymes in *P. gingivalis* that cleave proteins into peptides and therefore increase the energy sources for *F. nucleatum*, which does not possess high endopeptidase activity (Grenier, 1994).

The formation of biofilms, as observed in this study, might be a strategy used by *F. nucleatum* to overcome high oxidative stress, possibly because of the more reduced microenvironments inside the biofilm, which could in turn benefit *P. gingivalis*. The intricate networks formed by *F. nucleatum* in these biofilms are not surprising, considering the shape of the micro-organism, which increases its length in an oxygenated environment.

It is worth noting that haem-limiting conditions were used in this study for the growth of *P. gingivalis*. This choice was made in order to avoid the possible protective effect of haemin excess under oxidative stress conditions, as it has been suggested that the binding of haemin dimers to the surface of *P. gingivalis* would serve as a catalase-like buffer system (Smalley *et al*., 2000). The contribution of this mechanism to the ability of *P. gingivalis* to overcome oxygen stress could be determined by growing the organism under haemin-excess conditions. In this context, the study by Bradshaw *et al*. (1998), showing that *P. gingivalis* numbers were greatly reduced when *F. nucleatum* was omitted from a 10-member oral bacterial consortium subjected to aerated

conditions, was conducted under haemin-excess conditions.

In conclusion, additional to the known direct potential involvement of *F. nucleatum* in the disease process (Han *et al*., 2000; Yoshimura *et al*., 1997), this study suggests that *F. nucleatum* could have an important indirect role in the aetiology of periodontal diseases by supporting the growth of *P. gingivalis* and possibly other oral anaerobes in oxygenated and CO_2-depleted environments. Protection against the deleterious effects of oxygen might be important both in the early stages of plaque development and in periodontal pockets, where micro-organisms have to face the constant presence of residual oxygen levels.

From an ecological point of view, the identification of micro-organisms that, like *F. nucleatum*, support the growth of other periodontopathogens is important because control of such species might radically alter the pathogenic ecosystem.

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