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Identification Of Factors That Influence The Cell Density Dependent Growth Of Porphyromonas Gingivalis

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Identification Of Factors That Influence The Cell Density Dependent Growth Of *Porphyromonas Gingivalis*

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OUTLINE

1.INTRODUCTION

Periodontal diseases are complex, multifactorial, polymicrobial infections characterized by the destruction of tooth-supporting tissues. Initiation of the disease is triggered primarily by the microbiome that resides in the oral cavity. In an attempt to counteract the microbial insult, the host immune inflammatory response is activated, resulting in release of inflammatory mediators like cytokines, and prostaglandins which eventually cause destruction of connective tissue and bone and clinical signs of disease. The complex interplay between the microbial challenge and the host response determines the progression of the disease (***Page & Kornman 1997***).

1.1. THE ORAL MICROBIOME

The oral mucosa is persistently colonized by microorganisms growing in unique ecological niches. The oral cavity harbors at least five different habitats: teeth, which are non-shedding surfaces; saliva; the dorsal and lateral surfaces of the tongue; the gingival sulcus and the periodontal pocket; and the remaining epithelial surfaces of the oral mucosa (***Aas et al 2005***).

The oral microbiome is very diverse with more than 700 bacterial species present. In addition, the microbiome also contains protozoa, fungi, viruses and archaea which further adds to its polymicrobial complexity. One of the most important databases of taxa present in the oral cavity is the Human Oral Microbiome Database (HOMD) **(*Dewhirst et al 2010*).** The HOMD has information on 688 bacterial taxa, of which 343 are named species, 101 are unnamed cultivated microorganisms and 243 are uncultured phylotypes (***Yang et al 2014***).

The phyla of the domain Bacteria that are present in the oral microbiome include Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Tenericutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes and two currently unnamed phyla, SR1 and TM7, in addition to methanogenic species of the Methanobrevibacter genus from the domain Archaea. Several hundred, distinct species are contained within these divisions representing the highly diverse microbial communities of the mouth. The subgingival microbiota is particularly heterogeneous, and over 400 species have been described in this habitat alone using a 16S rRNA gene amplification, cloning and Sanger sequencing approach ***(Dewhirst et al. 2010***).

1.2 FORMATION OF ORAL BIOFILMS

Bacteria get established in the oral cavity, within few days after birth and they continue to exist in distinct niches in the form of biofilms. The non-shedding enamel surface supports the growth and maturation of a complex microbial biofilm. Biofilm communities are complex and dynamic structures that accumulate through the sequential and ordered colonization of multiple taxa ***(Kolenbrander et al., 2002),*** and are characterized by colonies of bacteria embedded in an extracellular matrix of polymeric substances. The nutrient foundations of the microbiota surviving on mucosae or within the tooth biofilm are the proteins and glycoproteins of saliva, and the carbohydrates, proteins and lipids of dietary food. Gingival crevicular fluid (GCF), which bathes the gingival sulcus, is an additional source of nutrition in the subgingival environment.

A first step in the development of dental plaque biofilms is the coating of uncolonized surfaces with polysaccharides and proteins forming an acquired pellicle which enhances attachment of initial colonizing bacteria. These primary colonizers such as *Streptococcus* and *Actinomyces* spp. grow and become enmeshed in a matrix of extracellular polymeric substances

(EPS) (***Sutherland et al 2001***). As the micro-colonies develop, additional species, ie, late colonizers such as *P. gingivalis*, are recruited through coaggregation which is mediated between adhesin proteins and polysaccharide receptors (***Kolenbrander et al 1982***) and non-specific aggregation interactions (***Busscher & van der Mei 1997***) increasing the species complexity. *Fusobacterium nucleatum* plays an important role in this coaggregation process, as it has the ability to aggregate with the primary as well as the secondary colonizers, thus serving as a bridging link. This successional process allows species that cannot adhere to the acquired pellicle to become part of the biofilm (***Rickard et al. 2003).*** Additionally, coaggregation, which promotes cellular juxtaposition between the colonizing species, facilitates enhanced cell–cell interactions, such as the exchange of metabolites (***Egland et al. 2004***) and cell signaling molecules (***Rickard et al. 2006***).

1.3. MICROBIAL SHIFTS FROM HEALTH TO PERIODONTITIS

An early study of the subgingival environment, in which the presence of 40 bacterial species was assessed by DNA- DNA hybridization, provided strong evidence for the existence of sets of co-occurring organisms (color-coded "complexes") which were associated with clinically defined periodontal health states ***(Socransky et al 1998***). The yellow complex, comprising Mitis-group streptococci, was associated with relatively healthy sites; the orange complex, comprising *Fusobacterium*, *Prevotella* and *Campylobacter*, was identified as a group possibly required for the colonization of periodontitis-associated microorganisms; and the red complex, comprising *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola*, was associated with the most severely compromised pockets and correlated with bleeding sites. These complexes were then used as a template for examining periodontal treatment efficacy in 461 patients **(*Haffajee et al 2006).***

Reductions in the numbers of bacteria of the red complex and of the orange complex were identified 12 months after starting treatment, which corresponded to clinical measures of treatment efficacy. For example, across all locations where attachment loss was greatly reduced through therapy, the numbers of bacteria of the red and orange complexes were also reduced. Conversely, in sites where attachment loss increased despite therapy, no changes were observed in these complexes.

More recently, higher advancement in molecular technologies have paved way for a better understanding of the microbiome transition from health to disease, which is characterized by shifts in the whole community. Studies by ***Griffin, et al* (2012) , *Abusleme et al*. (2013), *Hong et al.* (2015)** and ***Schincaglia et al.* (2017)** have shown that health-associated communities are enriched in Gram-positive bacteria like *Rothia* and *Actinomyces*, while gingivitis communities are associated with *Prevotella, Selenomonas and Fusobacterium* species. As periodontitis develops, there is a more pronounced shift with establishment of a highly diverse microbiota, with a trend towards increase in Gram-negative organisms among them *P. gingivalis, Tanerella forsythia* and *T. denticola*. However, it is also important to emphasize that though distinct species are associated with disease, health associated species can be detected in disease and disease associated species in health, although at low proportions and frequency. Also certain bacteria are constantly found in health and disease, which are known as the core species. These studies showed that dysbiosis results from a microbial succession without replacement process with periodontitis-associated species becoming the dominant biomass components in disease and health-associated species remaining part of the subgingival biofilm. Since most periodontitis-associated species can be found in a significant proportion of samples from healthy subjects, dysbiosis is not the consequence of de novo colonization ***(Abusleme et al. 2013)*.**

1.4. *PORPHYROMONAS GINGIVALIS* - A MAJOR PERIODONTAL PATHOGEN

Despite the polymicrobial nature and high diversity of the microbiome associated with disease, studies have shown that *P. gingivalis* plays a cardinal role in the etio-pathogenesis of periodontitis. In a study involving ligature induced periodontitis in *Macaca fascicularis*, the animals were super- infected with *P. gingivalis*, and then immunized with cysteine protease purified from *P. gingivalis* ***(Page et al 2007***). Alveolar bone loss was measured by digital subtraction radiography. It was observed that the onset and progression of alveolar bone loss was inhibited by approximately 50% in the immunized animals compared to controls, showing *P. gingivalis* as an important inducer of disease.

P. gingivalis is a non- motile, asaccharolytic, Gram-negative obligate anaerobic rod which forms black-pigmented colonies on blood agar plates. It has an absolute requirement for iron in the form of heme for its growth. It was formerly named *Bacteroides gingivalis* prior to its reclassification in a new genus, *Porphyromonas* ***(Nisengard and Newman, 1994)***. The name *Porphyromonas* comes from the Greek adjective '*porphyros'* meaning purple and the Greek noun *monas* meaning unit. Hence, the word *Porphyromonas* means porphyrin cell as the colonies on blood agar plates turn black after 6 to 10 days due to heme accumulation ***(Shah and Collins, 1988).***

1.5 SUBGINGIVAL COLONIZATION OF *P. GINGIVALIS*

The major habitat of *P. gingivalis* is the subgingival sulcus of the human oral cavity. Specific growth requirements and properties of *P. gingivalis* such as a need of iron in the form of hemin; an inability to ferment carbohydrates; production of potent proteases capable of degrading

host components; and an obligately anaerobic respiration, makes the subgingival sulcular environment characterized by the nutritional provision by GCF and reduced oxygen tension, a conducive milieu for the colonization of *P. gingivalis* ***(Bostanci and Belibasakis, 2012).***

P. gingivalis depends on nitrogenous substrates for energy. Most reports suggest that *P. gingivalis* can only utilize peptides efficiently for growth ***(Gharbia and Shah, 1991).*** Among peptides efficiently utilized are glutamylglutamate and aspartylaspartate ***(Takahashi et al. 2000).*** However, ***Dashper et al. (2001)*** identified an uptake system for the amino acids serine and threonine, suggesting *P. gingivalis* can also utilize certain free amino acids. Therefore an environment high in nitrogenous compounds such as the subgingival sulcus is conducive to the growth of *P. gingivalis*.

Since *P. gingivalis* requires iron in the form of hemin, an inflamed subgingival environment is propitious for its growth. *P. gingivalis* is thought to obtain hemin in vivo via the proteolysis of haemoglobin, haptoglobin and haemopexin, which are hemin-carrying plasma proteins (McKee et al. 1986). Another nutritional requirement of *P. gingivalis*, at least of some strains, is exogenous vitamin K (menadione or 2-methyl-1,4-naphtoquinone) which is required as an electron shuttle between respiratory complexes ***(Meuric et al. 2010)***. In the subgingival environment vitamin K may be provided by other microbiome members.

1.5.1. ADHESION AND INITIAL COLONIZATION OF *P. GINGIVALIS*

Although *P. gingivalis* cells adhere to the salivary pellicle on the tooth surface, the requirement for anaerobic conditions will tend to delay colonization until the initial colonizers have reduced the oxygen tension. *P. gingivalis* can adhere to many of these early plaque organisms, including streptococci and *Actinomyces* (***Goulborne et al 1996***). The fimbriae and a

35-kDa membrane protein of *P. gingivalis*, interact with the proteins of *Streptococcus* (***Lamont et al 1994).***

Fimbriae are thin, proteinaceous surface appendages that protrude from the outer membrane of a bacterial cell. These 3–25 nm long structures are harbored by most of the *P. gingivalis* strains. *P. gingivalis* expresses two distinct fimbriae on its cell surface: one consists of a subunit protein (named FimA or fimbrillin) encoded by the *fimA* gene (termed long, or major fimbriae), while the other subunit Mfa protein is encoded by the *mfa1* gene (termed short, minor or Mfa1 fimbriae). Even though the two fimbriae are antigenically distinct and differ in their amino acid composition, they both are believed to contribute to colonization and the progression of periodontal inflammation ***(Amano et al., 2010).***

Binding to *A. naeslundii* also involves fimbrillin and a 40-kDa membrane protein of *P. gingivalis* along with a high-molecular-weight carbohydrate of *Actinomyces* (***Goulborne et al 1996***). *P. gingivalis* can also bind other later colonizers such as *Fusobacterium nucleatum* and *Treponema denticola* ***(Yao et al 1996).*** *F. nucleatum* outer membrane proteins 30-kDa and 42-kDa participate in binding to galactose-containing carbohydrates on *P. gingivalis*. These kinds of inter-species binding interactions may not only favor colonization but also promote nutritional interrelationships and intercellular signaling mechanisms ***(Kolenbrander et al 1998).*** Eukaryotic cells in the oral cavity also provide substrates for *P. gingivalis* attachment. *P. gingivalis* can bind to epithelial cells, fibroblasts, and erythrocytes, and to components of the extra-cellular matrix, namely laminin, elastin, fibronectin, type I collagen, thrombospondin and vitronectin **(*Lamont et al 1998).***

The capsule of *P. gingivalis* also plays a role in initial colonization. Studies by ***Dierickx et al. (2003)*** revealed that the presence and type of capsule had a significant influence on the initial

adhesion of *P. gingivalis* to human periodontal pocket epithelial cells. Co-aggregation between *P. gingivalis* and another periodontal pathogen, *Fusobacterium nucleatum* has been shown to be capsular dependent ***(Rosen and Sela, 2006).*** Studies using mouse subcutaneous infection models have revealed that, encapsulated *P. gingivalis* strains are more virulent than non-encapsulated strains. The capsule was shown to promote virulence in a mouse model by reducing phagocytosis and thereby increasing bacterial survival within host cells, and eventually promoting a longer inflammatory response ***(Singh et al., 2011).*** On the contrary, in terms of invasion efficiency, the capsule of *P. gingivalis* makes it less efficient in invading gingival fibroblasts compared to the non-capsular strains ***(Irshad et al., 2012).*** In addition, the capsule may also contribute to increased survival by reducing the bactericidal effect of antimicrobial peptides such as defensins ***(Igboin et al., 2011).***

1.5.2 ROLE OF ENZYMES IN COLONIZATION AND SURVIVAL

The ability of *P. gingivalis* to secrete numerous hydrolytic, proteolytic, and lipolytic enzymes along with toxic metabolites, is one of the virulence characteristics that allows these bacteria to thrive in the oral cavity. These enzymes usually come into close proximity with the host cells. While some enzymes are found within the periplasmic space, others are transported from the outer membrane into outer membrane vesicles during growth. Proteases in particular appear to be strongly implicated in periodontal disease progression. Among these proteases are trypsin-, thiol-, caseinolytic proteinases, and peptidases ***(Curtis et al., 2001).***

There are two distinct families of proteases produced by *P. gingivalis*. One of them is the cysteine proteinase family or also known as “trypsin-like” enzyme and the other one is serine proteinase ***(Bostanci and Belibasakis, 2012).*** The “trypsin- like” enzymes cleave polypeptides at

the C-terminal end after arginine or lysine residues. These proteinases are commonly known as gingipains, namely gingipain R and K, that cleave after arginine and lysine, respectively. They collectively account for 85% of the extracellular proteolytic activity of *P. gingivalis* at the site of infection ***(de Diego et al., 2014).*** There are two types of gingipain R, namely RgpA and RgpB, while there is one type of gingipain K, Kgp. Gingipain R degrades extracellular matrix components, including the integrin–fibronectin-binding, cytokine, immunoglobulin and complement factors ***(Curtis et al., 2001).*** It is also vital for the processing and maturation of the major fimbriae (FimA) ***(Kristoffersen et al., 2015).***

P. gingivalis proteases are involved in the degradation of extracellular matrix proteins such as collagen, activation of the host matrix metalloproteinases, inactivation of plasma proteinase inhibitors, cleavage of cell surface receptors, and deregulation of the inflammatory response ***(Potempa et al., 2000; Imamura et al., 2003).*** They are also important additive agents on the growth of *T. forsythia* and *A. actinomycetemcomitans* in a mixed-species biofilm with *P. gingivalis* ***(Bao et al., 2014).***

Gingipains were found to degrade fibrinogen contributing to inhibition of blood coagulation, and also degrade host heme proteins, thereby enhancing the availability of hemin for bacterial growth ***(Sroka et al., 2001).*** Gingipains are also important for the degradation of antibacterial peptides, such as neutrophil-derived α-defensins, complement factors, such as C3 and C4, and T cell receptors, such as CD4 and CD8 ***(Hajishengallis et al., 2013; Bao et al., 2014)***

P. gingivalis cells bind to and degrade human plasma fibronectin, laminin, fibrinogen and collagen. The adhesion and degradation processes involve the activities of fimbriae and of the Arg-X-specific and Lys-X-specific cysteine proteinases ***(Lantz et al 1996).*** Hydrolysis of fibronectin

or other matrix proteins such as collagen by the *P. gingivalis* Arg-X proteinases RgpA and RgpB enhances the binding of fimbriae to these substrates. The proteinases are able to expose sequences within host matrix protein molecules that carry C terminal Arg residues, thus promoting adhesion of the organism through a fimbrial-arginine interaction ***(Kontari 1996).***

P. gingivalis, in addition produces at least eight hemagglutinating molecules. Since *P. gingivalis* utilizes heme for growth, binding of bacterial cells to erythrocytes may serve a nutritional function. Hemagglutinin activities expressed by *P. gingivalis* include those complexed with lipopolysaccharide and lipid on the cell surface and a released 40-kDa form of activity designated exo-hemagglutinin ***(Inoshita et al 1986).***

Invasive *P. gingivalis* cells inhibit secretion of interleukin 8 (IL-8) by gingival epithelial cells and, moreover, antagonize IL-8 secretion following stimulation by common plaque constituents ***(Darveau et al 1998).*** Inhibition of IL-8 accumulation, or proteolytic destruction by *P. gingivalis* could have a debilitating effect on innate host defense in the periodontium where bacterial exposure is constant. The host would be rendered incapable of directing leukocytes for bacterial removal. The ensuing overgrowth of bacteria could then contribute to a burst of disease activity ***(Hajishengalis et al 2015).***

Thus *P. gingivalis* has a repertoire of virulence factors that enables it to establish in the subgingival environment. *P. gingivalis*, however, only becomes an abundant member of plaque once inflammatory periodontal disease is established. It therefore appears that the periodontal pocket provides the most suitable conditions for *P. gingivalis*'s growth.

1.6 PREVALENCE AND ABUNDANCE OF *P. GINGIVALIS* IN HEALTH AND DISEASE

The association of *P. gingivalis* with periodontitis has been evidenced by the relatively high numbers in disease and minimal numbers in health. In a study comparing the prevalence of *P. gingivalis* in health and disease, it has been demonstrated using a highly sensitive PCR assay that *P. gingivalis* can be detected in 25% of the subjects with minimal periodontal disease but is detected in 79% of subjects affected by periodontitis ***(Griffen et al 1998)***. The odds ratio for being infected with *P. gingivalis* was 11.2 times greater in the periodontitis group than in the healthy group.

In order to elucidate the natural history of *P. gingivalis* colonization, a study by ***Lamell et al. (2000)*** examined for the presence of *P. gingivalis* with a PCR-based assay in a cohort of 222 children between ages 0 and 18 years. *P. gingivalis*, was detected in 36% of the subjects at the first sampling. 101 of the original subjects were recalled after 1 to 3 years to determine the continuous presence of *P. gingivalis*, with 43% of the subjects testing positive for *P. gingivalis* at the second visit. However, in most children, *P. gingivalis* appeared to colonize only transiently, with low concordance between colonization in the first and second sampling. *P. gingivalis* became more stable in the late teenage years (17-22 age group), with concordance between the two samplings seen in around 30% of subjects. Therefore, *P. gingivalis* is a common inhabitant of the oral cavity of children at any age but it only transiently colonizes. However, *P. gingivalis* appears to become more stable in the late teenage years, possibly as deeper pockets develop.

More recently, a meta-analysis of 16S rRNA gene-based studies of the subgingival microbiome conducted by ***Abusleme et al. (accepted for publication in Periodontology 2000)*** evaluated the prevalence and relative abundance of *P. gingivalis* in subjects with periodontal health, gingivitis and periodontitis. It was found that the prevalence of *P. gingivalis* was very low

in health and higher in both gingivitis and periodontitis. However, when comparing the relative abundance in different disease states, *P. gingivalis* showed a remarkable increase only in periodontitis, whereas it exhibited very low abundance in health and gingivitis. Altogether it appears that *P. gingivalis* has difficulty establishing under conditions of health, but its colonization is facilitated by the presence of inflammation, and its growth is promoted only in periodontal pockets.

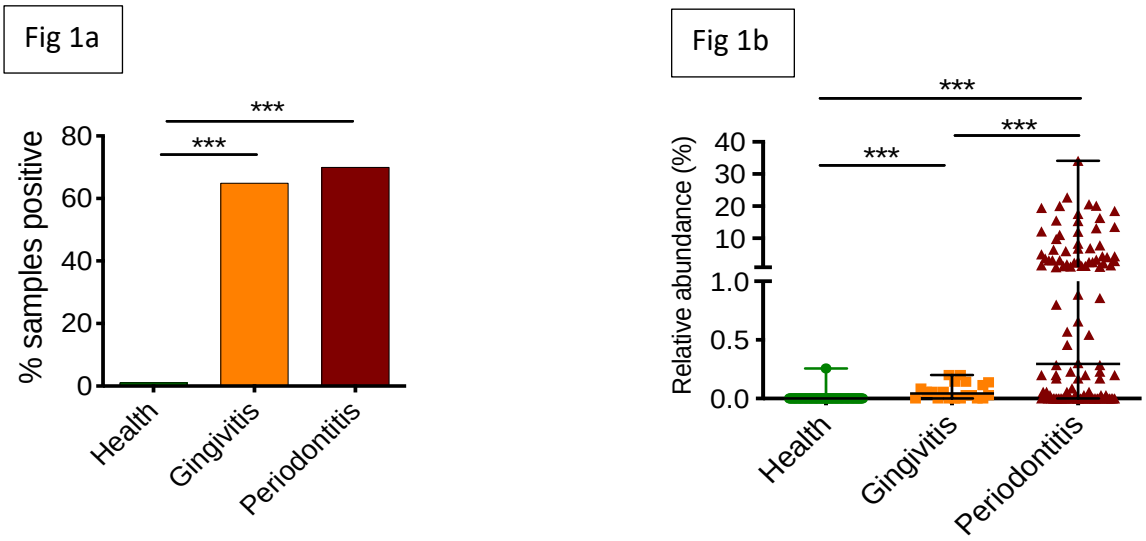


FIGURE 1: Prevalence of *P.gingivalis* (Fig 1a) and relative abundance (Fig 1b) in health, gingivitis and periodontitis. Data derived from meta- analysis of 16S RNA gene-based studies of the subgingival microbiome (*Abusleme* et al. Periodontology 2000, accepted for publication).

1.7. GROWTH OF *P. GINGIVALIS* IS CELL DENSITY DEPENDENT

Unpublished work form Dr. Diaz's lab shows that a cell-density-dependent mechanism controls the in vitro growth of *P. gingivalis*. Experiments have shown that *P. gingivalis* inoculated in batch anaerobic conditions at the low cellular concentrations of 10^5 or 10^6 cells/mL failed to grow, but when inoculated at 10^7 or 10^8 cells/mL was able to grow (Figure 2a). This dependency on inoculum size occurred in two growth media tested (Brain Heart Infusion – BHI- supplemented

with cysteine, hemin and menadione; and a hog gastric mucin-based medium, supplemented with hemin and 10% heat-inactivated human serum) (Figure 2b). To further delineate if this effect was mediated by a soluble factor produced by the bacteria, spent medium from stationary phase cultures was added to *P. gingivalis* inoculated at low cell density. It was observed that in comparison to the negative control (no spent medium), the spent medium restored growth of low cell density inocula. (Figure 3)

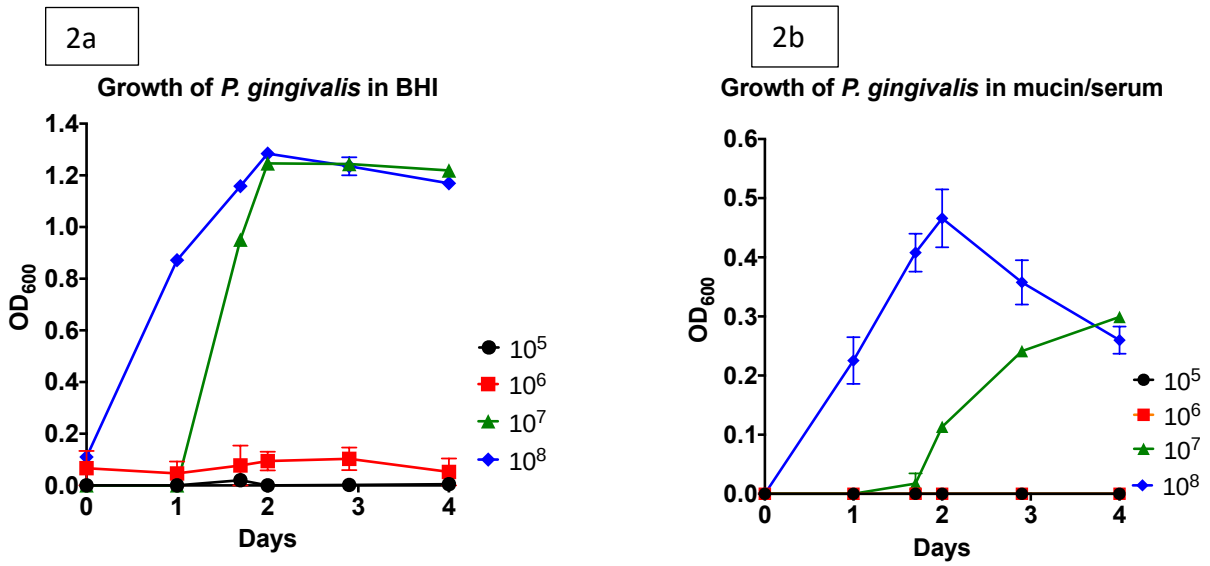


FIGURE 2: Effect of inoculum density on the growth of *P. gingivalis*. P. gingivalis was inoculated in BHI/cysteine/ hemin/menadione medium (2a) and mucin- serum medium (2b) at different cellular concentrations. Graphs show the growth curve of *P. gingivalis* plotted with OD against time showing the cell density dependent growth.

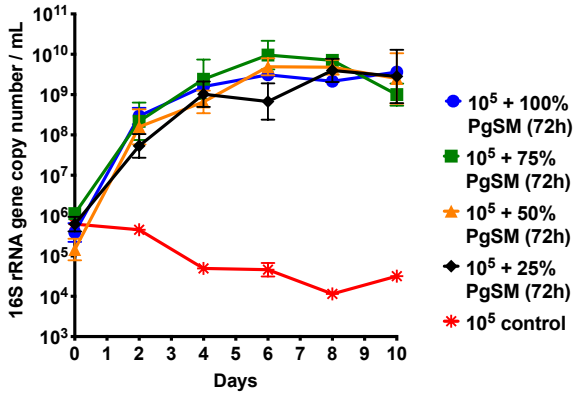


FIGURE 3: Effect of mucin- serum spent medium on the growth of *P. gingivalis*. Data show the growth of *P. gingivalis* when inoculated at a cellular concentration of 10^5 cells/ml in mucin-serum supplemented with spent medium prepared from a stationary growth phase culture of *P. gingivalis*. *P. gingivalis* growth was evaluated via qPCR for the 16S rRNA gene.

Subsequent experiments evaluated if other early colonizing microorganisms could support the growth of low density *P. gingivalis*. These experiments showed that *Veillonella parvula* was able to support the growth of *P. gingivalis* from low cell-density inocula (Figure 4a). The experiment was carried out with 3 different strains of *V. parvula* (ATCC 10790, PK1941, PK1910) and it was observed that irrespective of the strain, *V. parvula* supported the growth of low-cell density *P. gingivalis*. The effect of *V. parvula* was independent of the presence of the bacteria themselves as evidenced by the restoration of growth of *P. gingivalis* when inoculated with a 24 hour spent medium of a culture of *V. parvula*. (Figure 4b).

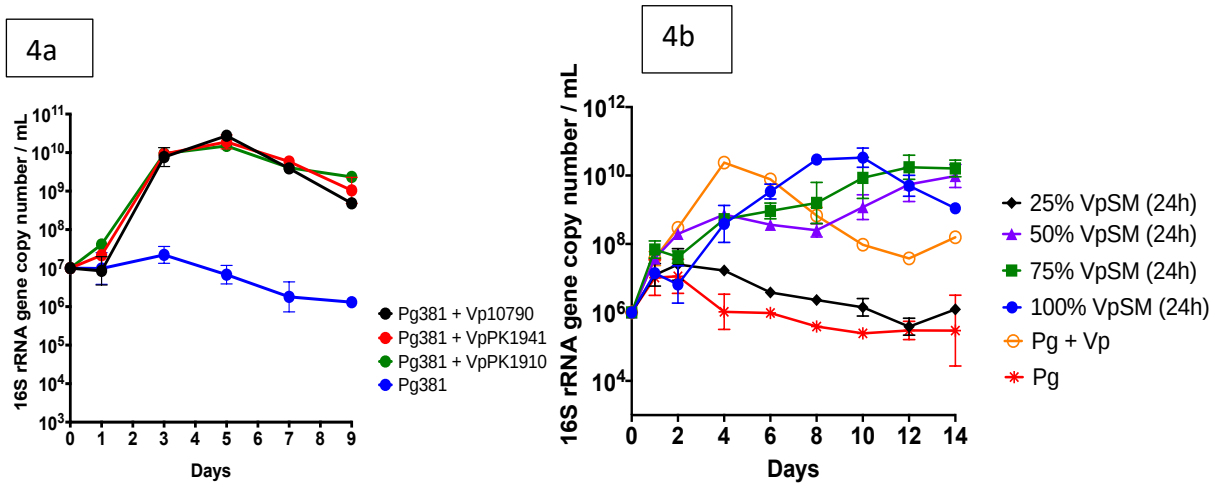


Figure 4: Effect of *V. parvula* on the growth of low cell density *P. gingivalis*. Figure 4a: Growth curve of *P. gingivalis* in mucin/serum medium when co-inoculated (at 10^5 cells/ml) with different strains of *V. parvula*. Figure 4b: Graph shows the growth of *P. gingivalis* when inoculated at a cellular concentration of 10^5 cells/ml in mucin/serum medium supplemented with spent medium prepared from a stationary growth phase culture of *V. parvula*.

Further experiments were conducted in Dr. Diaz's lab to characterize the spent medium from stationary liquid phase cultures of *P. gingivalis* and *V. parvula*. In these experiments, spent media was obtained, filtered through a 1 Kd spin filter and the <1 Kd filtrate, which supported *P. gingivalis* growth, was analyzed by gas chromatography (GC) time of flight (TOF) mass spectrometry. Fresh medium treated in the same manner was also analyzed in order to obtain a list of candidate small molecules enriched in the spent medium compared to the fresh medium and possibly required for the growth of *P. gingivalis*. The small molecules enriched in both the spent medium of *P. gingivalis* and *V. parvula* in comparison to the fresh medium are shown in Figure 5. Among the identifiable compounds in this analysis, glutamine, 4-hydroxybutyric acid, inosine, glycyl-proline and uridine have been tested but they did not allow growth of low cell-density *P. gingivalis* when exogenously added to fresh medium. The candidate compound that has not been evaluated yet is tyrosine.

BinBase name	m/z	Fold Vp SM / FM	Fold Pg SM / FM	Tested concentrations (mM)
glutamine	156	27.14	125.37	4.1, 0.4, 0.041
4-hydroxybutyric acid	233	5.15	11.65	0.31, 0.031, 0.0031
inosine	230	7.38	8.92	12.5, 1.25, 0.125
glycyl-proline	174	10.82	8.43	0.29, 0.029, 0.0029
uridine	224	2.40	6.51	0.65, 0.065, 0.0065
136	227	33.55	115.26	NT
16549	227	26.72	106.84	NT
592	142	3.41	14.29	NT
3143	155	4.24	12.05	NT
17505	324	8.37	10.02	NT
17537	258	2.65	7.18	NT
14682	159	27.75	7.14	NT
17233	170	2.53	6.80	NT
110021	205	3.01	6.23	NT
225276	183	4.13	5.76	NT
3618	328	2.29	4.90	NT
210231	145	2.99	3.81	NT
12296	124	3.21	3.38	NT
227209	271	3.33	3.16	NT
98027	217	2.34	3.12	NT
16675	217	6.30	2.85	NT
210891	276	2.94	2.69	NT
223259	437	2.13	2.50	NT
histidine	154	2.21	2.36	1, 0.1, 0.01
tyrosine	218	2.55	2.29	

FIGURE 5: Mass spectrophotometric analysis of the spent medium from *P. gingivalis* and *V. parvula*. Data represents the fold change of the compound in spent medium in comparison to fresh medium.

These experiments show that *P. gingivalis* requires a certain cell density threshold to grow and that a soluble factor present in its own stationary phase cultures allows it to initiate growth. These experiments also showed that a specific inter-species interaction with *V. parvula* supports growth of low cell density *P. gingivalis*. These findings may have implications for the survival of *P. gingivalis* in the oral environment and the regulation of its growth at the different stages of plaque development. In the initial stages of plaque formation, when environmental conditions are unfavourable for growth of *P. gingivalis*, *V. parvula* which is an abundant member of early stage biofilms may foster the growth of *P. gingivalis*. As plaque develops and total plaque biomass increases, a cell-density threshold is reached, and with the concomitant change in the environment

subsequent to the microbial succession and inflammatory response, *P. gingivalis* may be able to grow independently, initiating and establishing a dysbiotic milieu.

2. RATIONALE

At the moment the identity of the soluble factor facilitating the growth of low cell-density *P. gingivalis* is unknown. The factor is provided by the early colonizer *V. parvula*. The factor is also produced (or made available) by *P. gingivalis* if present at high cell density. At the moment it is also not clear if spent medium from *P. gingivalis* grown in BHI/cysteine/hemin/menadione restores growth of low cell-density inocula in a similar manner to spent medium from mucin-serum grown cultures. If spent medium from a *P. gingivalis* culture grown under different conditions (not in mucin-serum) also supports growth of a low cell-density inoculum, this would show the effect of the spent medium is robust and not dependent on the growth substrate. A better characterization of the spent medium released by *P. gingivalis* and *Veillonella* is required, which could show candidate compounds that promote growth.

Since inflammation promotes *P. gingivalis* colonization, it is possible that the local environment, including inflammatory components, also provides the required factor to initiate growth of low cell density *P. gingivalis*. Bleeding on probing is a sign of gingival inflammation. The increased proliferation and vasodilation of gingival capillaries along with the necrotic sulcular epithelium increases the propensity to bleed in sites of inflammation. Association of *P. gingivalis* with sites positive for bleeding on probing has been shown in studies by ***Socransky et al 1998.*** Although it is clear that hemin and menadione, which are present in blood, are not the factors required to initiate growth from low cell density, other

compounds present in blood may be able to promote the growth of *P. gingivalis*.

Polyamines are compounds involved in the regulation of inflammatory reactions (***Moinard 2005***). Spermine, a polyamine is released by damaged cells and favors cell migration and mammalian cell growth at sites of local inflammation. Increased levels of polyamines have been found in gingivitis (**Shearer 1997**). The effect of inflammatory components released upon tissue damage namely blood and other host-derived components, such as polyamines, on the growth of *P. gingivalis* has not been tested. Identifying the factor that promotes growth of low cell-density *P. gingivalis* could lead to a better understanding of its oral colonization requirements.

3. HYPOTHESES

- We hypothesize that the type of culture medium does not influence the ability of *P. gingivalis* to release a soluble factor that supports its own growth from low cell density inocula.
- We hypothesize that host-derived inflammatory components namely blood and polyamines are able to support the growth of *P. gingivalis* from low cell density inocula.
- We also hypothesize that tyrosine, one of the components present in the spent medium of *P. gingivalis* and *V. parvula* in higher amount than in fresh medium is able to support the growth of low cell density *P. gingivalis*.

4. AIMS

- Aim 1: to evaluate the effect of spent medium from *P. gingivalis* cultures grown in BHI/cysteine/hemin/menadione on the cell-density dependent growth of *P. gingivalis.*

- Aim 2: to evaluate the effect of inflammatory components/host-derived products on the growth of low cell density *P. gingivalis*. Compounds tested will include blood/blood products and polyamines.

- Aim 3: to evaluate the effect of tyrosine, a factor present in the spent medium of *P. gingivalis* and *Veillonella* for its support of growth of low cell-density *P. gingivalis.*

5. MATERIALS AND METHODS

5.1. PREPARATION OF LIQUID MEDIUM AND AGAR PLATES

A liquid culture of *P. gingivalis* was established in Brain Heart Infusion Medium (BHI/cysteine medium) supplemented with hemin and menadione. BHI medium was prepared by dissolving 18.5 g Brain Heart Infusion and 0.2 g cysteine in 500 ml distilled water, followed by autoclaving. The supplements, namely hemin (final concentration 5 μg/ml) and menadione (final concentration 1 μg /ml), were freshly added prior to inoculation. BHI/cysteine/hemin/menadione agar plates were prepared by adding 7.5 g of agar to 500 ml of BHI + cysteine medium, followed by autoclaving, cooling to 56°C, addition of hemin and menadione supplements and pouring. BHI blood agar plates were prepared in a similar manner plus the addition of 5% fresh blood. Mucin-serum liquid medium (1 L) was prepared by dissolving hog gastric mucin (2.5g) , KCl (2.5g), yeast extract (1g), proteose peptone(2g), trypticase peptone (1g) and cysteine (0.1 g) in distilled water (***Kiniment et al 1996***). 10% heat-inactivated human serum (inactivated by heating to 56°C for 30 min) and hemin to a final concentration of 5 μg/ml were freshly added.

5.2 GROWTH AND MAINTENANCE OF *P. GINGIVALIS*

Throughout this study, *Porphyromonas gingivalis* 381 was used. The strain was grown from frozen stocks into BHI blood agar plates, prepared as described in 5.1. Cultures grown on agar were passaged every 4-5 days for a maximum of 5 passages and then discarded. Liquid cultures were established in BHI/cysteine/hemin/menadione medium. Microorganisms were grown in an anaerobic chamber at 37°C, in a mixture of CO_2, nitrogen and hydrogen. Cell culture purity was routinely checked under a light microscope equipped with a phase-contrast lens.

5.3. PREPARATION OF INOCULUM AT THE OPTIMAL CONCENTRATION

An optical density of 0.4 of *P. gingivalis* as measured with a spectrophotometer (600 nm), corresponds to 5.5 x 10^8 cells/mL (as determined by counting a diluted culture using a Petroff-Hausser counting chamber). To add *P. gingivalis* at a concentration of 10^5 cells/mL, 2 μL of OD_{600} 0.4 was added to a solution of 12 mL of fresh medium. To achieve a cellular concentration of 10^8 cells/ml, 2 mLs of OD 0.4 were centrifuged, the supernatant was discarded and the resulting cell pellet was resuspended in 100 μL of fresh medium, and added to 12 mLs of the culture medium.

5.4. METHODS RELATED TO AIM 1

5.4.1 Preparation of spent medium

P. gingivalis was inoculated in BHI medium supplemented with hemin and menadione, at a cell density of 10^8 cells/ml, and incubated anaerobically for 48 hours. Then the spent medium was prepared by centrifugation and passage of the supernatant twice through 0.2 μm vacuum micropore filters. The resultant filtrate was collected as the spent medium.

5.4.2 Evaluation of effect of spent medium on the liquid growth of low cell density *P. gingivalis*

P. gingivalis at a cellular concentration of 10^5 cells / ml was inoculated in different concentrations of spent medium (0, 25, 50, 75 and 100%) diluted in BHI/cysteine. All cultures were supplemented with hemin and menadione. The growth of *P. gingivalis* was assessed by measuring the optical density daily over a period of 6 days.

5.4.3 Evaluation of effect of spent medium on the solid growth of low cell density *P. gingivalis*

To assess the effect of BHI spent medium on the growth of *P. gingivalis* on a solid medium, BHI agar plates supplemented with hemin and menadione were prepared. *P. gingivalis* was first grown in liquid broth. Different stock concentrations of *P. gingivalis* between the orders of 10^1 to 10^7 cells/mL were prepared by resuspending cells in the spent medium. 100 µl of each of these serially-diluted solutions was then inoculated on the BHI plates. The plates were incubated in the anaerobic chamber at 37°C for a week. The resulting colonies were then counted. BHI agar plates inoculated with *P. gingivalis* at different cellular concentrations (10^1- 10^7) resuspended in fresh BHI medium served as the negative control.

In order to evaluate if the spent medium from mucin-serum medium (hog gastric mucin medium supplemented with hemin and 10% heat inactivated serum) could promote the growth of *P. gingivalis* on solid medium, similar methodology of preparing the spent medium was employed, wherein an anaerobic culture of *P. gingivalis* in mucin-serum medium was established for 3 days, and then filtered twice through a vacuum filter. *P. gingivalis* was then resuspended in the spent

medium, and different concentrations of *P. gingivalis* were prepared by serial dilution, followed by inoculation on BHI plates (100 ul per plate). Plates were incubated in an anaerobic environment for one week. Growth was assessed by counting the number of colonies.

5.5. METHODS RELATD TO AIM 2

5.5.1. Effect of whole blood on the growth of *P. gingivalis* on solid media

To evaluate if whole blood could support the growth of a low cell density inoculum of *P. gingivalis*, the bacterium was inoculated on BHI/blood agar plates (BHI supplemented with hemin, menadione and 5% blood) at varying quantities (10^2, 10^3, 10^4, 10^5 and 10^7). The plates were incubated anaerobically and the resulting colonies counted.

Further, the effect of spent medium on the growth of *P. gingivalis* in the presence of blood was also studied. For this, a stationary liquid growth of *P. gingivalis* in BHI/hemin/menadione medium was established and the spent medium was prepared. A normalized culture of *P. gingivalis* (OD_{600} adjusted to 0.4)was resuspended in the spent medium and inoculated on the blood agar plates in different amounts. After incubation for a week, the growth of *P. gingivalis* was evaluated by assessing the number of colonies and compared to control plates without spent medium.

5.5.2. Effect of whole blood on the growth of *P. gingivalis* in liquid media

A mono culture of a low cell density inoculum (10^5cells/ml) was established in BHI/cysteine/hemin/menadione medium supplemented with 5% whole blood. Similarly, *P. gingivalis* was inoculated in mucin/serum medium containing 5% whole blood. *P. gingivalis* inoculated at 10^8 cells /ml served as a positive control, while a monoculture of *P. gingivalis* at a

concentration of 10^5 cells/ml in the absence of blood served as the negative control.

The growth of *P. gingivalis* was examined by visual inspection of a black sediment on the liquid medium. The biomass of *P. gingivalis* was also quantitatively measured using real time PCR for the 16S rRNA gene. Sampling was done every day wherein 250 ul aliquots from the culture were obtained and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the resulting precipitate was stored at -80°C for further quantitative assessment of the number of bacteria, including the sequential process of DNA isolation, qPCR amplification and quantification.

DNA isolation involved mixing the TE-resuspended sample with lysozyme followed by incubation at 37°C for 30 min. This was further followed by addition of buffer AL and Proteinase K and incubation at 56°C overnight. Samples were then incubated at 95°C for 5 min, treated with ethanol and DNA isolation carried out using a commercial kit (Qiagen- DNA Easy kit) as per the manufacturer's instructions. The DNA obtained was stored at -20°C until further use.

Amplification of the 16S rRNA gene was performed using a PCR mixture containing SYBR Green master mix, forward primer (AGGCAGCTTGCCATACTGCG) and reverse primer (ACTGTTAGTAACTACCGATGT), DNA template, and water up to a volume of 20 µl. Thermal cycler conditions included an initial denaturation step at 95°C for 20s, followed by 40 cycles of denaturation at 95°C for 3s and annealing/extension at 58 °C for 30s. The fluorescence obtained was monitored throughout the reaction and compared against a standard curve generated from a known copy number of *P. gingivalis*.

5.5.3. Effect of blood components on the growth of *P. gingivalis* in liquid media

To elucidate the specific components of blood that could have an influence on the cell density dependent growth of *P. gingivalis*, non-heat inactivated human plasma and non-heat inactivated human serum were evaluated. These blood fractions were added to the mucin-serum medium, substituting the heat inactivated serum, and a low cell density inoculum of *P. gingivalis* (10^5 cells/ml) was added. Growth was monitored for 12 days.

5.5.4. Effect of polyamines on the growth of *P. gingivalis* in liquid media

In order to evaluate the role polyamines could play on the growth of a low cell density of *P. gingivalis,* 3 candidate compounds namely Spermine, Cadaverine, Putrescine were selected. Stock solutions of the compounds were prepared by dissolving in distilled water (Spermine-50 mg/mL, Putrescine-100mg/mL, Cadaverine- 50 mg/mL). *P. gingivalis* was inoculated in mucin serum medium at a cell density of 10^5 cells/ml, and the compounds were added to the medium at different concentrations (concentration range 0.01- 1 mg/mL). The culture was incubated in an anerobic chamber and observed for smell and a black precipitate over 10 days

5.6. METHODS RELATED TO AIM 3

A stock solution of l-tyrosine was prepared and subsequently diluted to obtain stock solutions at different concentrations. Tyrosine stocks were used to supplement mucin-serum medium to final concentrations of 5, 0.5, 0.05 and 0.005 uM. *P. gingivalis* was inoculated at a concentration of 10^5 cells/ml in mucin–serum medium supplemented with different concentrations of l-tyrosine. The culture was maintained anaerobically for 10 days. Sampling was done every day wherein 250 ul aliquots from the culture were obtained and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the resulting precipitate was stored at -80°C for further analysis.

Quantification of *P. gingivalis* by 16S rRNA gene qPCR was done as per the protocol already described in 5.5.2

6. RESULTS

6.1. RESULTS RELATED TO AIM 1

6.1.1. Effect of BHI/cysteine/hemin/menadione spent medium on the liquid growth of *P. gingivalis*

P. gingivalis was inoculated at a cell density of 10^5 cells/ml in BHI/cysteine/hemin/menadione supplemented with spent medium at different concentrations (0, 25, 50, 75 and 100%). The spent medium restored the growth of the low cell density inoculum. This effect was seen in the presence of 25%, 50% and 75% spent medium, but when the BHI fresh medium was completely replaced by spent medium, the spent medium did not support growth (Fig. 6). These results contrasted with the previous experiments in Dr Diaz's lab, wherein *P. gingivalis* was inoculated with spent medium from mucin-serum, and it was observed that the mucin-serum spent medium at all concentrations tested restored the growth of low cell density inoculum (Fig 3).

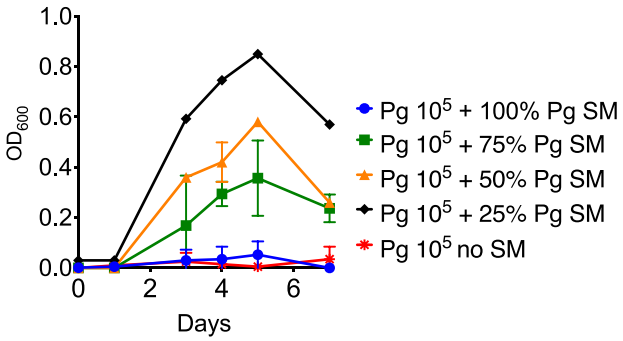
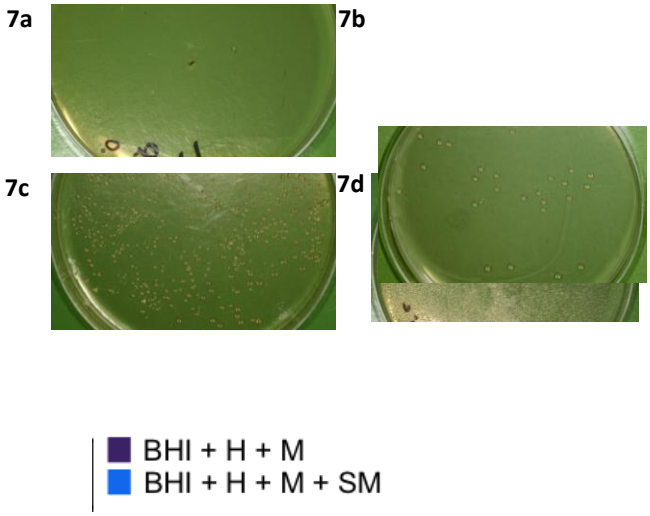


FIGURE 6: Effect of BHI spent medium on the growth of *P. gingivalis* in BHI liquid medium. *P. gingivalis* was inoculated at 10^5 cells/ml in BHI/cysteine/ hemin/menadione medium supplemented with different concentrations of spent medium, which was prepared from a stationary phase culture of *P. gingivalis* grown for 48 hours in BHI/ cysteine/hemin/menadione medium. Data shows the growth curve obtained by plotting OD_{600} against time, data obtained from 2 experiments, graph showing the standard deviation.

6.1.2. Effect of BHI/cysteine/hemin/menadione spent medium on growth of *P. gingivalis* on agar plates

The cell density restricted growth of *P. gingivalis* was evident on BHI/ cysteine/hemin/ menadione where no growth was seen when the inoculum was less than 1000 cells (Fig. 7). However, in the presence of spent medium, *P. gingivalis* exhibited growth when inoculated at a concentration as low as 10 cells per plate. This experiment was performed including replicate plates and repeated 3 times. The difference in the CFUs with and without spent medium was analyzed by t-tests, reaching statistical significance in the test group, especially in the 10^4 cells/ml inoculum.



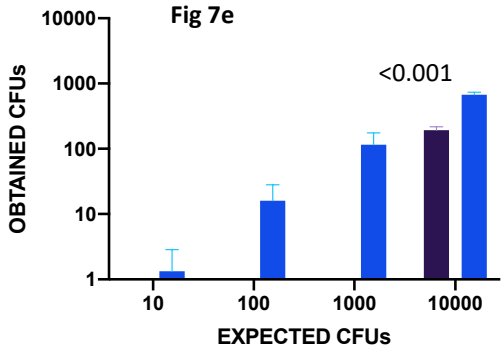


FIGURE 7: Effect of BHI spent medium on the growth of *P. gingivalis* on BHI agar plates. Figs. 7a-d show CFUs on BHI/ Cysteine/ hemin/ menadione agar plates when inoculated at cell density of 10^3(a) and 10^4(b) in the absence of spent medium and when inoculated at 10^3(c) and 10^4 (d) in the presence of spent medium. Fig 7e: Bar graph plotted comparing the expected CFUs to the observed CFUs. Data analysed by t-test.

6.1.2. Effect of mucin serum spent medium on growth of *P. gingivalis* on agar plates

A similar kind of effect of spent medium on restoring the growth of a low cell density inoculum was seen when the spent medium was prepared from a stationary liquid culture of *P. gingivalis* grown in mucin-serum medium supplemented with hemin (Fig. 8).

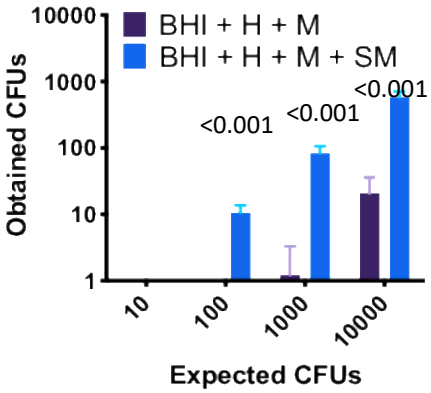


FIGURE 8: Effect of mucin serum spent medium on the growth of *P. gingivalis* on BHI agar plates. *P. gingivalis* was inoculated at different concentrations after resuspension in either fresh medium or spent medium (SM), which was prepared from a culture of *P. gingivalis* grown in

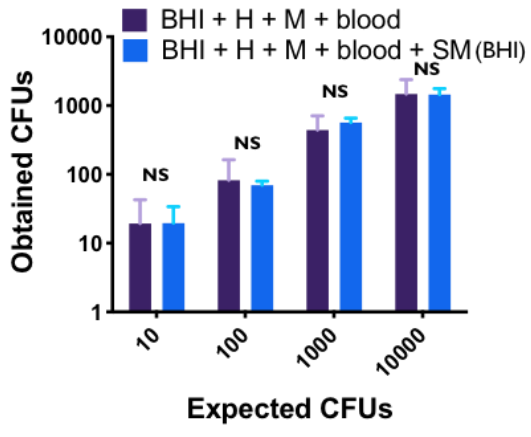
mucin-serum medium. The bar graph compares the expected CFUs and the observed CFUs (CFU- Colony Forming Unit). Data analysed by t- test.

6.2. RESULTS RELATED TO AIM 2

6.2.1. Effect of whole blood on the growth of *P. gingivalis* on solid media

P. gingivalis was inoculated on blood agar plates (BHI/cysteine/hemin/menadione supplemented with 5% whole blood). It was observed that the blood agar supported the growth of *P. gingivalis* from concentrations as low as 10 cells. Inoculation of 10 cells resulted in approximately 10 CFUs, 100 resulted in about 100 colonies, 1000 in about 1000 colonies and 10000 cells resulting in around 3000 colonies (due to crowding effect) (Fig. 9). When spent medium, prepared from BHI/ cysteine /hemin/ menadione/ medium or mucin-serum medium was added, there was no difference observed in the growth in comparison to that in the absence of spent medium (Fig 9a&b). Blood seemed to replace the growth promoting effect of spent medium.

9a



9b

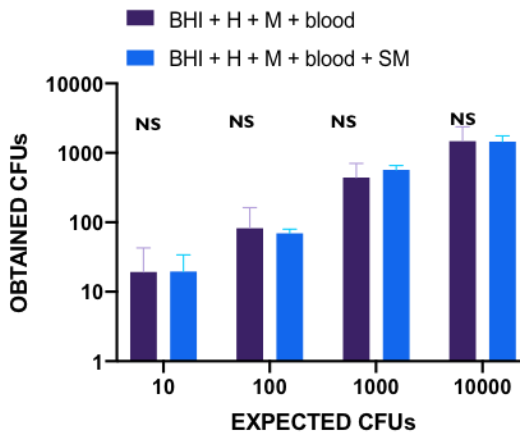


FIGURE 9: Blood supports the growth of low cell density P. gingivalis and abolished the effect of the spent medium. Graphs show obtained and expected CFUs when P. gingivalis was inoculated at different concentrations on blood agar plates. In Fig 9a *P. gingivalis* was resuspended in fresh medium or in BHI-derived spent medium. In Fig 9b *P. gingivalis* was resuspended in fresh medium or in mucin-serum spent medium. Bar graph represents the data from 3 experiments. The graph shows the comparison of the expected number of colonies over the observed colonies (CFU-Colony Forming Unit)

6.2.2. Effect of whole blood on the growth of *P. gingivalis* in liquid medium

When *P. gingivalis* was inoculated in BHI/cysteine/hemin/menadione liquid medium supplemented with 5% blood, it was seen that low cellular concentration of *P. gingivalis* (10^5 cells/ml) exhibited growth, which was evident on measuring the 16S RNA gene copy number (Fig 10a). A similar effect was seen in the mucin- serum liquid medium supplemented with blood, wherein *P. gingivalis* when inoculated at 10^5 cells/ml, exhibited growth in comparison to an inoculum in mucin – serum medium only (Fig 10b). Therefore, the growth restoring effect of blood was evident on both solid and liquid medium.

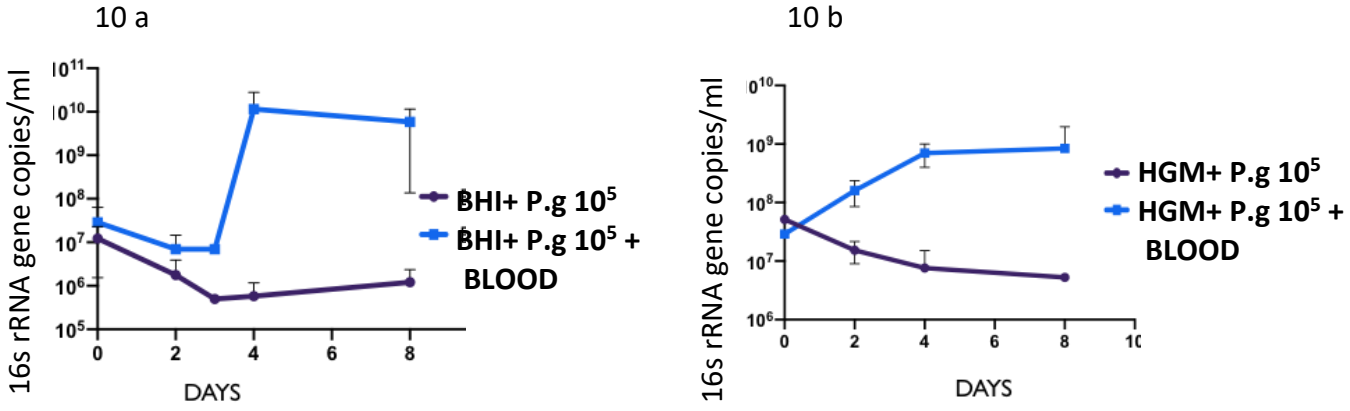


FIGURE 10: Effect of blood on the growth of a low cell density inoculum of *P. gingivalis*. *P. gingivalis* was inoculated at a cell density of 10^5 cells/ml in BHI/cysteine/hemin/ menadione liquid medium(10a) and mucin- serum medium (10b) and supplemented or not with 5% blood. Graph shows the growth curve plotted with 16S RNA gene copies against time, data collected from two experiments with the graph showing the mean and standard deviation.

6.2.3. Effect of blood components on the growth of *P. gingivalis* in liquid media

Since the previous experiments in mucin serum in which P. gingivalis exhibited no growth all involved heat-inactivation of the serum, and since non-heat inactivated whole blood supported growth of *P. gingivalis*, we next performed experiments to evaluate if non-heat inactivated blood components, namely serum and plasma, supported growth of *P. gingivalis*. In this evaluation no growth was observed when *P. gingivalis* was inoculated at 10^5 cells/ml in mucin medium supplemented with these non-heat inactivated blood components. The absence of growth was confirmed by the absence of smell and lack of a black precipitate in the anaerobic liquid culture tube.

	P. gingivalis 10^8 cells/ml	*P. gingivalis* 10^5 cells/ml	Mucin- serum medium+ non-heat inactivated plasma	Mucin- serum medium+ non-heat inactivated serum
Smell	positive	negative	negative	negative
Black Precipitate	positive	negative	negative	negative

TABLE 1: Effect of non heat inactivated plasma and serum on the growth of a low cell density inoculum of *P. gingivalis*. *P. gingivalis* was inoculated at a cell density of 10^5 cells/ml. Data represents the results from visual observation, and smell of the liquid culture observed for 10 days.

6.2.4 Effect of polyamines on the growth of *P. gingivalis* in liquid medium

To assess if polyamines could support the growth of *P. gingivalis*, an experiment was done with two replicate tubes, and repeated twice. The polyamines tested were spermine, putrescine and cadaverine. The candidate compounds were tested at different concentrations. However, it was seen that the compounds did not support the growth of *P. gingivalis* when exogenously added to mucin serum medium at any concentration tested (0.01-1 mg/mL).

	P. gingivalis 10^8 cells/ml	*P. gingivalis* 10^5 cells/ml	Mucin-serum medium+ spermidine (0.01-1 mg/mL)	Mucin-serum medium+ cadaverine (0.01-1 mg/mL)	Mucin-serum medium+ putrescine (0.01-1 mg/mL)
Smell	positive	negative	negative	negative	negative
Precipitate	positive	negative	negative	negative	negative

TABLE 2: Effect of spermidine, putrescine, and cadaverine on a low cell density inoculum of *P. gingivalis*. *P. gingivalis* was inoculated at a cell density of 10^5 cells/ml in mucin-serum medium supplemented or not with different concentrations of polyamines (0.01-1 mg/mL)). Data represents the results from visual observation, and smell of the liquid culture as observed for 10 days.

6.3. Effect of l-tyrosine on the growth of *P. gingivalis* in liquid medium

P. gingivalis was inoculated in mucin- serum medium supplemented with different concentrations of l-tyrosine. It was observed that tyrosine restored the growth of a low cell density inoculum of *P. gingivalis* (Fig. 11). There was no dose response seen as tyrosine influenced the growth of low cell-density *P. gingivalis* in a positive manner at all concentrations tested.

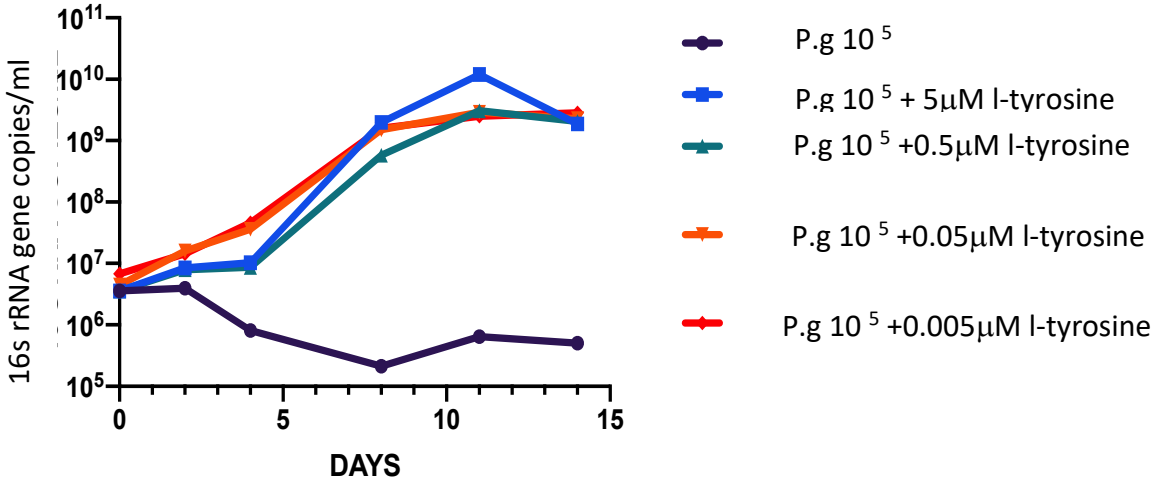


FIGURE 11: Effect of l-tyrosine on the growth of *P. gingivalis*. *P. gingivalis* at a cell density of 10^5 cells/ml inoculated with different concentrations of l-tyrosine in mucin-serum liquid medium. Growth curve plotted with 16S rRNA copies against time.

7. DISCUSSION

P. gingivalis exhibits a definite cell density dependent growth. This effect was seen on 2 different liquid media as per the experiments previously conducted in Dr. Diaz's lab (Figs 2a&2b). While trying to elucidate if this effect could be replicated on a solid surface, it was found that on solid medium (BHI/cysteine/hemin/menadione + agar), *P. gingivalis* also showed a cell density dependent growth (Fig 6). This may be of clinical relevance, considering *P. gingivalis* exists in the oral environment attached to a non- shedding tooth surface on which the biofilm gets established. The ubiquitous nature of the cell density dependent growth in liquid and solid medium, which was also independent of the medium in which *P. gingivalis* in grown, shows that the requirement for a certain cell density is a robust phenomenon, with possible relevance to *P. gingivalis* growth in oral

biofilms. This cell density dependent growth phenomenon may partially explain why *P. gingivalis* cannot easily establish in the oral cavity in health (Fig 1). The cell density dependent growth could be related to quorum sensing, wherein there is a requirement of a quorum (minimum cell threshold) for a microbial population to behave in a certain manner (***Kenneth et al 1990***).

The phenomenon of quorum sensing is based on three basic principles, as reviewed by ***Rutherford and Bassler (2012***). First, the members of the community produce autoinducers (AI), which are the signaling molecules. At low cell density (LCD), AIs diffuse away, and, therefore, are present at concentrations below the threshold required for detection. At high cell density (HCD), the cumulative production of AIs leads to a local high concentration. When the critical threshold signal concentration is reached (i.e., when the population is 'quorate'), the binding of the signal to a response regulator protein results in the switching of genes enabling a response ***(Kaplan et al 1985).*** However, the size of a 'quorum' is not fixed but will be determined by the relative rates of production and loss via degradation or diffusion of the signaling molecules. Secondly, AIs are detected by receptors that exist in the cytoplasm or in the membrane. Third, in addition to activating expression of genes necessary for cooperative behaviors, detection of AIs results in activation of AI production ***(Seed, Passador et al. 1995)***. This positive feedback mechanism loop promotes synchrony.

Quorum sensing was first described by ***Kenneth Nealson*** and his colleagues who demonstrated the bioluminescence of *Vibrio fischeri* at high cell density and also that the supernatant from the high cell density cultures could stimulate the light production in low cell density explaining the phenomenon of autoinduction. Subsequent proteomic work led to the characterization of the signaling molecule and the target gene and the discovery that other bacteria were also capable of autoinducer synthesis and detection ***(Engebrecht et al 1984).***

Oral microorganisms have been shown to produce quorum sensing mediators such as Autoinducer-2 (AI-2). *S. gordonii, F. nucleatum, Aggregatibacter actinomycetemcomitans* , *P. gingivalis* and *Streptococcus oralis* have been shown to produce AI-2 ***(Frias, Olle et al. 2001)***. Early colonizers *S. oralis* and *A. naeslundii* achieved mutualistic growth in an AI-2 concentration dependent manner ***(Rickard, Palmer et al. 2006).*** The addition of *F. nucleatum* AI-2 enhanced the mono- species biofilm of *P. gingivalis* and *T. forsythia* in a dose-dependent manner (***Jang et al. 2013).*** Additionally, LuxS/AI-2 signaling in *P. gingivalis* is involved in regulating the acquisition of hemin and growth under hemin-limited conditions, and the expression of proteases and stress-related genes. Further work needs to be conducted to evaluate the involvement of LuxS/Auto Inducer system in the regulation of the cell-density dependent growth in *P. gingivalis*.

In bacteria, quorum sensing is generally seen to affect cell behaviors rather than promote growth. However, recently, in a study by ***Cho et al 2016***, use of quorum sensing inhibitors namely Furane and D-Ribose in a mouse infection model showed a reduction of bone breakdown and a decrease in the number of bacteria in vivo. The phenomenon of a prolonged lag phase is known to exist in several bacterial species, with some reports showing that autocrine factors are able to stimulate growth ***(Weichart et al 2001; Mukamolova et al. 1998)***. It is not clear, however that the same described quorum-sensing autoinducers are the factors involved in growth stimulation of low cell density bacterial cells. In the fungus *Candida albicans*, however, there is a significant lag in growth at low cell density. The lag is abolished by addition of spent medium from a high density culture. The quorum sensing molecule tyrosol, which has been shown to be released continuously in the medium during growth, was shown to be the factor regulating the initiation of growth ***(Chen et al. 2004).***

In the presence of blood, both in liquid and solid media, *P. gingivalis* exhibited growth even at low cellular concentrations (Fig. 10a&b). Spent medium prepared from either BHI/cysteine/hemin/menadione or mucin – serum medium, did not have an additive effect on the growth on blood. However, this growth promoting effect was not shared by plasma or serum (as shown in liquid cultures) (Table 1).

Blood is classified as a connective tissue and consists of two main components; plasma which constitutes 55% of the total volume and cell elements which include erythrocytes, leukocytes and platelets. The absence of cell promoting effect in the presence of plasma or serum and in the presence of whole blood suggests the cellular elements may play a role in supporting the growth of *P. gingivalis*.

Polyamines are small polycations that are well conserved in all the living organisms except Archaea, Methanobacteriales and Halobacteriales. The most common polyamines are putrescine, spermidine and spermine. They are usually derived from diet or produced by de novo synthesis in mammalian cells. They are involved in a variety of cellular processes such as gene expression, cell growth, survival, stress response and proliferation (***Thomas et al 2001***).

Classical polyamines (putrescine, spermidine and spermine) are involved in multiple aspects of cell physiology, interacting with cell components such as nucleic acids, cytoskeleton proteins and membranes, and are involved in functions such as regulation of gene expression, ion channel function and cell cycle progression. Polyamines levels in cells are tightly controlled by strict regulation of key enzymes of their synthesis and catabolism and by regulation of their

transport but could also be modified by controlling their exogenous supply. Their role in cell proliferation means that they not only play important role in the normal functions of tissues with high rates of cell renewal, such as the digestive and immune systems, but also are involved in carcinogenesis.

Polyamines are also involved in the regulation of inflammatory reactions (***Moinard 2005***)· Spermine, one of the polyamines released by damaged cells favors cell migration and growth at the site of local inflammation. Increased levels of polyamines have been found in gingivitis compared to periodontal health (**Shearer 1997**). Polyamines are known to be necessary for critical physiological functions in bacteria, such as growth, biofilm formation, and other surface behaviors, and production of siderophores.

The localised increase of polyamines in the gingival sulcus at the site of inflammation and the role of polyamines on microorganisms led to the hypothesis that probably these compounds could influence the growth of *P. gingivalis*. However, it was seen that the polyamines did not support the growth of a low cell density inoculum of *P. gingivalis* (Table 2).

The identification of tyrosine in the spent medium of *P. gingivalis and Veillonella*, and the ability of exogenously added tyrosine to support growth of low cell density inocula suggest that tyrosine may be the factor required for growth (Fig 11). However, this experiment was only performed one time and therefore needs to be repeated to further confirm and corroborate the role tyrosine could play in the growth of *P. gingivalis.* After a search in the KEGG database, it was seen that *Veillonella* has the genes required to synthesize tyrosine de novo. However, *P. gingivalis* does not have a complete pathway for tyrosine biosynthesis. Therefore, *P. gingivalis* may be releasing tyrosine from the growth media via its potent proteases. This search also revealed that *P.*

gingivalis does not metabolize tyrosine for energy production as it does not have the gene machinery required for this function.

Tyrosine could be an important building block for bacterial enzymes such as tyrosine kinases and tyrosine phosphatases, which could affect bacterial behaviour. Bacterial tyrosine (BY) kinases are structurally distinct from eukaryotic tyrosine kinases, but are well conserved among bacterial species (***Cozzone 2009; Grangeasse et al. 2012)***. The best documented role for BY kinases is in the synthesis of extracellular polysaccharide where they function in polymerization and transport (***Whitfield 2006)***. However, proteomic studies have revealed a wide range of potential substrates for BY kinases and they are increasingly recognized as regulators of a variety of bacterial functions (***Whitmore et al 2012***). BY kinases have also been shown to be involved in stress responses, antibiotic resistance lysogeny, and DNA metabolism; and phosphorylation can affect the location as well as the activity of substrate proteins (***Whitmore & Lamont, 2012).***

Tyrosine could be used by *P. gingivalis* as a building block for tyrosine phosphatase and tyrosine kinase. The Ptk1 bacterial tyrosine (BY) kinase of *P. gingivalis* is required for maximal community development and for the production of extracellular polysaccharide. Complementation of a ptk1 mutant with the wild type ptk1 allele in trans could restore community development between *P. gingivalis* and *S. gordonii*, and extracellular polysaccharide production by *P. gingivalis* ***(Liu et al 2017).*** A possible role of tyrosine in promoting growth of *P. gingivalis* has not yet been established.

However, the influence of nutritional conditions, including tyrosine availability, on bacterial physiology has been evaluated on *Pseudomonas aeruginosa*. Aromatic amino acids,

including tyrosine, have been shown to influence the cell-cell signaling and antimicrobial activity in *P. aeruginosa* during growth in a sputum-like medium ***(Palmer et al. 2007)***. Tyrosine is important for production of the Pseudomonas quinolone signal, PQS, which is one of three quorum sensing molecules produced by *P. aeruginosa* ***(Palmer et al. 2005)***. These studies could provide further cues to elucidate the role of tyrosine on *P. gingivalis* cell-density dependent growth.

8. CONCLUSIONS

The present study showed that the cell density dependent growth of *P. gingivalis* occurs in liquid and solid media. The spent medium obtained from a stationary liquid phase culture of *P. gingivalis* supported the growth of low cell density *P. gingivalis* and was independent of the medium in which *P. gingivalis* initially grew. Blood supported the growth of a low cell density inoculum of *P. gingivalis,* in both BHI and Mucin-Serum medium, however it is not clear which component in blood is responsible for the effect. Tyrosine, which was previously detected in the spent medium, supported the growth of a low cell density inoculum of *P. gingivalis.*

However, there are several missing links that need to be established in understanding the whole process such as mechanisms underlying the cell density dependent growth, elucidation of the factor present in blood that promotes growth of *P. gingivalis*, decoding the cellular mechanism in which tyrosine may influence growth. Further studies need to be done to provide a better understanding of the phenomenon. Understanding of this cell density dependent growth in turn will throw light on the oral colonization requirements of *P. gingivalis*, which will in turn help in developing avenues for control of the growth of this microorganism associated with destructive periodontitis.

REFERENCES

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Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. Journal of clinical microbiology. 2005; 43:5721–5732.

Amano, A. (2010). Bacterial adhesins to host components in periodontitis. *Periodontology* 2000, 12–37

Bao, K., Belibasakis, G. N., Thurnheer, T., Aduse-Opoku, J., Curtis, M. A., and Bostanci, N. (2014). Role of *Porphyromonas gingivalis* gingipains in multi- species biofilm formation. *BMC Microbiol.* 14:258.

Belibasakis, G. N., Bostanci, N., and Reddi, D. (2010). Regulation of protease- activated receptor-2 expression in gingival fibroblasts and Jurkat T cells by *Porphyromonas gingivalis*. *Cell Biol. Int.* 34, 287–292.

Bostanci, N., Thurnheer, T., Aduse-Opoku, J., Curtis, M. A., Zinkernagel, A. S., and Belibasakis, G. N. (2013). *Porphyromonas gingivalis* regulates TREM-1 in human polymorphonuclear neutrophils via its gingipains. *PLoS ONE* 8:e75784.

Bos, M. P., and Tommassen, J. (2004). Biogenesis of the gram-negative bacterial outer membrane. *Curr Opin. Microbiol.* 7, 610–616.

Brunner, J., Scheres, N., El Idrissi, N. B., Deng, D. M., Laine, M. L., van Winkelhoff, A. J., et al. (2010). The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts. *BMC Microbiol.*

Chen H, Fujita M, Fung Q, Clardy J,.Tyrosol is a quorum sensing molecule in *Candida albicans*. *Proc Natl Acad Sci 2004*, 101(14), 5048-5052

Cho, Song, Amara,(2016). In Vivo Inhibition of *Porphyromonas gingivalis* Growth and Prevention of Periodontitis With Quorum-Sensing Inhibitors. J *Periodontol* 2016; 87:1075-1082.

Curtis, M., Aduse-Opoku, J., and Rangarajan, M. (2001). Cysteine proteases of *Porphyromonas gingivalis*. *Crit. Rev. Oral Biol. Med.* 12, 192–216.

Darveau RP, Belton CM, Reife RA, Lamont RJ. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. *Infect Immun* 1998: 66: 1660–1665.

Darveau, R. P., Pham, T.-T. T., Lemley, K., Reife, R. A., Bainbridge, B. W., Coats, S. R., et al. (2004). *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infect. Immun.* 72, 5041–505

Dashper SG, Brownfield L, Slakeski N, Zilm PS, Rogers AH, . Sodium ion-driven serine/threonine transport in Porphyromonas gingivalis. *J Bacteriol.* 2001 Jul;183(14):4142-8

de Diego, I., Veillard, F., Sztukowska, M. N., Guevara, T., Potempa, B., Pomowski, A., et al. (2014). Structure and mechanism of cysteine peptidase Gingipain K (Kgp), a major virulence factor of *Porphyromonas gingivalis* in periodontitis. *J. Biol. Chem.* 289, 32291–32302

Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, et al(2010). The Human Oral Microbiome. Journal of bacteriology; 192:5002–5017.

Díaz. L., Hoare. A., Soto. C., Bugueño, I., Silva, N., Dutzan, N., et al. (2015). Changes in lipopolysaccharide profile of *Porphyromonas gingivalis* clinical isolates correlate with changes in colony morphology and polymyxin B resistance. *Anaerobe* 33, 25–32.

Dierickx, K., Pauwels, M., Laine, M. L., Van Eldere, J., Cassiman, J. J., van Winkelhoff, A. J., et al. (2003). Adhesion of *Porphyromonas gingivalis* serotypes to pocket epithelium. *J. Periodontol.* 74, 844–848.

Engebrecht, J. and M. Silverman (1984)..Identification of genes and gene products necessary for bacterial bioluminescence.*Proc Natl Acad Sci* U S A **81**(13): 4154-4158.

Goulbourne PA, Ellen RP. Evidence that *Porphyromonas (Bacteroides) gingivalis* fimbriae function in adhesion to Actinomyces viscosus. J Bacteriol 1991 :173: 5266–5274.

Glenister, D.A.; Salamon, K.E.; Smith, K.; Beighton, D.; Keevil, C.W. (1988): Enhanced growth of complex communities of dental plaque bacteria in mucin-limited continuous culture. *Microbiol Ecol Health Dis* 1:31-38.

Griffen, Mitzi R. Becker, Sharon R. Lyons, Melvin L. Moeschberger, Eugene J. Leys,.(1998) Prevalence of *Porphyromonas gingivalis* and Periodontal Health Status . *Journal Of Clinical Microbiology*, Nov., P. 3239–3242

Gonzales, J., Groeger, S., Johansson, A., and Meyle, J. (2014). T helper cells from aggressive periodontitis patients produce higher levels of interleukin-1 beta and interleukin-6 in interaction with *Porphyromonas gingivalis*. *Clin. Oral Investig.* 18, 1835–1843.

Haffajee AD, Teles RP, Socransky SS. The effect of periodontal therapy on the composition of the subgingival microbiota. *Periodontology 2000*. 2006; 42:219–258

Hajishengallis, G., Abe, T., Maekawa, T., Hajishengallis, E., and Lambris, J. D. (2013). Role of complement in host–microbe homeostasis of the periodontium. *Semin. Immunol. (Elsevier)* 25, 65–72.

Igboin, C. O., Tordoff, K. P., Moeschberger, M. L., Griffen, A. L., and Leys, E. J. (2011). *Porphyromonas gingivalis*-host interactions in a *Drosophila melanogaster* model. *Infect. Immun.* 79, 449–458.

Imamura, T., Travis, J., and Potempa, J. (2003). The biphasic virulence activities of gingipains: activation and inactivation of host proteins. *Curr. Protein Pept. Sci.* 4, 443–450.

Inoshita E, Amano A, Hanioka T, Tamagawa H, Shizukuishi S, Tsunemitsu A.
Isolation and some properties of exo-hemagglutinin from the culture medium of Bacteroides gingivalis381. Infect Immun 1986:52: 421–427.

Irshad, M., van der Reijden, W. A., Crielaard, W., and Laine, M. L. (2012). In vitro invasion and survival of *Porphyromonas gingivalis* in gingival fibroblasts; role of the capsule. *Arch. Immunol. Ther. Exp.* 60, 469–476.

Jang, Y. J., Y. J. Choi, S. H. Lee, H. K. Jun and B. K. Choi (2013). Autoinducer 2 of Fusobacterium nucleatum as a target molecule to inhibit biofilm formation of periodontopathogens." Arch Oral Biol **58**(1): 17-27.

Jenkinson, H. F., and Lamont, R. J. (2006). "Oral microbial ecology," in *Oral Microbiology and Immunology*, eds R. J. Lamont, R. A. Burne, M. S. Lantz, and D. J. LeBlanc

Kaplan, H. B. and E. P. Greenberg (1985). Diffusion of autoinducer is involved in regulation of the Vibrio fischeri luminescence system. J Bacteriol **163**(3): 1210-1214.

Kolenbrander PE, London J. Adhere to-day, here tomorrow: oral bacterial adherence. *J Bacteriol* 1993:175: 3247–3252.

Kinniment,S. Wimpenny.J, Adams.D. Marsh. (1996). Development of a steady-state oral microbial biofilm community using the constant-depth film fermenter. *Microbiology* 142, 631-638

Kontani M, Ono H, Shibata H, Okamura Y, Tanaka T, Fujiwara T et al. Cysteine protease of *Porphyromonas gingivalis* 381 enhances binding of fimbriae to cultured human fibroblasts and matrix proteins. Infect Immun 1996:64: 756–762.

Kristoffersen, A. K., Solli, S. J., Nguyen, T. D., and Enersen, M. (2015). Association of the rgpB gingipain genotype to the major fimbriae (fimA) genotype in clinical isolates of the periodontal pathogen *Porphyromonas gingivalis*. *J. Oral Microbiol.* 7:29124.

Liu, C, Miller, D, Wang y, Merchant, M. Lamont.(2017) Structure-function aspects of the *Porphyromonas gingivalis* tyrosine kinase Ptk1 . *Mol Oral Microbiol*. August ; 32(4): 314–323.

Liu, R., Desta, T., Raptis, M., Darveau, R. P., and Graves, D. T. (2008). *P. gingivalis* and *E. coli* lipopolysaccharides exhibit different systemic but similar local induction of inflammatory markers. *J. Periodontol.* 79, 1241–1247.

Lamell, Griffen, McClellan, Leys. Acquisition and Colonization Stability of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in Children. *J Clin Microbiol. 2000* Mar; 38(3): 1196–1199

Lamont RJ, Gil S, Demuth DR, Malamud D, Rosan B. Molecules of *Streptococcus gordonii* that bind to *Porphyromonas gingivalis. Microbiology 1994:140: 867–872.*

Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis* . *Microbiol Mol Biol Rev* 1998:62: 1244–1263.

Lantz MS, Allen RD, Duck LW, BlumeJL, Switalski LM, Hook M. Identification of *Porphyromonas gingivalis* components that mediate its interactions with fibronectin. J Bacteriol 1991:173:4263–4270.

Lepine G, Progulske-Fox A. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. Oral Microbiol Immunol 1996:11: 65–78.

Maeba, S., Otake, S., Namikoshi, J., Shibata, Y., Hayakawa, M., Abiko, Y., et al. (2005). Transcutaneous immunization with a 40-kDa outer membrane protein of *Porphyromonas gingivalis* induces specific antibodies which inhibit coaggregation by *P. gingivalis*. *Vaccine* 23, 2513–2521.

Michael. Polyamine function in archaea and bacteria**.** *J Biol Chem.* 2018 Nov 30;293(48):18693-18701

Moinard C, Cynober L, de Bandt JP. Polyamines: Metabolism and implications in human diseases. *Clin Nutr 2005*;24:184-197.

Mukamalova G, Kaprelyants A, Young D, Young M, . A bacterial cytokine . *Proc Natl Acad Sci* 1998, 95, 8916-8921

Ogawa, T., and Yagi, T. (2010). Bioactive mechanism of *Porphyromonas gingivalis* lipid A. *Periodontology* 2000, 71–77

Ozer, Elgun, Ozdemir, Pervane Arginine–Nitric Oxide–Polyamine Metabolism in Periodontal Disease. *J Periodontol* 2011; 82:320-328.

Palmer, K, Mashburn L, Singh P and Whiteley M. Cystic Fibrosis Sputum Supports Growth and Cues Key Aspects of *Pseudomonas aeruginosa* Physiology . *Journal Of Bacteriology*, Aug. 2005, p. 5267–5277

Palmer KL, Aye LM, Whiteley M. Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum. *J Bacteriol.* 2007 Nov;189(22):8079-87.

Page, RC, Kornman, KS. The pathogenesis of human periodontitis: an introduction. *Periodontol 2000* 1997: 14: 9– 11.

Page RC, Lantz MS, Darveau R, Jeffcoat M, Mancl L, Houston L, Braham P, Persson GR. Immunization of Macaca fascicularis against experimental periodontitis using a vaccine containing cysteine proteases purified from Porphyromonas gingivalis. *Oral Microbiol Imunol 2007*: 22: 162–168

Potempa, J., Banbula, A., and Travis, J. (2000). Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontology* 2000, 153–192.

Rosen, G., and Sela, M. N. (2006). Coaggregation of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* PK 1594 is mediated by capsular polysaccharide and lipopolysaccharide. *FEMS Microbiol. Lett.* 256, 304–310.

Rutherford, S. T. and B. L. Bassler (2012). "Bacterial quorum sensing: its role in virulence and possibilities for its control." Cold Spring Harb Perspect Med **2**(11).

Saito, S., Hiratsuka, K., Hayakawa, M., Takiguchi, H., and Abiko, Y. (1997). Inhibition of a *Porphyromonas gingivalis* colonizing factor between *Actinomyces viscosus* ATCC 19246 by monoclonal antibodies against recombinant 40-kDa outer-membrane protein. *Gen. Pharmacol.* 28, 675–680.

Schincaglia GP, Hong BY, Rosania A, Barasz J, Thompson A Sobue T[1], Panagakos F, Burleson JA, Dongari-Bagtzoglou A Diaz PI·Clinical, Immune, and Microbiome Traits of Gingivitis and Peri-implant Mucositis. *J Dent Res.* 2017 Jan;96(1):47-55

Shah, H. N., and Collins, M. D. (1988). Proposal for reclassification of *Bacteroides asaccharolyticus, Bacteroides gingivalis*, and *Bacteroides* endodontalis in a new genus, *Porphyromonas*. *Int. J. Sys. Bacteriol.* 38, 128–131.

Sharma, A., Sojar, H. T., Lee, J. Y., and Genco, R. J. (1993). Expression of a functional *Porphyromonas gingivalis* fimbrillin polypeptide in *Escherichia coli*: purification, physicochemical and immunochemical characterization, and binding characteristics. *Infect. Immun.* 61, 3570–3573.

Shibata, Y., Hiratsuka, K., Hayakawa, M., Shiroza, T., Takiguchi, H., Nagatsuka, Y., et al. (2003). A 35-kDa co-aggregation factor is a hemin binding protein in *Porphyromonas gingivalis*. *Biochem. Biophys. Res. Commun.* 300, 351–356.

Singh, A., Wyant, T., Anaya-Bergman, C., Aduse-Opoku, J., Brunner, J., Laine, M. L., et al. (2011). The capsule of *Porphyromonas gingivalis* leads to a reduction in the host inflammatory response, evasion of phagocytosis, and increase in virulence. *Infect. Immun.* 79, 4533–4542.

Shearer JD, Richards JR, Mills CD, Caldwell MD. Differential regulation of macrophage arginine metabolism: A proposed role in wound healing. *Am J Physiol* 1997;272:E181-E190

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998; 25:134–144.

Sroka, A., Sztukowska, M., Potempa, J., Travis, J., and Genco, C. A. (2001). Degradation of host heme proteins by lysine- and arginine-specific cysteine proteinases (Gingipains) of *Porphyromonas gingivalis*. J.*Bacteriol.* 183, 5609– 5616.

Takahashi N, Sato T, Yamada T.Metabolic pathways for cytotoxic end product formation from glutamate- and aspartate-containing peptides by Porphyromonas gingivalis. *J. Bacteriol*. 2000 Sep;182(17):4704-10.

Thomas T, Thomas TJ. Polyamines in cell growth and cell death: Molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* 2001;58:244- 258

Van Steenbergen, T. J., Delemarre, F. G., Namavar, F., and De Graaff, J. (1987). Differences in virulence within the species *Bacteroides* gingivalis. *Antonie Van Leeuwenhoek* 53, 233–244.

Weichart D, DouglassK,(2000). Characterisation of an auto stimulatory substance produced by *Eschericisa coli*. *Microbiol* 2000.147, 1875-1885

Yang L, Chen T, Izard J, Tanner AC, Wade WG, Paster BJ, et al. The Human Oral Microbiome Database: Updates and New Features. *Journal of dental research*. 2014:93

Yao ES, Lamont RJ, Leu SP, WeinbergA. Interbacterial binding among strains of pathogenic and commensal oral bacterial species. Oral Microbiol Immunol 1996:11: 35–41.

Yoshimura, A., Hara, Y., Kaneko, T., and Kato, I. (1997). Secretion of IL-1 beta, TNF-alpha, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *J. Periodontal Res.* 32, 279–286.

Zenobia, C., and Hajishengallis, G. (2015). *Porphyromonas gingivalis* virulence factors involved in subversion of leukocytes and microbial dysbiosis. *Virulence* 6, 236–243.

Zhang, T., Hashizume, T., Kurita-Ochiai, T., and Yamamoto, M. (2009). Sublingual vaccination with outer membrane protein of *Porphyromonas gingivalis* and Flt3 ligand elicits protective immunity in the oral cavity. *Biochem. Biophys. Res. Commun.* 390, 937–941

Zhang, W., Ju, J., Rigney, T., and Tribble, G. D. (2011). Fimbriae of *Porphyromonas gingivalis* are important for initial invasion of osteoblasts, but not for inhibition f their differentiation and mineralization. *J. Periodontol.* 82, 909–916.