Characterization of a novel quorum sensing system in *Porphyromonas gingivalis*

by

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ABSTRACT

Oral health is closely connected with the overall systemic health. Oral biofilms are dynamic and present a very specific bacterial community which affects oral health. Oral biofilms are directly associated with the major oral infections including caries and periodontal disease. Understanding the oral microbial community is important for preventing oral disease and maintaining oral and overall health. In the first part of this study, I have explored the pathogenic load for three microorganisms *Porphyromonas gingivalis, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* in companion cats and dogs. Results showed detectable levels of *P. gingivalis* and *T. forsythia* in both healthy and diseased cats and surprisingly the number was minimal in healthy dogs. *A. actinomycetemcomitans* was not detected in any healthy or diseased samples as this bacterium is found only in aggressive stages of Periodontitis.

Quorum sensing (QS) system is a cell-cell signaling system that enables bacteria to interact with their neighboring cells and facilitates coordinated bacterial behavior in response to environmental changes. Gram-negative bacteria contain several quorum sensing systems in which different molecules are used as signals, including acyl-homoserine lactones (AHLs), cyclic dipeptides, autoinducers 2 and 3. The LuxI/LuxR-type quorum sensing system consists of a LuxI-like autoinducer synthase that produces AHLs as signals which are detected by a LuxR-type receptor upon exceeding a threshold concentration. The AHLs are detected through binding or activating cytoplasmic receptor proteins, which then dimerize to regulate transcription of downstream genes.

P. gingivalis which demonstrated the strongest association with periodontal disease in our study of cats and dogs. *P. gingivalis* does not produce AHL but possesses many LuxR like homologs on its chromosome. One of these LuxR homologs is PGN_1373 which has been shown to affect the interaction between *Streptococcus gordonii* and *P. gingivalis*.

Dialkyresorcinols encoded by *dar* gene has been previously demonstrated to bind to another luxR homolog PauR in *Photorabdus asymbiotica*. *Porphyromonas gingivalis* encodes for DarB which synthesizes Cyclohexanediones (CHDs). We hypothesized that CHD may bind to PGN_1373 and regulate virulence. Hence, I investigated the presence of a novel quorum-sensing molecule CHD synthesized by DarB and its effect on virulence factors and phenotype of *Porphyromonas gingivalis.* The High-Performance liquid chromatography results show that *P. gingivalis* secretes molecules like commercially available 1,3 cyclohexanediones. The addition of CHD extracellularly influenced expressed bacterial virulence genes at 100 µM and biofilm formation at 10 µM. The new DarB/PGN_1373 quorum sensing circuit may add to the existing pool of mechanisms regarding intra and interspecies communication in *Porphyromonas gingivalis*.

KEYWORDS: *Porphyromonas gingivalis*, Cyclohexanediones, Quorum sensing, PGN_1373

LIST OF ABBREVIATIONS

AHL - Acyl homoserine lactones

CFU - Colony forming unit

CHD - Cyclohexanedione

DAR - Dialkyresorcinols

DNA - Deoxyribonucleic acid

gDNA - Genomic DNA

HPLC - High-Pressure liquid chromatography

mRNA – Mitochondrial RNA

NAM- N-acetylmuramic acid

PBS- Phosphate buffer solution

qPCR- Quantitative polymerase chain reaction

QS- Quorum sensing

RNA- Ribonucleic acid

rRNA- Ribosomal RNA

RT-PCR – Reverse transcriptase polymerase chain reaction

TAE- Tris-acetate EDTA

Tris- HCl - Tris-hydrochloric acid

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1. Introduction

Periodontal disease is the 6th most prevalent disease worldwide (Tonetti, Jepsen, Jin, & Otomo-Corgel, 2017).Given the population trends and changing risk factors, the global burden of periodontal disease has increased to 57.3% from 3440 in 1990 to 5410 in 2010 (Jin et al., 2016; Kassebaum et al., 2014). Periodontal diseases are mainly caused by multi-species dental plaque which is defined by the WHO as "specific but of highly variable structural entity resulting from sequential colonization and growth of microorganisms on the surfaces of teeth and restorations consisting of microorganisms of various strains and species embedded in self-produced extracellular matrix, composed of bacterial metabolic products and substances from serum, saliva, and blood (Kassebaum et al., 2014). The tenacious organized bacterial communities are known as biofilms. Oral biofilms are unique because the microorganisms that colonize then follow a pattern of succession and preferential aggregation. These have been termed as "complexes" as classified by Socransky in 1968 (Socransky, 1968).

Members of the yellow complex (*Streptococcus spp*.) and the purple complexes (*Actinomyces odontolyticus* and *Veillonella parvula*) are the early colonizers of the dental plaque. Members of the green (*Eikenella corrodens*, *Aggregatibacter actinomycetemcomitans*, and *Capnocytophaga spp*.), orange (*Fusobacterium, Prevotella, and Campylobacter spp.),* and red (*P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) complexes are the secondary colonizers of the dental plaque. Colonizing organisms have demonstrated neutral (*P. intermedia* and *F. nucleatum*) (Takahashi, 2003) antagonistic (*S. sanguinis* and *S. mutans; S.mutans and T.denticola*) (Kemp, Robrish, Curtis, Sharer, & Bowen, 1983) and synergistic (*P. gingivalis* and *T. denticola*) (Grenier, 1992) association. Two species of bacteria that have been readily identified with periodontal disease, both chronic and aggressive, are *P.*

gingivalis associated with the “Red” complex and *A. actinomycetemcomitans* is associated with “Green” complex respectively.

While several different species have been recognized as inhabitants of the periodontal environment, it is now acknowledged that the ‘red complex’ has been found to be most strongly associated with chronic periodontitis and is considered a hallmark of the destructive periodontal disease. *P. gingivalis* is a key member of the Red complex and is involved in periodontal tissue destruction and bone loss. Previous studies suggest that *P. gingivalis* is associated with periodontal attachment loss of 5mm or more and a pocket depth of 4mm and more (Edwardsson et al., 1999; Van Winkelhoff et al., 1999). Porphyromonas is often undetectable (>5% of culturable microflora) in healthy individuals; however, during periodontal disease, their numbers increase dramatically. Detection of *Porphyromonas gingivalis* is also considered a risk for periodontal disease because of its ability to produce significant numbers of potential virulence molecules. The partnership between periodontopathic bacteria is very specific and has been studied extensively. Understanding the direct and indirect interaction between red complex pathogens and the role of Porphyromonas in such an association can have significant effects on managing periodontal diseases.

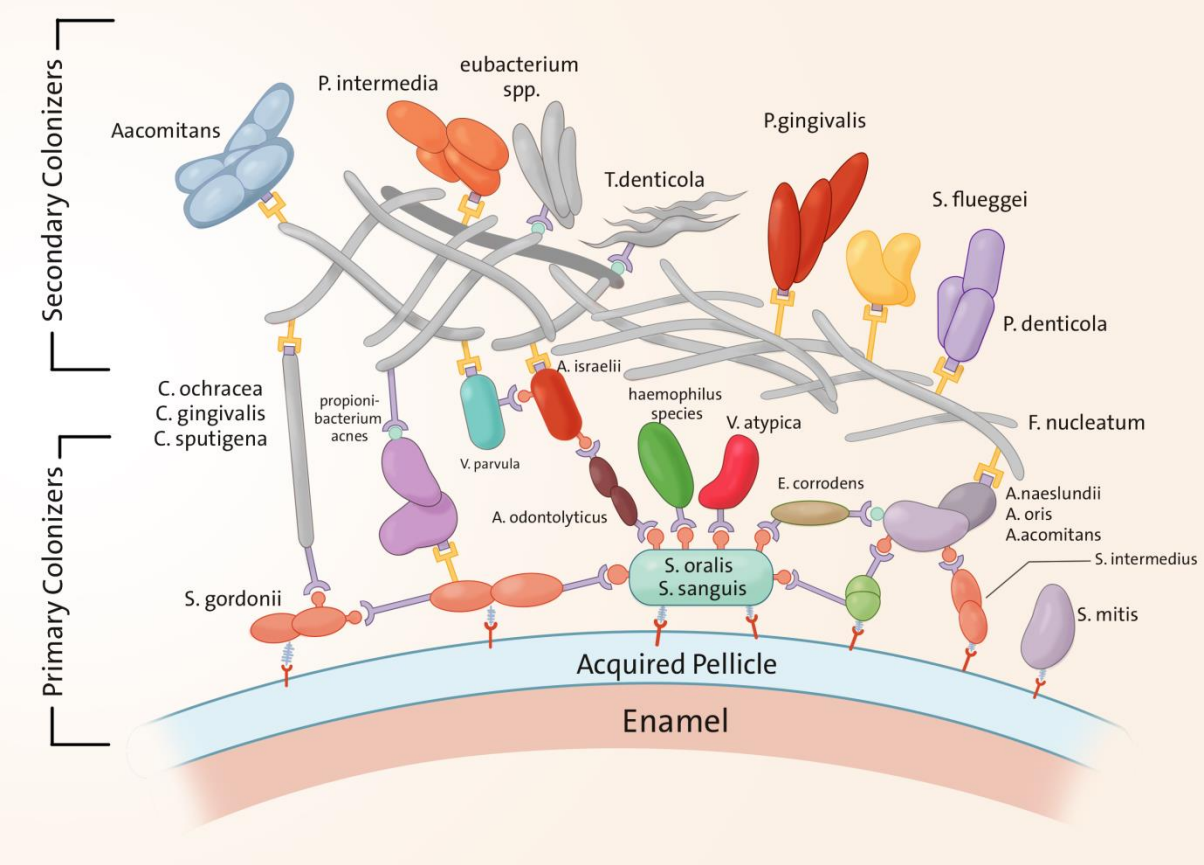


Figure 1. Schematic representation of the primary and secondary colonizers of dental plaque.

Primary colonizers bind by specific or non-specific physio-chemical interactions with components of acquired enamel pellicle. Secondary colonizers bind to primary colonizers by a process called co-aggregation. The figure is adapted and redrawn from (Kolenbrander *et al*., 2002)

1.1. Periodontal diseases in domestic cats and dogs

Cats and dogs are the most common household pets in the world. As per Canada veterinary medical association (CVMA) approximately 35% of households have dogs and 38% have cats. The prevalence of periodontal diseases is around 80% (Lund, Armstrong, Kirk, Kolar, & Klausner, 1999) Although studies have shown that there is minimal transfer of bacteria between the companion animal and its owner, the phylogenetic analysis shows similarity in the species of bacteria present in them (Deng & Swanson, 2015; Oh *et al.*, 2015). The close relationship between humans and their pets may constitute a mode of microbial transfer across the species. Interestingly, cats and dogs have been found to possess similar disease-causing oral bacteria (Harris et al., 2015). Dental plaque or tartar in dogs and cats can form because of many other factors such as food, hair and foreign particles from chewing. Thus, collected foreign particles can result in accumulation of bacteria and initiate the inflammatory sequences in the host. The dental plaque formed can convert into hard-shelled rigid calculus in as little as 48 h. Food habits also affect the oral health of cats and dogs equally. Bacteria such as *P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans*, *T. denticola*, *P. intermedia*, *E. corrodens* and *C. rectus* have been related to several forms of periodontal diseases in animals and humans (Valdez, Haines, Riviere, Riviere, & Thomas, 2000). One study noted an association between severity of periodontitis and azotaemic canine kidney disease (Glickman et al., 2011) Prevalence of periodontitis in companion dogs has been found to correlate with height, body weight, and being fed soft textured food. In another study, a strong association was found between the severity of periodontitis and the risk of endocardiac disease (Glickman, Glickman, Moore, Goldstein, & Lewis, 2009).

In companion dogs with periodontitis, black-pigmented anaerobic bacteria are commonly isolated in routine microbial cultures (Boyce *et al*., 1995; Forsblom, Love, Sarkiala-Kessel, & Jousimies-Somer, 1997) Many of these have been identified as different members of the

Porphyromonas species such as; *P. asaccharolytica, P. circumdentaria, P. endodontalis, P. levii, P. gulae, P. macacae, P. catoniae,* and *P. salivosa*. Of these, *P. gingivalis* has been implicated as a key periodontal pathogen. *A. actinomycetemcomitans* a green complex pathogen and *T. forsythia* were found to associate with advanced periodontal diseases along with *P. gingivalis* as per study (Oh *et al*., 2015). Interestingly, these microbes correspond very terms of their disease association with their human counterparts. Thus, understanding their inter-relationship is key to designing therapeutics, and strategies to prevent any zoonotic transmission.

1.2. Interdependence between *P. gingivalis, T. forsythia,* and *A. actinomycetemcomitans*

Though periopathogens can cause tissue disease and destruction in isolation as well, they are found together as Red-Red (*P. gingivalis-T. denticola*; *P. gingivalis-T. forsythia),* Red-Green (*P. gingivalis-A. actinomycetemcomitans*) or Red-Orange complexes (*P. gingivalis-F. nucleatum*; *F. nucleatum-T. forsythia*; *P. gingivalis-T. denticola-T. forsythia*). Synergistic inter-species associations between bacteria can provide robustness and sustainability in a dynamic environment. Knowledge of these interactions, specifically those of antagonistic nature can be employed to form a barrier to colonization by opportunistic endogenous as well as invading exogenous pathogens. Such non-pathogenic, endogenous antagonistic bacteria may offer the capability for probiotic action, which may be used to protect against periodontitis (Holt & Ebersole, 2005).

P. gingivalis

P. gingivalis is a rod –shaped, non-spore forming bacteria mainly present in subgingival and periodontal pocket lesions, observed predominantly beneath the spirochete layer. Upon growth on a suitable culture media, they grow as a white to cream colored raised colonies which darken to a deep red to black color in 4 to 8 days (How, Song, & Chan, 2016).

P. gingivalis is highly pathogenic bacteria, contributing to some very aggressive forms of periodontal diseases. They have been assigned as the 'keystone pathogen' in periodontal diseases (Hajishengallis & Lamont, 2012). Oral microenvironment is highly dynamic and to survive within this ever-changing niche, *P. gingivalis* not only needs to sense the environmental conditions such as pH, nutrient availability, extracellular enzymes, temperature and oxygen availability, but also microbial density and the presence of other microorganisms(Inagaki, Onishi, Kuramitsu, & Sharma, 2006). Major virulence factors in *P. gingivalis* include phospholipase, alkaline and acid phosphatases, lipopolysaccharide, proteases, hemolysins and fimbriae, phospholipase (Lamont & Jenkinson, 1998). Some other virulence factors are hydrogen sulfide, methylmercaptan, dimethyl disulfide, butyrate, propionate, indole, and ammonia, which helps in coaggregation and cell adhesion (Holt & Ebersole, 2005; Sanghavi, Shah, Shah, & Sanghavi, 2014). Table.1 provides a list of key virulence factors in *P. gingivalis*. *P. gingivalis* also exhibits cysteine proteases such as the Arg-gingipain (Rgp) and Lys-gingipain (Kgp) as they can cause a dysfunction of immune response and inflammation and can result in tissue protein destruction (Li & Collyer, 2011). Rgp is encoded by genes *rgpA* and *rgpB,* whereas Kgp is encoded by *kgp* only. These gingipains have also been implicated in synergism between *P. gingivalis* and *T. forsythia* (Jung, Jun, & Choi, 2016). The pathogenic process of *P. gingivalis* involves adherence and colonization to periodontal tissues. This is followed by nutrient acquisition, proliferation, neutralization of host defenses, evading immune response by manipulation of inflammatory responses eventually resulting in tissue invasion and destruction. Perhaps the most important aspect of pathogenicity of *P. gingivalis* is its association with other members of its complex which are now discussed in detail(Mysak *et al*., 2014).

Table 1. Major virulence genes of *P. gingivalis* and their functions (How *et al.*, 2016; Mysak *et al*., 2014)

Virulence gene	**Name**	**Function**
rgpA	Arginine protease	Proteolysis
rgpB	Arginine protease	Proteolysis
Kgp	Lysine protease	Proteolysis
fimA	Major fimbriae protein	Cell attachment
Mfa1	Minor fimbriae protein	Cell aggregation, biofilm formation
galE	Synthesis of sugar nucleotides	Biofilm Formation
PGN_1373	Transcription factor	quorum sensing
dnaK	Chaperone system	Heat shock protein
htrA	Surface protein	Biofilm formation
Sod	Super oxide dismutase	Environment stress
Pgn 0178	Hypothetical protein	
Pgn 0181	Hypothetical protein	
0183	Major fimbrial subunit	
hagA	Haemagglutinin protein A	Cellular processes/ heterospecies biofilm formation
hagB	Haemagglutinin protein B	Adherence to cell
clpB	Caseinolytic protease	Biofilm formation
luxS	Quorum sensing gene	Quorum sensing, haem agglutination
ragB	Outer membrane protein	Tissue destruction
ragA	Outer membrane protein	Tissue destruction

T. forsythia

T. forsythia, a non-spore-forming fusiform rod frequently isolated with *P. gingivalis* in cases of progressing periodontitis located within, the surface layers of the subgingival plaque. *T. forsythia* is associated with virulence factors such as gingival pain, propionate, alkaline phosphatase, acid phosphatase, lipopolysaccharide, propionate, isovalerate, and acid phosphatase phenylacetate (Friedrich, 2015; Megson *et al.,* 2015). Other virulence factors are proteins involved in inhibition of the host's immune response and evasion of the host's immune response (Gomes *et al*., 2007; Holt & Ebersole, 2005). *T. forsythia* has been shown to exhibit a symbiotic nutrient utilization relationship with *P. gingivalis* (Yao, Lamont, Leu, & Weinberg, 1996). When cocultured *in vitro, T. denticola,* and *P. gingivalis* co-aggregated and formed colonies (Tan *et al*., 2014). It has also been observed that *P. gingivalis* attaches first as a primary colonizer followed by attachment of *T. forsythia* to form biofilms *in vitro.* Interestingly addition of *T. forsythia* to *P. gingivalis* significantly enhance the virulence of *P. gingivalis* as compared to a mono infection (Inagaki *et al.,* 2006). The surface proteins of these pathogens also play a major role in their concerted virulence. The unique S-layer of *T. forsythia* is involved in haemagglutination and invasion of epithelial cells, and assists in coaggregation with *P. gingivalis* (Zhu & Lee, 2016). These two bacteria also show synergistic effects on the formation of abscess as result of impaired immune-inflammatory responses and absence of humoral immune protection during periodontitis in rat models (Suzuki, Yoneda, & Hirofuji, 2013). *P. gingivalis* and *T. forsythia* are considered as the most important markers of aggressive periodontal disease (van Winkelhoff, Loos, van der Reijden, & van der Velden, 2002).Thus, it is critical to identify and manage the *P. gingivalis* coupled co-infection by *T. forsythia*.

A. actinomycetemcomitans

A. actinomycetemcomitans is Gram-negative, nonmotile, facultative anaerobic bacteria associated with aggressive forms of periodontitis. *A. actinomycetemomitans* has also been implicated in extra-oral infections such as infectious endocarditis (Gurcan, Unlu, Kuloglu, Karadenizli, & Kuskucu, 2016), septicemia (Figuero *et al*., 2014) and osteomyelitis (Sharma, Mudgil, Whitehall, & Gosbell, 2017). The primary foci of infection to these are oral periodontal lesions. *A. actinomycetemcomitans* infection results in an immune-inflammatory response, which causes periodontal tissue destruction. It does so by producing numerous factors such as leukotoxin (LtxA), GroEL, cytolethal distending toxin (CDT),peptidoglycan-associated lipoprotein (PAL), and lipopolysaccharide (LPS) (Zijnge, Kieselbach, & Oscarsson, 2012). Pathogenicity of *A. actinomycetemcomitans* involves impaired osteoblast function and thus causing impairment to normal bone remodeling processes, which ultimately results in an alveolar bone loss (Kayal, 2013). Most of the factors contributing to the adhesion and colonization of *A. actinomycetemcomitans* include invasins and bacteriocins (Benso, 2017). *A. actinomycetemomitans* produce outer membrane vesical proteins (OMV) that have been shown to be highly virulent These OMV can enter the bloodstream of the host and produce different proinflammatory responses (Kieselbach, Zijnge, Granstrom, & Oscarsson, 2015).

Though physiologically *P. gingivalis* and *A. actinomycetemcomitans* are remarkably different as pathogens there is evidence to suggest that they may act synergistically to potentiate mutual virulence. The presence of *P. gingivalis* and high colonization by *A. actinomycetemcomitans*, *T. denticola*, and *P. intermedia* are shown to be strong risk indicators of severe periodontitis (Torrungruang, Jitpakdeebordin, Charatkulangkun, & Gleebbua, 2015).

1.3. Communication in bacteria

Cell-cell interactions in bacteria may occur by at least two known mechanisms: one that involves physical contact between two cells and the second involves the use of communication mediated by small molecules (Miller & Bassler, 2001). Within dental biofilms, physical contact provides the site for adherence to the successive microorganism(Huang, Li, & Gregory, 2011). In such a mode of interaction, metabolites from nearby cells bring about the environmental changes favorable for the growth of pathogens (Bassler, 1999). Genetic exchange in neighboring cells by means of transduction, conjugation, and transformation, provides the microbial resistance against the antibiotics. Signaling molecules on the other hand help bacteria to regulate their behavior in response to changes in the environment (Papenfort & Bassler, 2016).

Interspecies communication plays a major role in the initiation and progression of periodontal disease. Thus, there exists interdependence among the bacteria present in the same or different complexes, for which an effective communication mechanism is essential. Communication among different bacterial species improves their chance of survival and has a major role in the etiopathology of the periodontal disease (Campbell, Lin, Geske, & Blackwell, 2009).

Gram-positive and Gram-negative bacteria differ in their signaling molecules. The Gram-positive bacteria produce autoinducing peptide (AIP) as signaling molecules. The final AIP is formed from a larger precursor peptide. AIP is then sensed by two-component signaling proteins that can subsequently regulate genes downstream to bring about a specific response (Verbeke *et al.*, 2017) (Reen *et al*., 2011).

Table 2. Quorum sensing molecules in gram negative bacteria.

Quorum Sensing molecule	Bacteria	Citation
C4-HSL, 3-oxo-C10-HSL, Pseudomonas quinolone signal(PQS)	*Pseudomonas aeruginosa*	(Kariminik, Majid, & Kheirkhah, 2017; Reen *et al*., 2011)
4-OH-C4-HSL/ Autoinducer 2	*Vibrio harveyii*	(Lorenz, Shin, & Jung, 2017)
C6-HSL	*Pseudomonas chloraphis*	
3-oxo-C8-HSL	*Agrobacterium tumefaciences*	(Lang & Faure, 2014)
C8-HSL	*Ralstonia solanacearum*	(J. S. Kumar, Umesha, Prasad, & Niranjana, 2016)
3-OH-C6-HSL	*Pectrobacterium caratovora*	(Garge & Nerurkar, 2016)
3-OH-C14:1-HSL, C8-HSL, 3-oxo-C8-HS	*Rhizobium leguminosarum*	(González & Marketon, 2003; Sanchez-Contreras, Bauer, Gao, Robinson, & Allan Downie, 2007)
Cyclic Dipeptides(Diketopiperazine)	*Pseudomonas aeruginosa, Pseudomonas fluorescence, Pseudomonas alcaligenes, Cronobacter sakazakii and Bacillus cereus*	(Campbell *et al.*, 2009)
Autoinducer 3	*Enteroheamorragic Escherichia. Coli 0157:H7*	(Moreira & Sperandio, 2016)
Diffusible extra cellular factor	*Xanthomonas campestris*	(He & Zhang, 2008)

1.3.1. LuxS/Autoinducer-2 (AI-2) quorum sensing system

LuxS/Autoinducer-2 (AI-2) quorum sensing system is perhaps one of the most investigated quorum sensing systems in *P. gingivalis*. First studied in the marine bacterium *Vibrio harveyi* LuxS/Autoinducer-2 (AI-2), QS system is dependent on the Autoinducer-2 family of signal molecules and the *luxS* gene. Biosynthesis of AI-2 is a three-step process (Fig. 2). It is a part of a methionine catabolism cycle which is called Activated Methyl Cycle (AMC). The first step is the removal of a methyl group from S-Adenosyl Methionine (SAM), catalyzed by SAM-dependent methyltransferases. This results in the formation of S-Adenosyl Homocysteine (SAH), which is then converted to S-Ribosyl Homocysteine (SRH) by the

enzyme SAH Nucleosidase. *luxS* encodes AI-2 synthase or S-Ribosylhomocysteinase also known as LuxS protein, that hydrolyzes S-ribosylhomocysteine into 4,5-dihydroxy-2,3-pentanedione (DPD). According to SCOP system of protein classification, this enzyme belongs to LuxS/MPP-like metallohydrolase superfamily. An extraordinary characteristic of this protein is that it is one of the few enzymes capable of cleaving thioether bonds without using a redox cofactor. 4,5-DPD further undergoes hydrolysis autocatalytically into various molecular ring-structure forms of AI-2 (Surette & Bassler, 1998).

AI-2 is one of the three sub-types of autoinducers: First, acylated homoserine lactones (AHLs), used by gram-negative bacteria (also referred as autoinducer-1 AI-1]); secondly, peptide signals, utilized by gram-positive bacteria; and thirdly, autoinducer-2 (AI-2), by both gram-negative and gram-positive bacteria. AI-2 is said to represent universal language. It is hypothesized that AI-2 inter-conversions allow bacteria to respond to endogenous AI-2 as well those produced by other bacteria in its surroundings. AI-2 by itself or in conjunction with other AHLs and homoserine lactones (HSLs) controls virulence in many bacteria including *P. gingivalis*.

LuxS/AI-2 signalling in *P. gingivalis* is involved in regulating the acquisition of hemin and growth under hemin-limited conditions (Burgess *et al*., 2002; James *et al*., 2006), and the expression of proteases and stress-related genes (Chung *et al.*, 2001; Yuan, Hillman, & Progulske-Fox, 2005). Analysis of a *luxS* mutant of *P. gingivalis* showed that genes encoding the TonB-linked hemin binding protein Tlr, the arginine-specific protease Rgp and lysine-specific protease Kgp, that degrade host heme-containing proteins, were down-regulated in the luxS knockout (*ΔluxS)* strains (Burgess *et al*., 2002; James *et al.*, 2006).

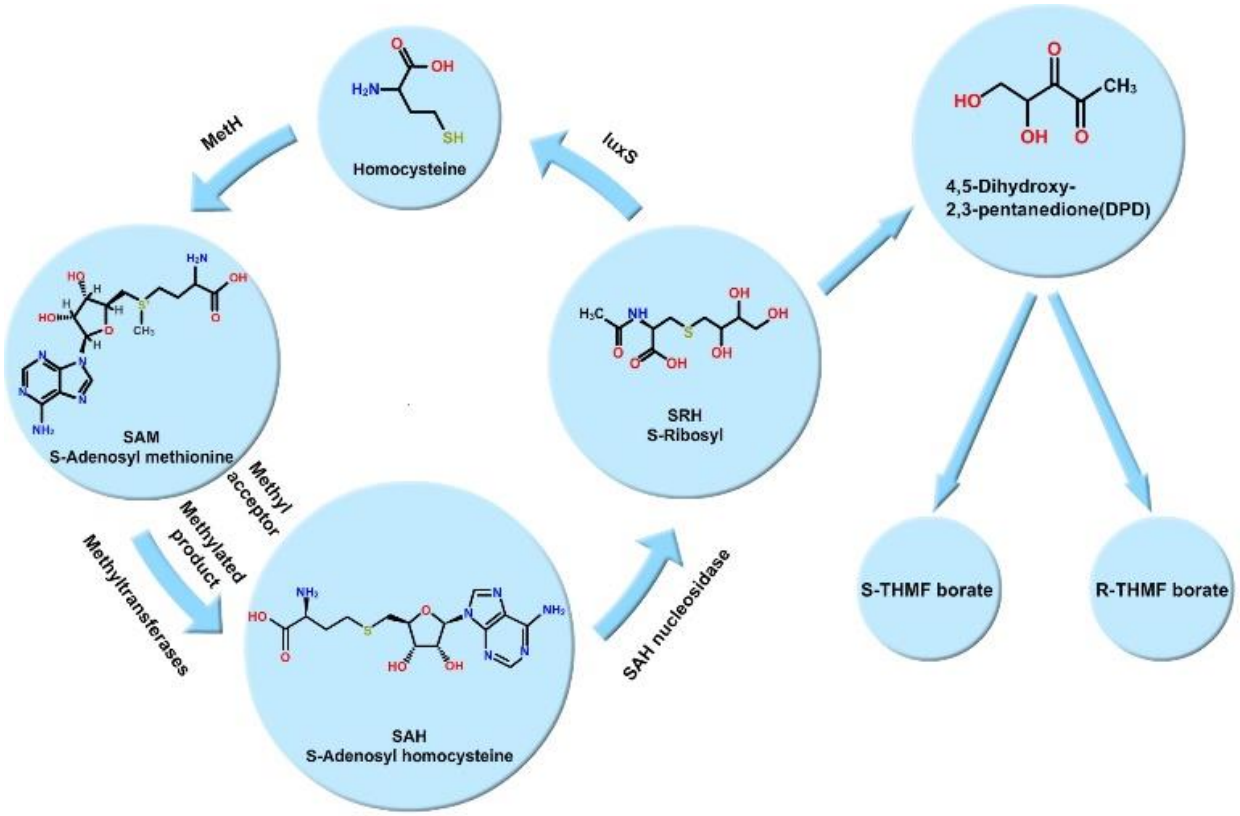


Figure 2. AI-2 biosynthetic pathway

RNA-Seq for studying the role of the role of LuxS/AI-2 in *P. gingivalis* revealed that expression of 57 genes and systems was influenced by LuxS(Scheres, Lamont, Crielaard, & Krom, 2015). Among these were genes involved in the hemin uptake regulon, flavodoxin FldA, tRNA-guanine transglycosylase, the YjgP/YjgQ family permease, *ssrA* RNA binding protein, *dpp*, and lipoyl synthase (Hirano, Beck, Demuth, Hackett, & Lamont, 2012). Though most of the down-regulated genes in Δ*luxS* could be restored by complementation by exogenously added DPDs, the enzymatic product of LuxS, the expression of two genes was not restored by addition of DPD. These findings suggest that in *P. gingivalis* LuxS is involved in regulation of gene expression in both an AI-2 dependent and an AI-2 independent system (Hirano *et al.*, 2012).

LuxS/AI-2 quorum sensing system has also been observed in other oral microorganisms such as *A. actinomycetemcomitans* and oral streptococci apart from *P. gingivalis* (McNab & Lamont, 2003). Quorum sensing *luxS* has also been shown to contribute towards activation of methyl cycle in the recycling of the toxic compound S-adenosyl-homocysteine (SAH)(Vendeville, Winzer, Heurlier, Tang, & Hardie, 2005; Winzer *et al*., 2002). AI-2 signaling has been shown to be essential to the formation of a *P. gingivalis*-*S. gordonii* mixed-species biofilm. AI-2 produced by *S. gordonii* (Vendeville *et al*., 2005) and *A. actinomycetemcomitans* (Fong, Chung, Lamont, & Demuth, 2001) can complement a *luxS* knockout in *P. gingivalis*. AI-2 produced by *F. nucleatum* has been shown to stimulate co-aggregation and the expression of adhesion molecules in *P. gingivalis*, *T. denticola*, and *T. forsythia* (Jang, Choi, Lee, Jun, & Choi, 2013). *Filifactor alocis* is another fastidious anaerobic bacterium strongly associated with chronic forms of periodontitis. LuxS/AI-2 signaling has been shown to be involved in the physical interaction between *P. gingivalis* and *F. alocis* (Spooner *et a*l., 2016). *AI-2* production by *P. gingivalis* helped maintain levels of *F. alocis* (Q. Wang, Wright, Dingming, Uriarte, & Lamont, 2013).

The role of LuxS/AI-2 quorum sensing system has not been explored extensively with regards to host interaction. It was noted that *luxS* mutant is impaired in its ability to induce an inflammatory response in PDL fibroblasts. This defect cannot be restored by chemical complementation with DPD (Scheres *et al*., 2015). *P. gingivalis* expresses Low Molecular Weight Tyrosine Phosphatases (LMWTP) through *Ltp1*. Ltp1 regulates the transcriptional activity of *luxS* and thus impacts AI-2-dependent signaling in biofilm communities (Maeda et al., 2008). It was observed that in Ltp1mutant, uptake of hemin is impaired and though the gingipain proteinases Kgp and RgpA/B remain phosphorylated in the Ltp1 mutant, Rgps are poorly secreted, whereas cell surface activity of Kgp is enhanced (Maeda *et al*., 2008).

Since LuxS is also a metabolic enzyme in the activated methyl cycle which is responsible for the generation of S-adenosyl-L-methionine, the major methyl donor in the cell, a non-quorum sensing role for LuxS is also postulated (Rezzonico & Duffy, 2008).

Thus, in conclusion, LuxS-signaling in *P. gingivalis* has a distinct role in *P. gingivalis* virulence towards host as well as towards interaction with other organisms.

1.3.2. LuxI/LuxR-type quorum sensing using AHL as signal molecules

Acyl-homoserine lactones (AHLs) are autoinducer (AI) molecules belonging to AI-1 class. The LuxI/LuxR-type quorum sensing system consists of a LuxI-like autoinducer synthase that produces AHLs as signals which are detected by a LuxR-type receptor upon exceeding a threshold concentration. The AHLs are detected through binding or activating cytoplasmic receptor proteins, which then dimerize to regulate transcription of downstream genes.

LuxI/LuxR-type quorum sensing is one such system. LuxI directs AHL synthesis while LuxR function as a transcriptional regulator that is capable of binding AHL signal molecules. AHL-type AIs are usually detected by a membrane-bound two-component hybrid protein.

Figure 3. LuxI/LuxR quorum sensing circuit

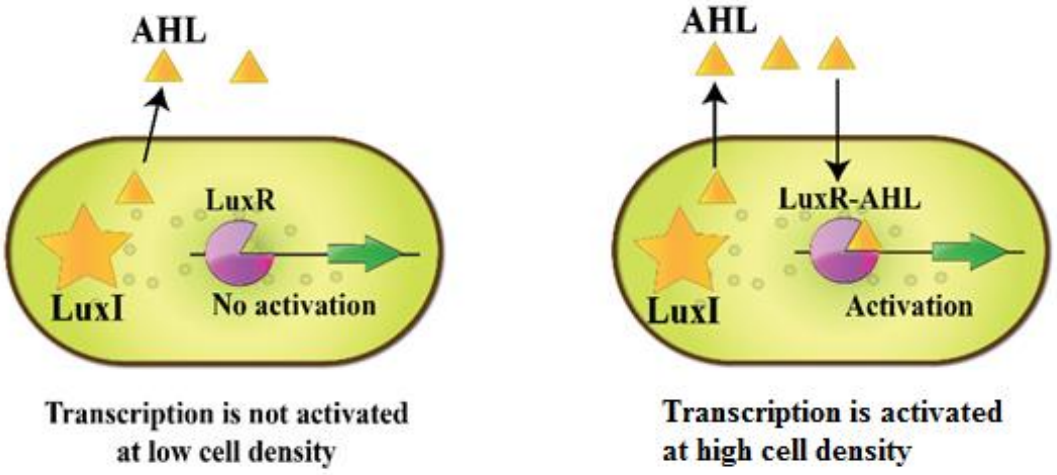


Figure 3. The figure represents the LuxI/LuxR and AHL quorum sensing circuit

The phosphoryl group is transferred from a sensor kinase to a histidine phosphotransfer protein and subsequently to a response regulator molecule. Often the luxI/luxR pairs are genetically clustered; however, sometimes these functional pairs are distantly located either on the bacterial chromosome or on plasmids. Often LuxR homologs may be found with no cognate LuxI autoinducer synthase or be present independently in addition to a functional LuxI/LuxR quorum sensing system. LuxR proteins are composed of an AHL binding amino-terminal domain and a DNA binding carboxy-terminal. The DNA binding domain contains a helix-turn-helix (H-T-H) motif and any truncations in this domain can affect the activation of the *luxICDABE* genes. In the absence of an AHL, the N-terminal domain folds over the H-T-H domain, thus, the target promoter DNA is unable to bind to it. However, in the presence of AHL, H-T-H is able to bind to other promoter sequences and activate transcription of target genes.

These LuxR homologs are designated as LuxR solos or LuxR orphans. LuxR solos allow bacteria to respond to endogenous as well as exogenous AHLs and even eukaryotic signals like hormones. This is significant since this type of sensing can allow for an efficient and quick adaptation to the external environment. LuxR solos have been shown to be involved in intra-and as well as inter-kingdom signaling.

P. gingivalis does not produce AHLs though the genome of *P. gingivalis* encodes for members of the LuxR family. Thus, these LuxR regulators are considered orphans or solos because they lack a cognate LuxI AHL synthase. Unpaired LuxR regulators have been shown to control properties as diverse as type IV secretion, antibiotic production, exopolysaccharide synthesis and biofilm formation (Patankar & Gonzalez, 2009; Subramoni & Venturi, 2009).

1.4. DAR-mediated QS system in bacteria

Dialkyresorcinols (DAR) were reported in the year 1975 in DB-2073 (I), as an antibiotic produced by *Pseudomonas* sp. B-9004, having a formula of $C_{15}H_{24}O_2$ (ME 236), further characterization indicated that it was a resorcinol antibiotic and it was elucidated as 2-n-hexyl-5-n-propylresorcinol (Kanda, Ishizaki, Inoue, Oshima, & Handa, 1975; Kitahara & Kanda, 1975; Schoner, Kresovic, & Bode, 2015). In 2015, Heerman, Bode, and colleagues reported DAR as a novel signaling system in *Photorabdus asymbiotica* (Brameyer, Kresovic, Bode, & Heermann, 2015). *P. asymbiotica* lacks AHL, which is mainly responsible for diseases in insects. The team discovered a new quorum sensing system operated by *luxR*-type receptor PauR, which recognizes a definite set of DAR molecules. Unlike other LuxRs, this receptor does not sense AHL but DARs and Cyclohexanediones(CHD). HPLC/MS analysis of numerous Photorabdus species showed the presence of DAR and their biochemical precursors CHD produced by *P. asymbiotica* exclusively. These signaling compounds were shown to be under the regulation of *darABC* operon (Burgess *et al*., 2002). PauR induces cell clumping by activating the expression of *pcfABCDEF* operon and regulates virulence (Chung *et al*., 2001). Apart from *P. asymbiotica*, this molecule has been discovered in 116 more pathogenic bacterial species which lack *luxL* -AHL synthesizing receptor. In nitrogen-fixing bacteria *Azoarcus sp.*BH72 DAR exists as a xanthomonadin-dialkylresorcinols hybrid (from Xanthomonas) called arcuflavin. Acruflavin is assumed to protect the cell from oxidative stress (Schöner, Fuchs, Reinhold-Hurek, & Bode, 2014).

Dar synthesis involves the darABC operon though, sometimes these may be found to exist separately. Of these DarA encodes for an aromatase, DarB encodes the 3-ketoacyl-ACP synthase III (KAS III) and DarC encodes for an acyl carrier protein (ACP) (Nowak-Thompson *et al*., 2003).

P. gingivalis ATCC 33277 strain contains only DarB gene encoding for CHD but not DarA or C (Schöner et al., 2014). DarB ketosynthase produces 2, 5-dialkylcyclohexane-1, 3-diones (CHDs) from fatty-acid-derived precursors, which can be further oxidized into DARs by the DarA. DAR has been shown to bind to luxR homolog PauR and activate *pcf* operon which activates cell clumping behavior in P. *asymbiotica* (Posch *et al*., 2011). LuxR solos such as PauR though possessing conserved motifs, also demonstrate variations and may reflect the diversity of signaling molecules they are capable of binding to (Papenfort & Bassler, 2016).

P. gingivalis 33277 LuxR orphan PGN_1373(*cdhR*) has been found to share homology with LuxR from *V. fisherii* and others as well as control transcription of the *hmu* operon in a cell density-dependent manner (Wu, Lin, & Xie, 2009). Classic LuxR-type receptors consist of an N-terminal signal-binding domain (SBD) for signal binding, binding specificity and shaping the ligand pocket and a C-terminal DNA-binding domain (DBD). The DBD contains a helix-turn-helix motif, the HTH LUXR motif.

CdhR (Community Development and Hemin Regulator), a LuxR homolog has been shown to control LuxS and AI-2 in *P.gingivalis* (Friedrich *et al*., 2015). Expression of *cdhR* was activated by *Streptococcus gordonii* and this requires the presence of tyrosine phosphatase, Ltp1. Mutants lacking CdhR exhibited enhanced expression of *mfa* gene. Mfa encodes for short fimbriae. Short fimbriae encode for an approximately 27 aa binding epitope of SspA/B termed DAR. DAR is involved in heterotypic community formation with *S. gordonii* (Lanza *et al*., 2016). CdhR binds upstream to Mfa and LuxS which in turn limits the binding of *S. gordonii* to *P. gingivalis*.

1,3-Cyclohexanedione is a ketosynthase important for fatty acid and polyketide biosynthesis. The molecular formula is $C_6H_8O_2$ (Fuchs *et al.*, 2013). In the Solid state, 1,3-cyclohexanedione has been found to exist in keto-enol forms with monomer units connected

by intermolecular hydrogen bonds (Calabrese *et al*., 2013). Sulcatrione or Mikado, a tri-ketone compound is well-known maize herbicide. This compound is chemically known as 2-(2-chloro-4-methylbenzoyl)-cyclohexane-1,3-dione synthesized from 1,3-cyclohexanedione and 2-chloro-4-methanesulfonyl-benzoyl chloride compounds. Orfadin is prescribed for the treatment of Tyrosinemia contains a compound called nitisinone which is 2-(2-nitro-trifluoromethylbenzoyl)cyclohexane-1,3-dione (NTBC) (Durairaj, 2005).

2. Scope of this study

LuxR solos in non-AHL-producing bacteria can bind AHLs, exogenous signaling molecules, or even other signals produced by eukaryotes, like hormones. LuxR solos have conferred bacteria the ability to communicate and benefit from their other bacterial neighbors and adapt their behavior accordingly. The ability to recognize signals from the eukaryotic host may equip the bacteria with information about the habitat to activate virulence and facilitate coordinated behavior. In the first part of this study, I have explored the pathogenic load for three specific microorganisms in companion cats and dogs. In this study, we looked at relative numbers of *P. gingivalis*, *T. forsythia,* and *A. actinomycetemcomitans. P. gingivalis* demonstrated the strongest association with periodontal disease.

P. gingivalis does not produce AHL but possesses many LuxR like homologs on its chromosome. One of this LuxR homolog is PGN_1373 which has been shown to affect the interaction between *S. gordonii* and *P. gingivalis*. DAR has been previously demonstrated to bind to another luxR homolog PauR in *P. asymbiotica*. *P. gingivalis* encodes for DarB which synthesizes 1,3 cyclohexanediones CHDs. We hypothesized that CHD may bind to PGN_1373 and regulate virulence. Thus, for the next part of my study, I investigated the presence of a novel quorum-sensing molecule CHD synthesized by DarB and its effect on virulence factors and attachment of *P. gingivalis*.

In this study, we have shown that *P. gingivalis* secretes molecules similar to commercially available CHDs. The addition of CHD extracellularly influences bacterial virulence genes and biofilm formation. The new DarB/PGN_1373 quorum sensing circuit may add to the existing pool of knowledge regarding intra and interspecies communication in *P. gingivalis*.

3. Hypothesis and objectives

This study had two hypothesis and objectives. The first hypothesis is that multiple periodontal pathogens influence the progression of periodontal disease in cats and dogs. To test this hypothesis are Quantitative Real-time Polymerase Chain Reaction (qPCR) based detection of periodontal pathogens *Tannerella forsythia, Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis* in domestic cats and dogs. This study showed a correlation between bacteria in diseased cats and dogs at healthy and disease stages.

The second hypothesis is that PGN_1373 and *darB* present a novel quorum sensing system in *P. gingivalis*. The objective of this study was to determine the presence and functional aspect of CHD as quorum sensing molecule *in P. gingivalis*.

4. Materials and methods

4.1. Bacteria used in this study

1) *Actinobacillus actinomycetemcomitans HK1651*

2) *Tannerella forsythia ATCC 43037*

3) *Porphyromonas gingivalis ATCC 33277*

Table.3. Primers used in this study

Primer	Sequence (5'-3")
A. actinomycetemcomitans 16s rRNA-F	ATTGGGGTTTAGCCCTGGTG
A. actinomycetemcomitans 16s rRNA-R	ACATGTCCACCGCTTGT
T. forsythia 16s rRNA-F	TACAGGGGAATAAAATGAGATACG
T. forsythia 16s rRNA-R	GGATAACGCTCGCATCCT
P. *gingivalis*16s rRNA -F	TGTAGATGACTGATGGTGAAA
P. *gingivalis*16s rRNA -R	CTGTTAGCAACTACCGATGT
rgpA- F	CTGCGAGCGGTATTAGTGGT
rgpA-R	CTACCAGCCCGTTTCCAACT
rgpB-F	GCTTCAGCTGTTTCGCTAC
rgpB-R	GCTCCGCTCCAGTATAACCC
fimA-F	AAGACAACGAGGCAAGAACCC
fimA-R	TCGTCATCGCCAACTCCAAA
Mfa1-F	ACTTCTCCCGATTCATGGTG
Mfa1-R	GCATTCGGGTCAGGGTTATT

galE-F	TCGGCGATGACTACGACAC
galE-R	CGCTCGCTTTCTCTTCATTC
PGN_1373-F	CGCAGACCAATCGCATAAG
PGN_1373-R	CAGAATAGCCATCGCACAGA
darB-F	TTGGGGCCTATCTCCCTGAA
darB-R	GACGCTCTTTGATCCCCACA
dnaK-F	GTGGCTTTTGTGGATGGTGG
dnaK-R	TCGTCTTGGTCGGATTGGTG
htrA-F	AAGCTTCGTGTGGGAGAGTG
htrA-R	GACCTTTTGCGCTGACGATG
Pgn 0178-F	CCACTTCTTCGGCTTCAC
Pgn 0178-R	CAATGGCGTATCCCTCGTAT
Pgn 0181-F	GACGATCACATGCTGGAAGA
Pgn 0181-R	ATTGGGCAGGTCAGTACACC
kgp-F	CGGGAAGCTTCTGCCTTCTT
kgp-R	ACTGCTACAACGCAGGGTC
hagB-F	GACTGTAGAAAATTTGCGTCTGC
hagB-R	TCGGGCAGTTTGGGATTTCAG
luxS-F	TATTTGGCCATCGGCAGGTT
luxS-R	GCAGGAGACTTTCCGCTTCA

(F-Forward; R-Reverse)

4.2. Culture Media, Chemicals, and Bacterial Growth Conditions

All the chemical reagents and antibiotics were purchased from Sigma, BD, Oxoid, and Difco. Reagents were weighed on a Denver S-602 digital balance, while smaller quantities were measured using a Denver SI-114 digital analytical balance. Microbiological media and solutions were prepared using Milli-Q water. Solutions were sterilized either autoclaved at 121°C for 15min at 4 atmospheres pressure in an autoclave, or 0.22 µm-filter sterilized, depending on their temperature stability. The recipe for the preparation of stock solutions is listed in the table below:

Table.4. Stock solutions

Chemicals (Sigma Aldrich, Canada)	Stock Solution	Preparation
Hemin (Cat # H9039)	5 mg/ml	50 mg Hemin in 5 ml of MilliQ water. Add 1 ml of 1M NaOH. Make up the volume to 10ml with water.
Vitamin K (Cat #M5625)	10 mg/ml	100 mg Vitamin K in 10ml of MilliQ water.
N-acetylmuramic acid (NAM) (Cat # A3007)	10 mg/ml	100 mg NAM in 10 ml of MilliQ water.
Vancomycin (Cat # 75423)	100 mg/ml	1g Vancomycin in 4 ml of Milli-Q Water Makes up the volume to 10 ml of water.
1,3-Cyclohexanedione (CHD) (Cat #C101605)	1 mM	1.21 mg 1,3 Cyclohexanedione in 10 ml of MilliQ water. Make up the volume to 10 ml of water.
CheleX-100 (Cat #C7901)	5%	2.5 g CheleX-100 in 50 ml of 20 mM Tris HCl, pH 8 solution (Merck, Darmstadt, Germany).
Proteinase K (Cat 70663)	20 mg /ml	100 mg Proteinase K in 5 ml of 50mM Tris (pH 8.0).

Culture of anaerobic bacteria

All the bacteria were grown anaerobically in Trypticase Soy Broth (TSB) with or without additives. For a generation of anaerobic conditions, campy gas pouches (Cat #260683, 260678 and 260679 BD Gaspak) were used. Cultures in broth were incubated at 37°C on a

shaker at 225 rpm. The supplemented TS agar plates were pre-reduced under anaerobic conditions overnight prior to use.

Supplemented TS broth/Agar (1L)

For the culture of anaerobic bacteria, supplemented TSB was prepared as follows: To 950 ml of deionized H_2O, 30 g of TSB (Cat # DF0370-17-3, Fisher Scientific, Canada) was added and stirred until dissolved; the volume of the solution was adjusted to 1 L with deionized H_2O. The medium was sterilized by autoclaving. The broth was cooled to 55°C and 50 ml of defibrinated sheep blood (# SD500, Quad five, VWR, Canada) was added. Hemin was added to the final concentration of 5 mg/ml and Vitamin K was added to the final concentration of 10 mg/ml. For TS agar plates, 15 g of agar (Cat # BP1423, Fisher Scientific, Canada) was added to the broth before autoclaving. For *T. forsythia,* NAM was added instead of vitamin K to the final concentration of 10mg/ml. For *A. actinomycetemcomitans* vancomycin was added to the final concentration of 9 µg/ml.

4.3. Periodontal sample collection from cats and dogs

Periodontal samples were collected from cats (62) and dogs (31) between 8 weeks to 7 years of age, from Winnipeg Humane Society (WHS). The samples were collected and provided by the WHS veterinary surgeons. The samples were obtained from the single site, as sterile paper points (Hygenic, Coltene, and Whaledent, USA) which were inserted to the bottom of the pocket and then stored aseptically for transport at 4°C or until further use. The cats and dogs included in this study were classified by criteria based on Canadian Veterinary Medical Association as shown in the table below.

Table.5. Classification criteria of clinical samples of dogs and cats

Condition	Dental Plaque	Symptoms
Healthy	No plaque	No physical symptoms
Diseased	plaque and calculus in sub-gingiva	Redness, edema, bleeding on probing, gum recession

4.4. Sample preparation and genomic DNA preparation

200 µl of 1X PBS was added to the 1.5 ml microcentrifuge tubes containing paper points and incubated at room temperature for 10 min. For DNA binding, CheleX-100. For each sample, 200 µl of 5% CheleX-100 and 20 µl of Proteinase K (10mg/ml) were added. The samples were then incubated in 100 °C water bath for 10 min. The tubes were cooled at room temperature for 10 min and vortexed followed by centrifugation at 13,000 rpm for 8 min. 100 µl of the sample was transferred to the fresh tube without disturbing the bottom layer. For *A. actinomycetemcomitans*, *T. forsythia*, and *P. gingivalis* gDNA isolation the bacteria were cultured in their respective media followed by harvesting by centrifugation at 5000 rpm for 10 minutes. The cells were washed with 1XPBS twice. The Absorbance was measured as Optical Density at 600nm (OD_{600}) using a spectrophotometer (Biotek, Canada). The OD_{600} for the harvest was adjusted to 1 and was serially diluted to determine the Colony Forming Units (CFUs). 25 µl of cell suspension with CFU were used for lysate preparation as described for the samples and the gDNA was isolated using CheleX-100. The samples were stored at -20 °C.

4.5. Quantitative real-time polymerase chain reaction (qPCR)

qPCR was carried out using Eco, Illumina Real-time PCR system (San Diego, USA). The Power-up SYBR green (Cat# A25741) and TaqMan FAST advanced master mixes (Cat # 44-

445-57) were purchased from Applied Biosystems (Thermo Fisher Scientific, Canada). Primers for qPCR were ordered from Sigma Aldrich and are listed in table 3.

4.6. PCR primers for absolute quantitation using qPCR

Designing of primers

For designing the PCR primers for bacterial detection, the variable region in the bacterial small subunit 16S rRNA gene (16S rDNA) was chosen. The 16S rRNA sequences of the genus *P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans*, were selected from the taxonomy database of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/). The strains and GenBank accession numbers are *A. actinomycetemcomitans* HK1651 (NZ_CP007502) and *P. gingivalis* ATCC 33277 (L16492). Sequences of *A. actinomycetemcomitans HK1651* and *P. gingivalis* ATCC 33277 were aligned with that of *E. coli (J01695)* by using the Ribosomal Database Project (Cole et al., 2014). The species-specific primers applied in the qPCR for *P. gingivalis* (*Escherichia coli position* 1039); *A. actinomycetemcomitans (Escherichia coli position 870)* and *T. forsythia*, (*Escherichia coli position* 1227) are listed in Table 2.1Pure cultures of *A. actinomycetemcomitans*, *T. forsythia*, and *P. gingivalis* were used for generating standard curves to quantitate their cell numbers in clinical samples. A multiplex PCR technique was used for determining *T. forsythia* and *A. actinomycetemcomitans* numbers in the standards as well as the samples.

Validation of primers

The specificities of the prospective primers were tested by the program FastA (Genetics Computer Group) against all existing DNA sequence information stored in GenBank-EMBL, and by program CHECK-PROBE (Loy *et al*., 2008) from the Ribosomal Database Project. No sequences completely homologous to prospective *A. actinomycetemcomitans* and *P.*

gingivalis forward primers were found in 16S rDNA for 95 other oral bacterial species in these databases. The combinations were checked for primer-dimer or internal hairpin configurations, melting temperature, and percent G+C values using OligoCalc (Kibbe, 2007) and Mfold software (Zuker, 2003). Melting curve analysis demonstrated further the absence of any PCR artifact formation for each of the primer sets.

The detection limit for this study was defined as the smallest number of bacteria in a sample that could be detected by qPCR. This was assessed by determining the Ct values of serial 10-fold dilutions of purified genomic DNA from *P. gingivalis* strain ATCC33277. PCRs were optimized by using a matrix of concentrations of the forward primer, the reverse primer, and the probe to determine the optimal concentration yielding the lowest threshold cycle (Ct) values and, the highest amplification efficiencies. The specificity of the real-time PCR assay for each bacteria was verified with purified genomic DNA from 3 different bacterial strains.

4.7. PCR reaction mix

a) For generating *T. Forsythia* and *A. actinomycetemcomitans* standard curves and clinical samples multiplex qPCR was used. All the reagents were thawed on ice. The total reaction volume of each sample was 10 µl consisting of 5 µl TaqMan FAST Advance qPCR Master mix 2×, 1 µl (0.4µM) of Primer mix of each bacteria and 0.5 µl Probe of each bacteria were added. Next, to each tube 1 µl template and rest 1 µl Nuclease-Free water was added.

b) For generating standard curve and quantitation of clinical samples for *P. gingivalis* the reaction mix was prepared at a final volume of 10 µl with 5 µl of TaqMan FAST Advance qPCR Master mix 2×, 1 µl each of primer mix (0.4µM) and DNA template and the remaining 3 µl with Nuclease-Free water.

c) The qPCR cycling parameters were carried out in eight steps. The first step, UDG incubation was set at 95°C for 2 min followed by polymerase activation at the same

temperature for 5 min. The PCR cycling was carried out in step three at 95°C for 10 sec, four at 58 °C for 30 sec and five for 15 sec at 72 °C repeated for 40 cycles. The last three steps of the program were set at 95 °C for 15 sec each for the melt curve.

4.8. Amino acid sequence alignment and molecular modeling

Amino acid sequence analysis of PGN_1373 (GenBank: BAG33892.1), *Vibrio fisherii luxR* (YP_206883.1) and Photorhabdus *part* (GenBank: AJW31137.1) were retrieved from the NCBI database, and multiple sequence alignment was performed using T-COFFEE multiple sequence programs. The domain organization of PGN_1373 was analyzed by SMART database (Schultz, Milpetz, Bork, & Ponting, 1998). In absence of a crystal structure, Homology-based modeling of PGN_1373 was carried out to predict its three-dimensional (3D) structure. Conserved domains on PGN_1373 were predicted using NCBI conserved domain (CDD) database (Marchler-Bauer et al., 2013). The molecular models of PGN_1373 were generated on the basis of the crystal structure of the response regulator *vraR* from *Staphylococcus aureus* (PDB ID: 4GVP) as the template using Swiss model (Schwede, Kopp, Guex, & Peitsch, 2003). The final structural prediction was selected by taking the lowest energy model as determined by a low-resolution Rosetta energy function. The backbone conformation of the modeled structure was estimated by analyzing phi (F) and psi (ψ) torsion angles using Rampage (V. Kumar et al., 2013).

4.9. *In vitro* amplification of *darB* gene by PCR

PCR technique was used for confirming presence and amplification of *darB* gene in *P. gingivalis*. *P. gingivalis* was cultured on TSA as described before and was harvested on day 3 followed by gDNA isolation. gDNA was isolated using Bacterial genomic DNA kit (Cat. # GBB100, Geneaid, Taiwan) as per manufacturer's instructions. 50 ng of gDNA was used for PCR. PCR was performed with 16S rRNA as PCR control, water in place of template served as negative control.

4.10. Extraction of cyclohexanediones from *P. gingivalis* supernatant

Rotary evaporation was carried out using BUCHI R-210 (BUCHI, Switzerland). P. *gingivalis* was inoculated in 250 ml of TSB broth and grown anaerobically in 37 °C to OD_{600} of 1.8. The culture was centrifuged for 15 min at 9000 rpm and the supernatant was added to a separatory funnel (Cat #4300-0250, Fisher Scientific, Canada). The supernatant was extracted twice using ethyl acetate (Cat # EX0240, HPLC grade, EMD) in 1:1 ratio. The obtained organic layer (top layer) was transferred to rotary evaporator flask. The solvent was extracted at 40° C -45°C temp water bath and pressure gradient of 242-140 mbar and at 30 rotations per min till 90% of the solvent was extracted. The remaining solution was transferred to a smaller evaporator flask the process was repeated till all the solvent was extracted completely and the residue remained in the flask. The residue was re-suspended in 1 ml of 20% acetonitrile and stored at -20 °C in aliquots (J. Wang, Quan, Wang, Zhao, & Fan, 2011).

4.11. High-performance liquid chromatography analysis for CHDs

HPLC was carried out by using Shimadzu (Mandel scientific, Ontario). CHD was used as a standard. The column used was a C-18 reverse phase of length 150 X 4.6 mm with a particle size of 5 microns (Alltima, Grace Discovery Sciences, Ontario). Two mobile phases were used isocratically. Solvent A was Acetonitrile (# A998 HPLC grade, Fisher Scientific) and solvent B was water (# W5, HPLC grade, Fisher Scientific) adjusted to a pH of 2.7 with sulphuric acid (# A300 ACS grade, Fisher Scientific) using a pH meter (Thermos Fisher, Canada). The oven temperature was set to 40 °C. The standard was prepared to a concentration of 0.5mg/ml in solvent B. The sample was then filtered using 0.2 µm sterile syringe filter (VWR, Canada) into a fresh HPLC 1.5 ml vial (Prominence, Shimadzu, Japan) and placed in the sample tray. The run was carried out by injecting 20µl of the sample and the flow was set to 1 ml/min for 60 min run time. The Ultraviolet visible spectroscopic detector

(UVVIs) set to 254 nm. Similarly, the crude extract sample was dissolved in 1ml of ethyl acetate (Cat # EX0240, HPLC grade, EMD, Canada) and was run using the same above parameters.

4.12. Preparation of 1,3- cyclohexanedione (CHD)

CHD stock solution was prepared as described. *P. gingivalis* cells were grown till OD_{600} of 0.5 for 2 days. The stock solution of CHD was prepared as described before. For assays, 4 concentrations were prepared from stock: 100 µM, 50 µM, 10 µM and 1 µM. For negative control, water was added instead of CHD.

4.12.1. Effect on growth of *P. gingivalis*

P. gingivalis cells were grown and induced with CHDs as described above at final concentrations of 100 µM, 50 µM, 10 µM and 1 µM. The cells were cultured under anaerobic conditions at 37 °C for 60 h. To study the growth, OD_{600} was recorded after every 12 h using Synergy H4 microplate reader (BioTek, Canada).

4.12.2. Effect on virulence gene expression in *P. gingivalis* by qPCR

mRNA isolation for *P. gingivalis*

For isolation of mRNA from *P. gingivalis,* RNeasy mini kit (Cat # 74104, Qiagen, Germany) was used. *P. gingivalis* cells were grown and induced with CHDs to final concentrations of 100 µM, 50 µM, 10 µM and 1 µM. *P. gingivalis* culture with solvent without CHDs was used as negative control. Two volumes of RNA protect was added to the culture and was vortexed and incubated at room temperature for 10min. The culture was centrifuged for 15 min at 9000 rpm. The supernatant was discarded and 1 ml of TE buffer containing lysozyme (Cat # 26876, Sigma Aldrich) was added and incubated at 37 °C for 1 h. Later, 1ml of RLT buffer containing β-mercapthoethanol (Cat # M6250, Sigma) was added and vortexed, and 1 ml of absolute ethanol (Cat # AC615095000, Fisher Scientific) was added and mixed by pipetting.

From this, 700 µl of the sample was transferred to an RNeasy mini spin column provided in the kit. The lysate was centrifuged at 12,000 rpm for 30 s and the supernatant was discarded. This step was repeated till all the sample was centrifuged.700 µl of RWI buffer was added to the column and centrifugation step was repeated. In the next step, 500 µl of RPE buffer was added to wash the RNA. This step was repeated twice. The column was dried and place in new 1.5 ml microcentrifuge tubes. Finally, the mRNA was eluted using 50 µl of RNase and DNase free water (Fisher Scientific) and stored at -20ºC. RNA quality and quantity were measured by NanoDrop ND1000 spectrophotometer (NanoDropTechnologies; Thermo Scientific™, LLC, Wilmington, DE, USA). All the samples used in this study exhibited an A260/A280 ratio of at least 2.0.

DNA digestion

Following the isolation of mRNA, DNA digest was used to eliminate any trace DNA present in the sample. All the reagents were thawed on ice. To 50 µl mRNA sample 5 µl of DNase1-RNase free enzyme (Thermo Fisher, # 89836) and 5 µl of 10X DNase1 reaction buffer (Thermo Fisher, # 89836) was added and mixed by short spin for 30 s. The samples were incubated at 37°C for 90 min. DNase 1 was inactivated by addition of 10 µl of 50 mM EDTA (Thermo Fisher, # 89836), incubated at 65°C for 10 min. The samples were stored at -80°C. The absence of genomic DNA contamination in the RNA preparation was verified by using DNA digested RNA for qPCR using 16s rRNA housekeeping gene along with gDNA as PCR control. The absence of detectable signal in RNA sample indicated a successful DNA digest and the sample was used for further experiments. PCR products were also analyzed on a 2% agarose gel containing SYBR safe DNA stain for the presence of amplicons. All RT-PCR amplifications were done at least thrice using RNA obtained from three separate extractions.

cDNA synthesis by reverse transcriptase PCR

RT-PCR was performed using a qScript cDNA synthesis kit (Cat #101414-096, Quanta, VWR, Canada). Reactions were performed in a total volume of 50 µl, which contained 1X Reaction mix containing a concentrated solution of an optimized buffer, magnesium, oligo (dT) and random primers, dNTPs, 100ng of template RNA, using a SCILOGEX DNA Thermal Cycler (Froggabio, Canada). The RT-PCR amplification program was set as follows. One cycle of 22 °C for 5mins, one cycle of 42 °C for 30 mins followed by 1 cycle of 85 °C for 5 mins. After completion of cDNA synthesis 1µl of the amplicon was used for qPCR.

Gene expression analysis using qPCR

Real-time quantitative PCR analysis was conducted in an Eco Illumina (Montreal Biotech, Canada) in combination with the SYBR Select PCR Master Mix, as recommended by the manufacturer. *P. gingivalis* ATCC33277 16s rRNA was used as the internal reference. Real-time quantitative PCR was performed three times for each sample. The data were analyzed according to relative gene expression by the $2^{-\Delta\Delta}$Ct method.

For the qPCR reaction mix for *P. gingivalis* gene expression analysis, the reagents were thawed on ice. The total reaction volume of each sample was prepared at a final volume of 10 µl with 5 µl of TaqMan FAST Advance qPCR Master mix 2×, 1 µl each of primer mix (0.4µM) and DNA template and the remaining 3 µl with Nuclease-Free water.

4.12.3. Effect on proteolytic activity

To test the proteolytic activity, 50 ml of supplemented TS agar plates with 1% skim milk and CHDs to the final concentration of 100 µM, 50 µM, 10 µM and 1 µM were added and the plates were pre-reduced under anaerobic conditions overnight prior to use. Supplemented TS agar with solvent without CHDs was used as negative control. *P. gingivalis* cells were grown to OD_{600} 0.5-0.7 and 5 µl of culture was plated on a respective agar plate in triplicates. The

plates were then incubated at 37 °C for 48 h. The diameters of zones of clearance were compared to study the effect on proteolysis activity of *P. gingivalis*.

4.12.4. Biofilm formation assay

P. gingivalis was cultured to O.D $_{600}$ of 0.5. The cultures were then seeded 1:100 in 96-well microplate (#655160, CellStar) in a 100 µl volume. CHDs were added to a final concentration in triplicates as described above. The plate was incubated at 37 °C for 5 days. Post incubation, the culture was pipetted out and discarded. The wells were washed 3 times with 100 µl 1X PBS (pH 7.4). The wells were stained with 200 µl of 0.1% crystal violet dye (Cat#C581, Fisher scientific). After 5 minutes, the dye was removed from wells and washed thrice with 1X PBS. The wash step was repeated to remove all the excess dye from the well. The biofilm was solubilized using 125 µl of 30% acetic acid in water per well. The plate was measured at $O.D_{595}$ using Synergy H4 microplate reader (Biotek, Canada).

4.13. Statistical analysis

Data are expressed as mean unless differently specified. The null hypothesis of data analyses for the study of periodontal samples from cats and dogs was that no difference in the frequency of detection of periodontal pathogens would be observed between healthy and diseased cats and dogs. Secondary outcomes included possible associations between detection of one or multiple periodontal pathogens and the disease stage for different age groups of the animals.

Between-group comparisons were performed with the nonparametric Mann-Whitney test. The statistical analysis of the phenotype data was performed using t- test or one way-ANOVA followed by multiple comparison tests using graph pad prism6 software. The level of statistical significance was set at a P value of *0.05.

5. Results

5.1 Optimisation of qPCR for quantitation of dental pathogens *P. gingivalis* and *T. forsythia*

To determine the correlation of *T. forsythia* and *P. gingivalis* cells in healthy vs diseased cats and dogs, a standard curve was generated with the qPCR procedure using *T. forsythia* and *P. gingivalis* specific primers. DNA from known amounts of *T. forsythia* and *P. gingivalis* was added in serial dilutions from 10^2 to 10^8 cells per qPCR to generate a threshold cycle (C_t) based standard curve. R^2 value is used in analyzing the relative values. An R^2 value > 0.99 provides good confidence in correlating two values. The reactions were carried out in an Eco Illumina real-time PCR thermocycler, and the fluorescence was monitored. The detection and quantitation were linear over the range of DNA concentrations examined.

Using the standard curve, a *T. forsythia* and *P. gingivalis* cell number was obtained for every sample of cats (n= 62) and dogs (n=31). The null hypothesis for this study was that no observable difference in the frequency of detection of *T. forsythia* or *P. gingivalis* would be observed between healthy and diseased cats and dogs. The detected cell numbers between *T. forsythia* and *P. gingivalis* between healthy and diseased group were compared using non-parametric Mann-Whitney analysis.

The total clinical samples used in this study were 93 out of which 62 were cats and 31 were dogs, which were 39 healthy and 23 diseased samples of cats and 13 healthy and 18 diseased dogs

Figure 4.a represent the standard curve for *P. gingivalis* and 4. b for *T. forsythia*. A statistically significant ($p<0.01$) difference was observed between *P. gingivalis* (Figure 5. a and b) and *T. forsythia* (Figure 5.c and d) cell numbers in samples from dogs with periodontitis as compared to the healthy ones. A statistically significant difference was

observable between detectable *P. gingivalis* cell number between healthy and diseased cats (Figure 5. a) and dogs (Figure 5. b) both *($p < 0.01$).* These results show that *P. gingivalis* is totally absent in 13 healthy dogs and is detectable in 7 out of 18 diseased dogs inferring to the less prevalence of this bacteria in dogs compared to cats. In cats, P. gingivalis was detectable in 7 of 39 healthy and 18 of 23 diseased whereas *T. forsythia* was detectable in 37 out of 39 healthy and in all 23 diseased cats whereas, in dogs, these bacteria were detectable in only 4 of 13 healthy and 15 of the total 18 dogs. *T. forsythia* which shows that these bacteria are less prevalent in healthy dogs. *P. gingivalis* showed low detectable levels in both cats and dogs which is a significant observation.

5.1.2. Standard Curve for the periodontal pathogens *T. forsythia* and *P. gingivalis*

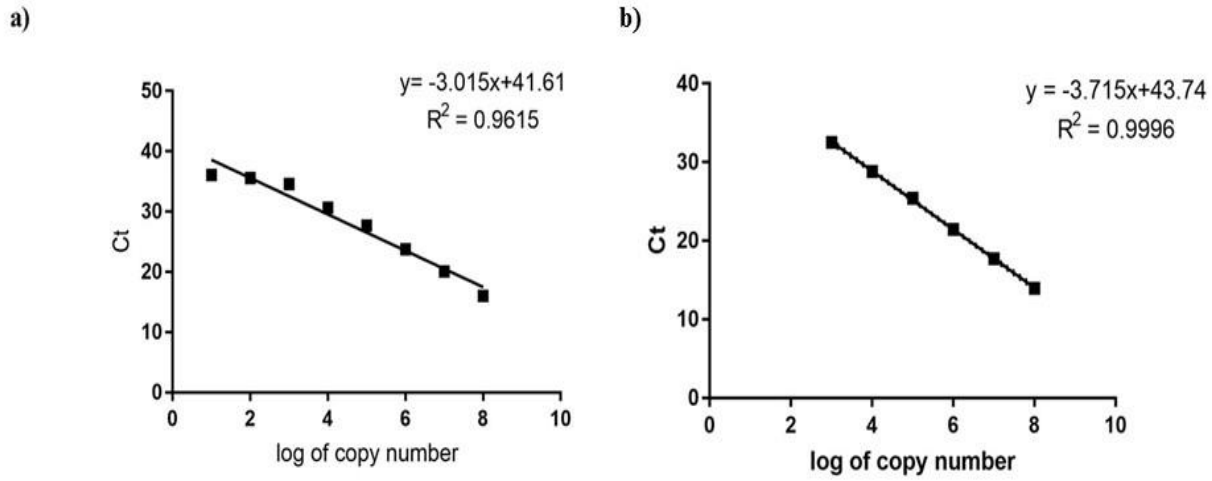


Figure 4. Standard curve for the periodontal pathogens *T. forsythia* and *P. gingivalis.*

The quantitative standard curve of P. gingivalis (a) and T. forsythia (b) cell number**.** Serial dilutions of bacterial cells with known cell numbers from *T. forsythia* were used as templates for real-time PCR. The relative fluorescence is the increase in reporter dye intensity relative to the passive internal reference dye. The amount of *T. forsythia* cell number in each sample is shown as log cell number on X-axis. The threshold fluorescence, or level at which the threshold cycle was determined, is shown as the C_t number on Y-axis. (correlation coefficient = 0.99).

5.1.3. Quantitation of *P. gingivalis* and *T. forsythia* cell number in clinical samples

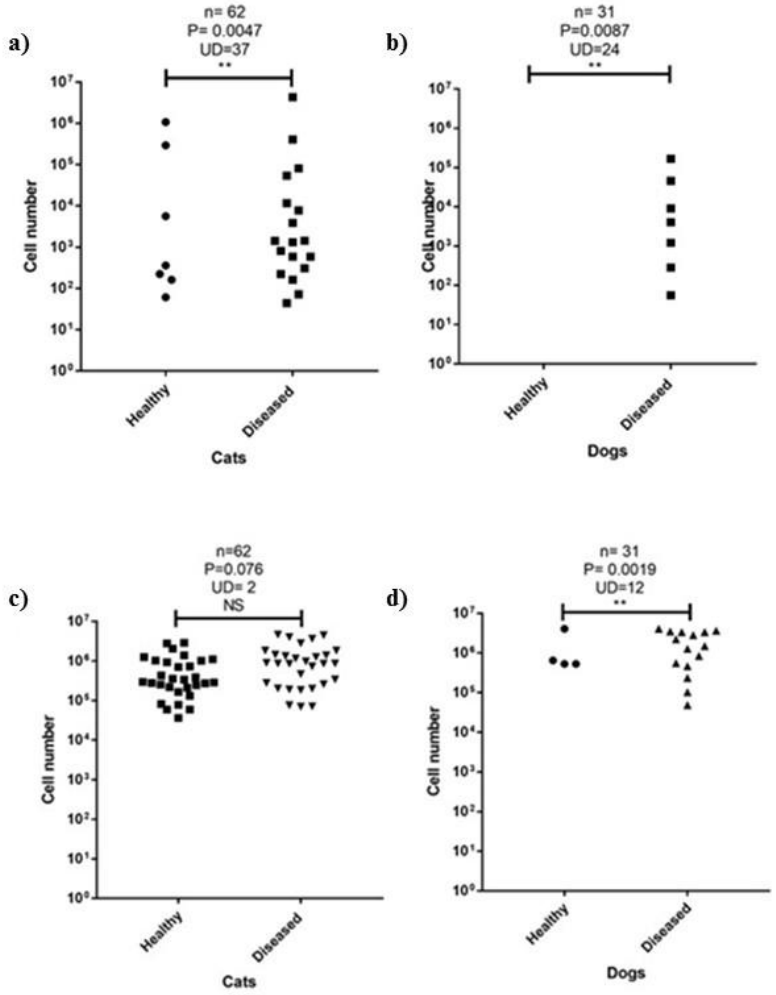
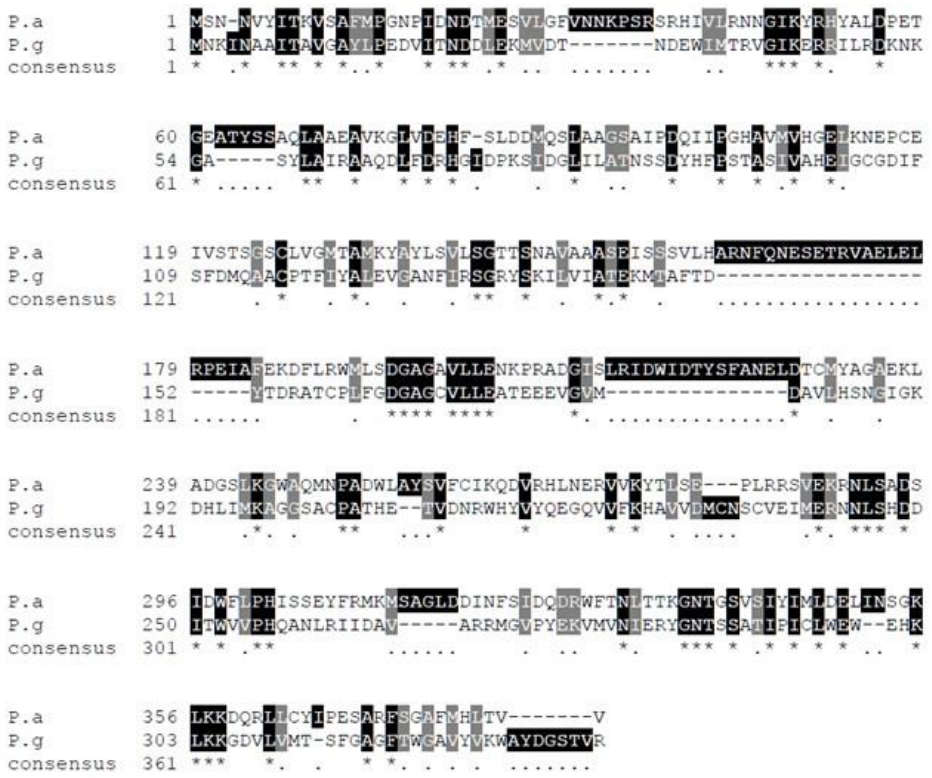


Figure 5. Quantitation of *P. gingivalis* and *T. forsythia* cell number in clinical samples. qPCR analysis of *P. gingivalis* (a and b) T. forsythia (c and d) cell numbers in clinical samples of diseased and healthy cats and dogs. Each square or triangle represents the log cell number of *T. forsythia* in an individual sample. A total number of samples analyzed for each group is shown as n. The significant difference group was analyzed using non-parametric Mann-Whitney analysis. The p-value and the undetected sample numbers are indicated on the top of each graph. UD indicated the total numbers of samples in which bacteria was undetectable. Non-significant (NS), $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

5.2. Bioinformatics analysis of DarB from *P. gingivalis* and *P. asymbiotica*, and confirmation by PCR.

Previously it has been shown that *P. gingivalis* encodes for DarB. DarB from *P. gingivalis* (PGN_0189) was aligned with DarB from *P. asymbiotica* (PAU_02401), using ClustalW (EMBO). As seen in the figure. 6a the DarB in *P. gingivalis* shares homology to the one in *P. asymbiotica*. A BLOSUM score of 75 was obtained for the protein aligned. A higher BLOSUM scores indicate related protein sequences. Primers were designed using *darB* from *P. gingivalis* using the sequence from NCBI and PCR was performed to confirm its presence. Lane L shows 100bp DNA Ladder, lane 1 is gDNA from *P. gingivalis* ATCC33277, lane 2 is gDNA from *P. gingivalis* W50 and lane 3 is a negative control. As seen in the gel, a 750 bp amplicon for DarB is present for both *P. gingivalis* ATCC33277 as well as W50.

a)



b)

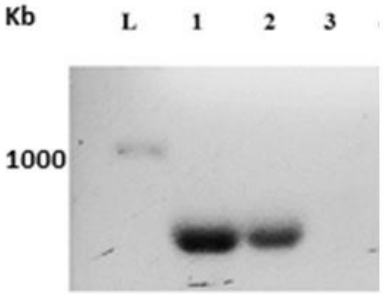


Figure 6. Amino acid sequence alignment of *darB* gene from *P. asymbiotica* and *darB* gene of *P. gingivalis* ATCC 33277. (a) using clustal omega tool b) PCR confirmation of *darB* gene in *P. gingivalis* ATCC 33277 genomic DNA.L= 1000bp Ladder, Lane 1 is the *P. gingivalis* ATCC 33277 genomic DNA, Lane 2 is the *P. gingivalis* W50 genomic DNA, Lane 3 is the negative control.

5.2.1. Amino acid sequence analysis of PGN_1373 with alignment of LuxR in *P. asymbiotica* and *P. gingivalis*

Previous studies have defined a role for PGN_1373 as influencing interspecies interactions. Thus, we wanted to confirm if PGN_1373 shares a homology with other known LuxR proteins and if it may serve as a receptor for quorum sensing molecules. Amino acid sequence analysis of PGN_1373 (NCBI accession ID: BAG33892.1), *Vibrio fischeri luxR (*NCBI accession ID: YP_206883.1) and Photorhabdus *pauR* (NCBI accession ID: AJW31137.1) were retrieved from the NCBI database, and multiple sequence alignment was performed using ClustalW 2.1 multiple sequence program. As seen in the figure. 6b PGN_1373 shares homology with other known LuxRs. A BLOSUM score of 58 was obtained for the proteins aligned. LuxR type proteins share a conserved set of motifs including a DNA binding domain and signal receiving domain. Thus, to further confirm if PGN_1373 did have those motifs, we performed a domain analysis. The domain organization of PGN_1373 was analyzed by SMART database. The LuxR type HTH domain is located 146-173aa. Within the SBD the six conserved amino acids, the WYDPWG-motif of AHL-sensors, are also present.

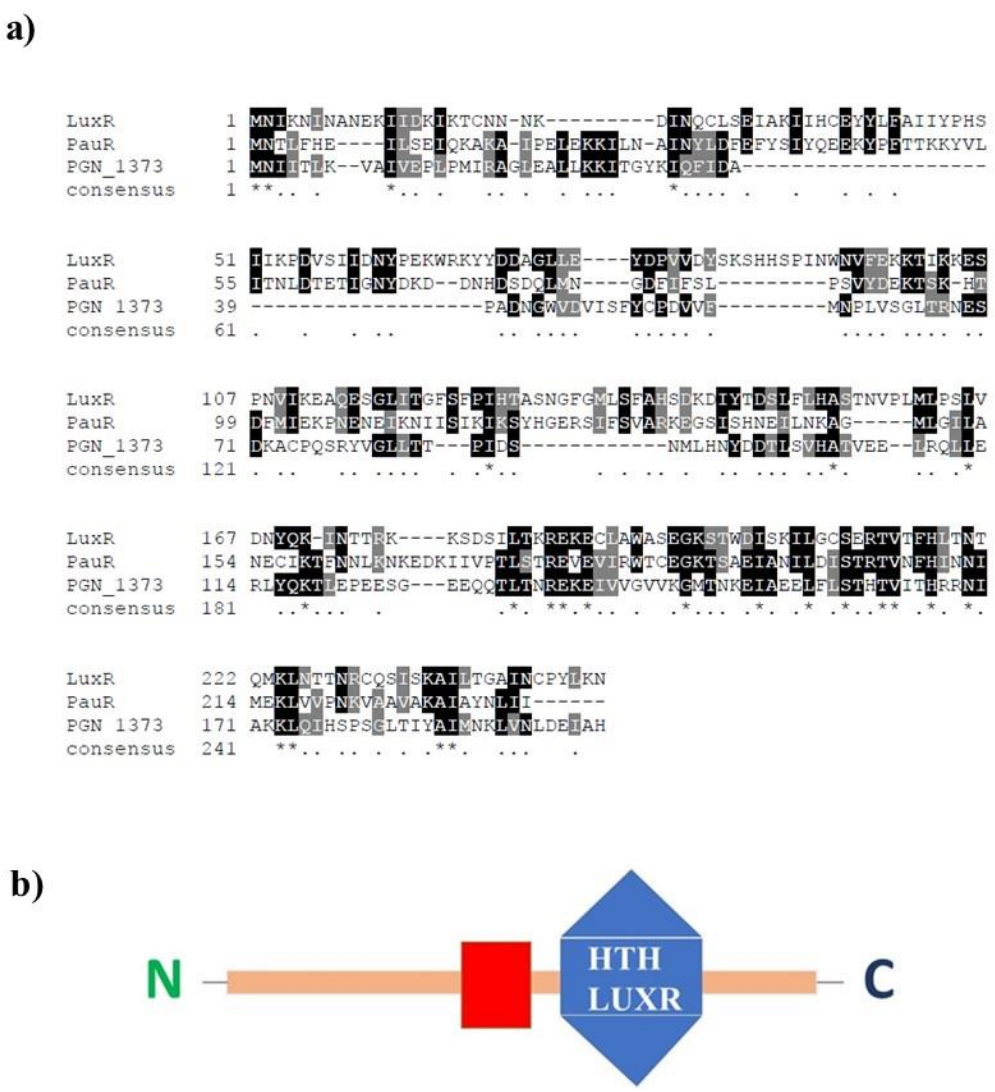


Figure 7. Sequence alignment of PGN_1373 and domain organization. Protein sequence alignment of PauR (*P. asymbiotica*), LuxR (*V. fisherii*) and PGN_1373 (*P. gingivalis* ATCC 33277) using T-COFFEE tool (a). Conserved domains are represented by an asterisk (*). (b) Graphical representation of the functional domain of PGN_1373 identified by Simple Modular Architecture Research Tool (SMART)

5.3. Liquid chromatography to detect the presence of cyclohexanediones in *P. gingivalis* supernatant extracts.

Sequence alignment analysis indicated that PGN_1373 was homologous to LuxR from other species. A blast search indicated that *P. gingivalis* genome only encodes for DarB. DarB is a ketosynthase that forms 1,3 cyclohexanediones (CHDs). Thus, to confirm if *P. gingivalis* secreted CHDs, an HPLC analysis was carried out. *P. gingivalis* was cultured anaerobically at 37°C to OD_{600} of 1.5. The filtered supernatant was extracted using ethyl acetate in 1:1 ratio. Commercial CHD purchased from Sigma Aldrich and used as the standard. Stock for CHD was prepared as described previously. A concentration of 0.5 mg/ml was used for HPLC analysis. The flow rate was adjusted to 1ml/min for at wavelength of 254nm. 1,3-Cyclohexanedione exists in three tautomeric forms 1) Dihydroresorcinol, 2) 3-hydroxycyclohex-2-enone and 3) 1, 3-Cyclohexanedione (Calabrese et al., 2013; Fuchs et al., 2013). Since we have used a reverse phase column which is made up of non-polar silica with water as the mobile phase, based on polarity, the compound to be eluted first are alcohols and aldehydes and then the ketones corresponding to the tautomer forms of CHDs, 1, 3-Cyclohexanedione and Dihydroresorcinol.

The retention times of standard peaks of Form B and Form A match closely within experimental error in the sample and standard in the following table:

Table.6. Retention time of HPLC

	Form B – 1,3 Dihydro resorcinol Retention time (min)	**Form A – 1,3 Cyclohexanedione Retention time (min)**
Standard	28.968	52.216
Sample	27.568	51.191

From this table, we can conclude that there is a proximity of the retention times of standard and sample for the tautomers. The difference of retention times of standard and crude are within experimental error.

5.4. Effect of exogenous addition of CHD on phenotypes of *P. gingivalis*

Liquid chromatography suggested the presence of CHD in *P. gingivalis*. It has already been established that bacteria regulate various phenotypes like motility, growth, biofilm, and regulation of virulence by modulating cell signaling via quorum sensing (González & Keshavan, 2006). Exogenously added QS molecules such as AHLs have shown to influence biofilm formation and phenotypes in marine bacteria *Pseudoalteromonas ulvae* TC14 (Mireille Aye *et al*., 2015). In our study, we believe that if *darB* is an active synthase with PGN_1373 as a QS receptor, then altering exogenous QS molecule levels should reflect changes in phenotype and gene expression. Thus, to test this hypothesis, we examined the effect of the addition of CHDs extracellularly to *P. gingivalis* cultures to study its effect on phenotype and virulence genes.

5.4.1. Effect of exogenous CHD on growth of *P. gingivalis*

To examine the exogenous addition of CHD affected the growth of *P. gingivalis*, we monitored the growth of *P. gingivalis* over 60 h at concentrations 1 µM,10 µM, 50 µM and 100 µM. *P. gingivalis* were grown anaerobically in supplemented trypticase soy broth induced with different concentrations of CHD. The growth was measured every 12 h for 3 days. Culture with no added CHD in the media was used as negative control. No significant differences in growth were observed at any concentration. The results showed that the exogenous CHD did not have any effect on growth.

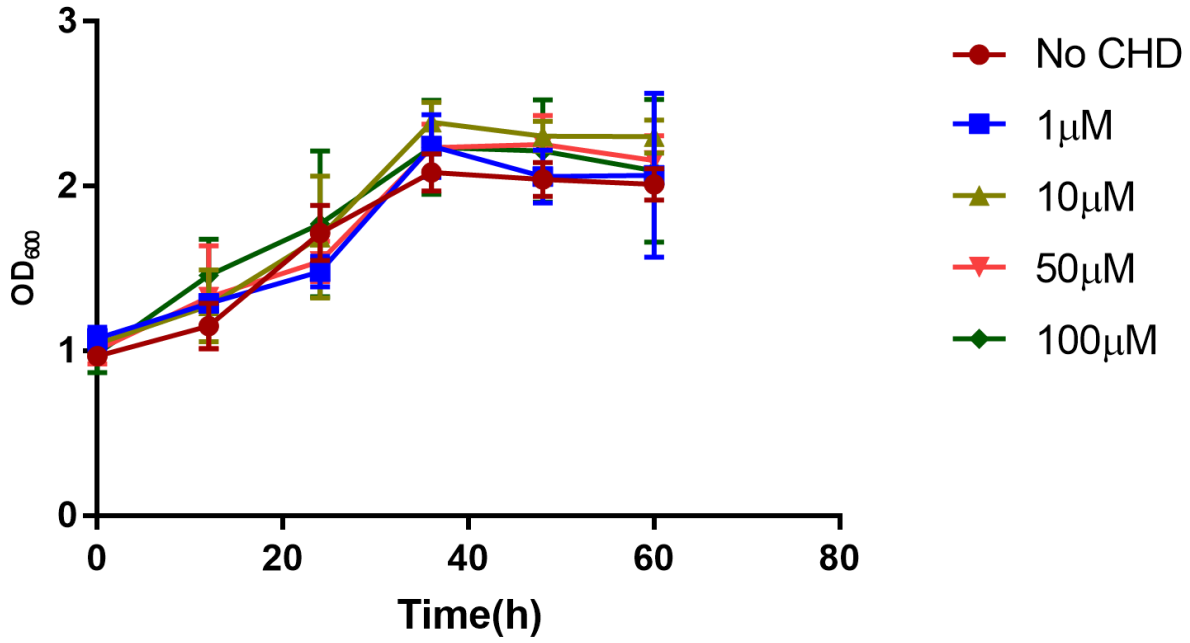


Figure 8. Effect of exogenous CHD on the growth of *P. gingivalis.* The graph shows growth curve of *P. gingivalis* in the presence of four concentrations (1 µM,10 µM, 50 µM and 100 µM) of CHD over 60h. Culture with no added CHD in the media was used as negative control.The growth curve was plotted by measuring OD_{600} for every 12 hours. Data are the means ± SD of values from three independent experiments.

5.4.2. Effect of exogenous CHD on *P. gingivalis* biofilm formation

Biofilm is a complicated, dynamic and multistage aggregation, maturation, and dispersal of cells. In *P. gingivalis* luxS/AI-2 and PGN_1373 have been shown to regulate, both homogeneous and heterogeneous biofilm formation. In this experiment, we tested exogenous CHD influence on mono-species biofilm formation. *P. gingivalis* culture induced with 10 µM concentration of CHD showed 2.5 -fold increase in biofilm formation compared to the control. The biofilm density increased at 1 µM and 10 µM concentrations and decreased at 50 µM and 100 µM**.**

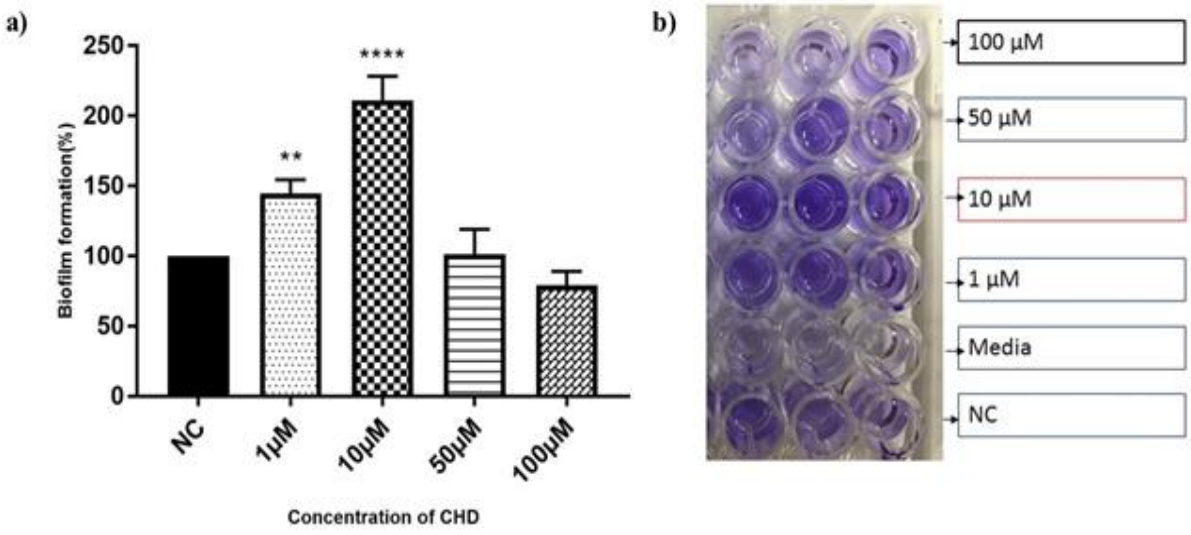


Figure 9. Effect of exogenous CHD on *P. gingivalis* biofilm formation (a) Biofilm formation by *P.gingivalis* induced with different concentrations of CHD. Culture with no added CHD in the media was used as negative control. The graph shows the percentage change in biofilms formed after 5 days. The absorbance of crystal violet-stained biofilms was measured at OD_{595}. Data were derived from three independent experiment. Data were analyzed using graph-pad prism software using one-way ANOVA. Not significant (NS) $p > 0.05$,**** $p < 0.0001$,***$p < 0.001$, **$p<0.01$, *$p < 0.05$. (b) *P. gingivalis* biofilm in 96-well transparent plate. The biofilms were stained with 0.1% crystal violet. The figure is representative of three independent experiments.

5.4.3. Effect of exogenous CHD on proteolytic activity of *P. gingivalis*

Gingipains are one of the most important host-targeted virulence factors responsible for invasion and persistence in *P. gingivalis*. Gingipains mainly consist of arginine and lysine cysteine proteases. The effect of CHD on protease activity was tested. The bacteria were grown and plated on supplemented trypticase agar plates with 1% skim milk with different concentrations of CHD. After 48 h, clear zones of inhibition were measured. Compared to non-CHD controls a higher zone of clearance was observed at 100 µM and 50 µM of CHD.

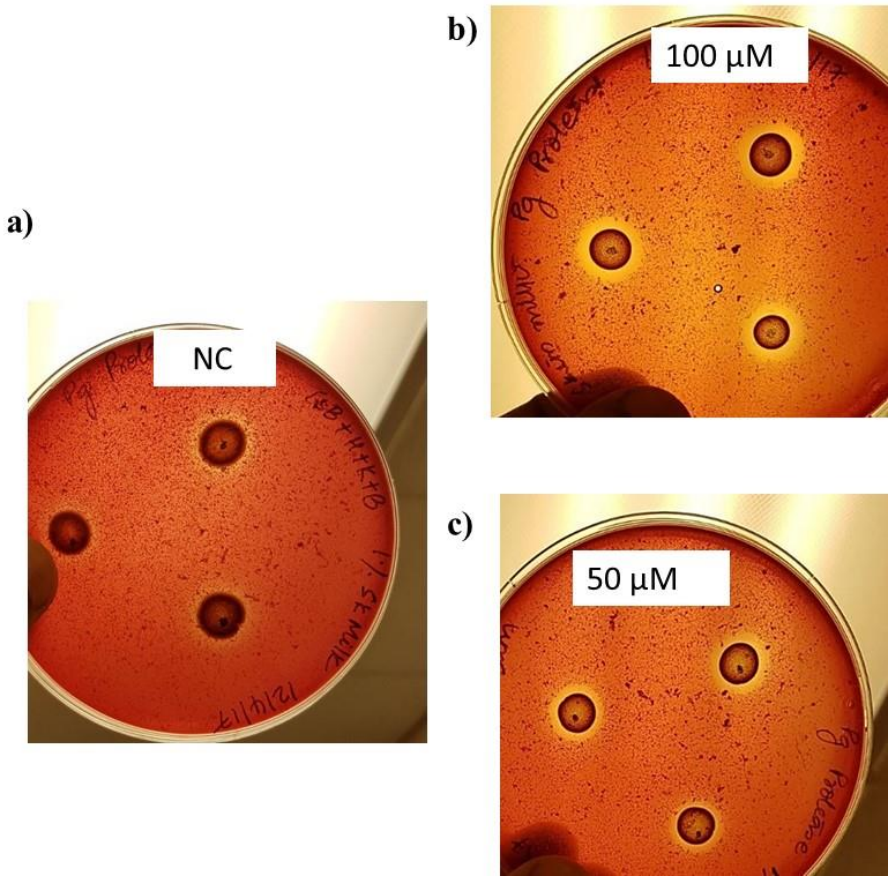


Figure 10. Effect of exogenous CHD on proteolytic activity of *P. gingivalis.* 1% skim milk plates were prepared with different concentrations of 100 µM, 50 µM, 10 µM and 1 µM CHD. Each plate represents one concentration of CHD. Zones of clearance can be seen as clear rings around the *P. gingivalis* colonies. The figure is representative of three independent experiments. a) Negative control (no added CHD), b) 100 µM CHD, c) 50 µM CHD.

5.4.4. Effect of exogenous CHD on mRNA expression levels of *P. gingivalis* genes

As mentioned previously, quorum sensing plays a major role in regulating the virulence factors of *P. gingivalis*. These virulence factors are encoded by various virulence genes. Hence, we have attempted to study the mRNA expression levels of some important virulence genes in *P. gingivalis.* The results showed upregulation in mRNA levels for genes activating biofilm formation at 10 µM concentrations and a second set of virulence genes activating host or inter-species pathogenicity at both 10 µM and 100 µM.

Figure 11 and 12 shows genes demonstrating expression pattern at 1 µM, 10 µM, 50 µM and 100 µM. *P. gingivalis* grown without CHD was used as negative control. Data are shown as percentage change relative to no-CHD control. *P. gingivalis* 16srRNA was used as the housekeeping gene.

Table 7. The table summarizes mRNA expression results of all the genes tested in terms of fold change increase with different concentrations of CHD.

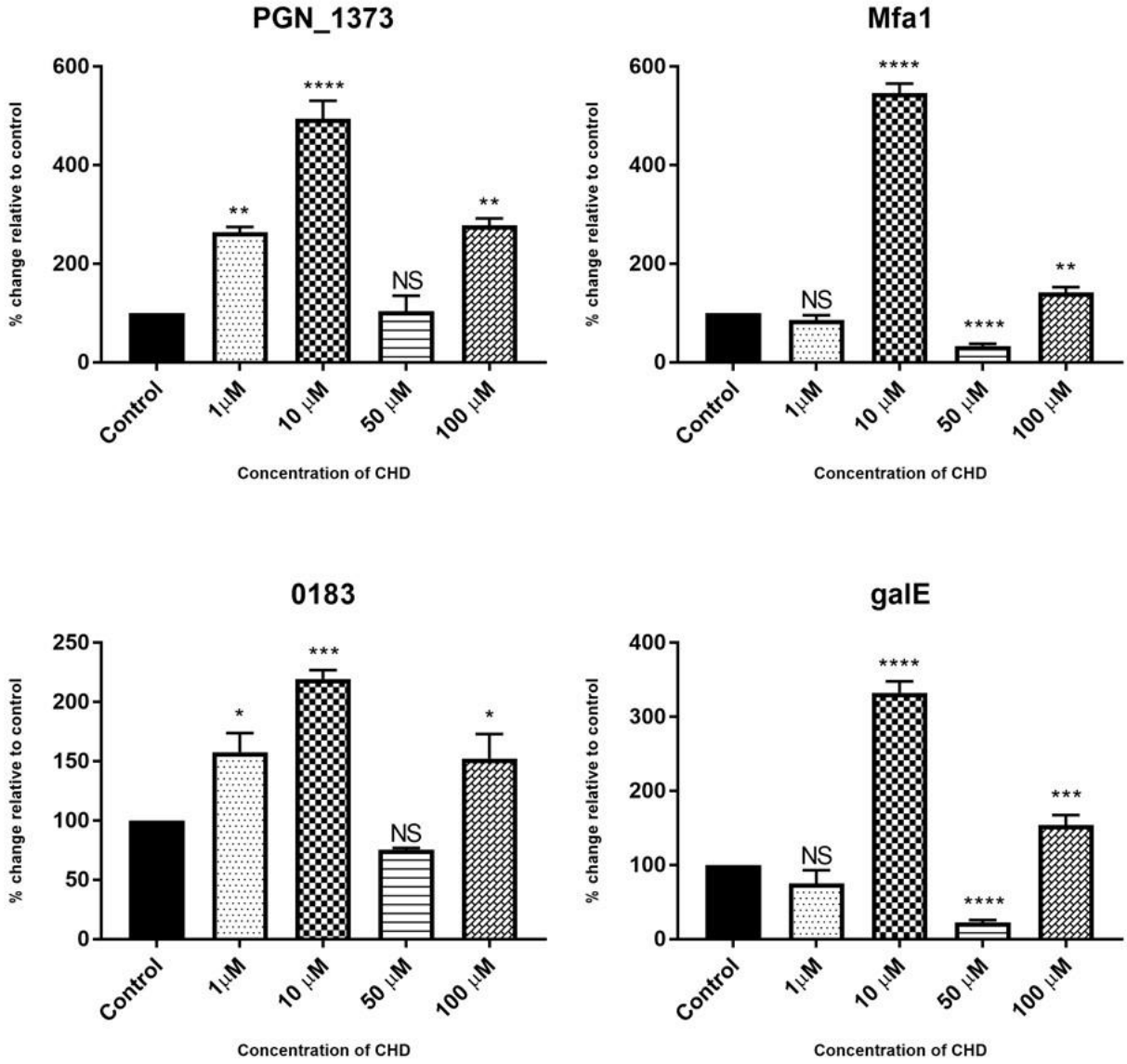


Figure 11. Quantification of mRNA expression. Expression of various biofilm forming genes at different concentrations of CHD. Culture with no added CHD in the media was used as negative control. Relative gene expression of each gene (mean ± SD) was normalized to the expression of the housekeeping 16s rRNA. Data are the means ± SD of values from three independent experiments.Data are presented as percent change in gene expression relative to no CHD control. Data were analyzed using graph-pad prism software using one-way ANOVA. Not significant (NS) $p > 0.05$,**** $p < 0.0001$,***$p < 0.001$,**$p<0.01$,*$p < 0.05$

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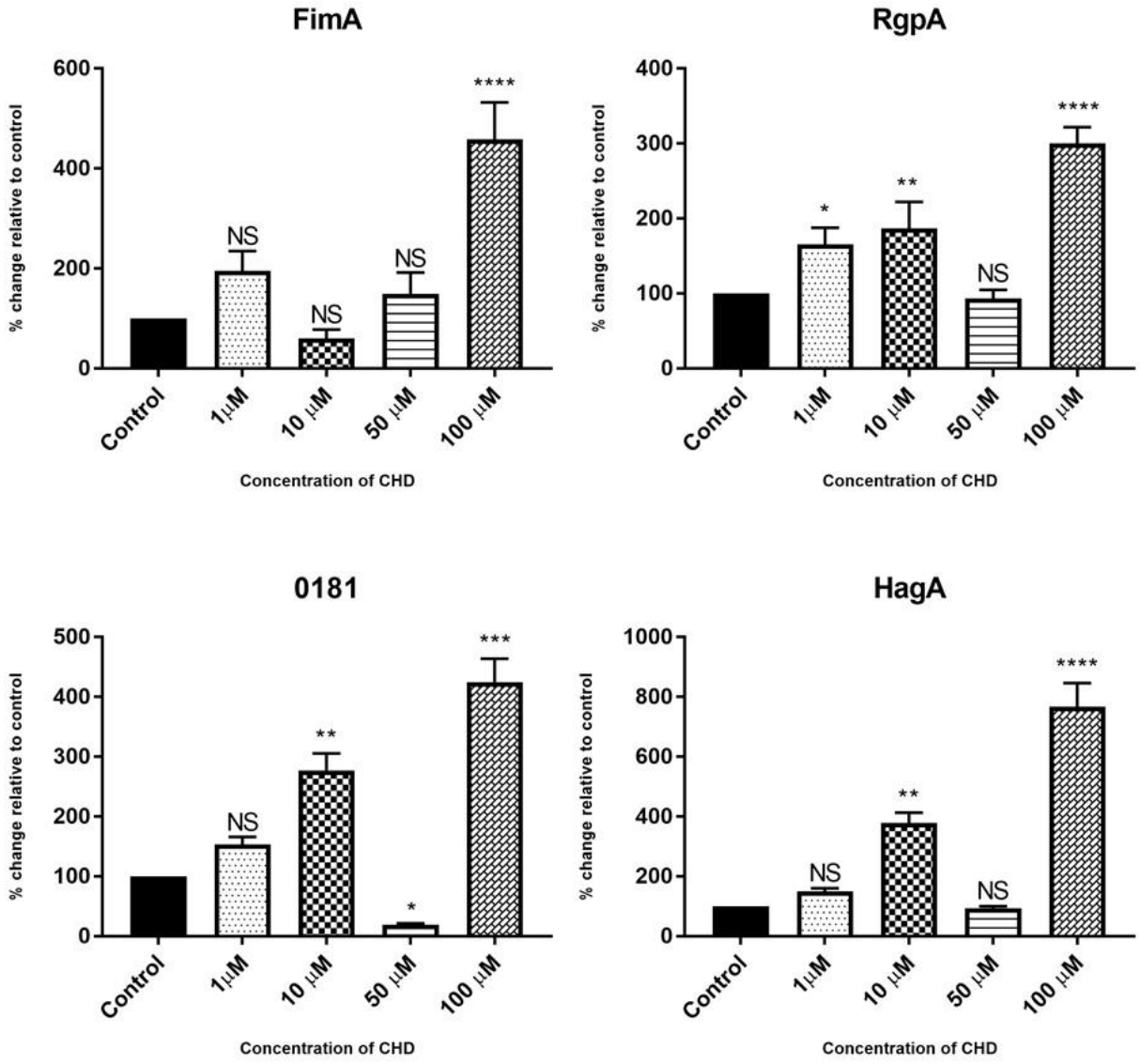

Figure 12. Quantification of mRNA expression. Gene expression of virulence regulating genes at different concentrations of CHD. Culture with no added CHD in the media was used as negative control. Relative expression of each gene (mean ± SD) was normalized to the expression of the housekeeping gene 16s rRNA. Data are the means ± SD of values from three independent experiments. Data are presented as percent change in gene expression relative to no CHD control. Data were analyzed using graph-pad prism software using one-way ANOVA. Not significant (NS) $p > 0.05$,**** $p < 0.0001$,***$p < 0.001$, **$p<0.01$, *$p < 0.05$.

Table.7. mRNA expression fold change at different concentrations of CHD in various virulence genes of *P. gingivalis*

	Fold Change			
Virulence genes	**1 µM**	**10 µM**	**50 µM**	**100 µM**
PGN_1373	+ 2.6	+ 4.5	NS	+ 2.8
mfa1	NS	+ 5.5	- 0.3	+ 1.4
galE	NS	+ 3.3	- 0.2	+ 1.5
PGN_0183	+ 1.6	+ 2.2	NS	+ 1.5
hagA	NS	+ 3.8	NS	+ 7.7
fimA	NS	NS	NS	+ 4.5
rgpA	+ 1.7	+ 1.9	NS	+ 3.0
PGN_0181	NS	+ 2.8	- 0.2	+ 4.3
htrA	NS	+ 1.7	- 0.4	+ 1.6
dnaK	NS	+ 1.9	- 0.4	+ 1.8
rgpB	+ 1.7	-0.1	- 0.4	+ 1.5
kgp	NS	+ 5.0	NS	+ 5.0
PGN_0178	NS	+ 2.6	- 0.2	+ 2.6

6. Discussion

Periodontitis is characterized by persistent, uncontrolled inflammation that causes the destruction of the supporting soft tissues surrounding the teeth, and resorption of the alveolar bone. While treatment and proper oral hygiene can slow the progression of periodontitis, the damage caused cannot be reversed (Kasuga, Ishihara, & Okuda, 2000).

Periodontitis is a significant infectious and progressive problem in cats and dogs but is entirely preventable. On average, by the age of three most cats and dogs demonstrate evidence of periodontal disease (AVDC, 1988; Marshall *et al*., 2014). Periodontitis begins with the accumulation of plaque and tartar at or below the gum-line. Dental plaque is a very organized and selected association of pathogens. Of these colonizing bacteria, the red complex bacteria such as *P. gingivalis* is highly pathogenic and is considered a hallmark of progressing periodontal disease. Others such as *A. actinomycetemcomitans*, *T. forsythia* have been recognized as key opportunistic pathogens is the development of periodontal disease.

Several molecular methods have been employed for detection and quantitation of these perio-pathogens in the past but they are limited by not being cost-effective or being too cumbersome. In this study, qPCR was pursued with TaqMan probes for the direct identification and quantitation of periodontal pathogens. The use of TaqMan probes provides added sensitivity to accurately detect and quantify microbial species in mixed populations.

Comparison of bacterial species in different stages of gingival health in various host species helps to inform the role of oral bacteria in different stages of disease progression. In the present study, the presence of three oral bacteria *P. gingivalis, A. actinomycetemcomitans* and *T. forsythia* in clinical samples of dogs and cats was determined. The TaqMan method identified the detection limit for this study was as low as 10 cells and highest was 10^7. In this

study, we have used a simple in-house technique for isolation of gDNA from mixed samples without the use of expensive kits.

The results show that *T. forsythia* was detectable in both healthy and diseased groups. This is not surprising since *T forsythia* is an opportunist and is also routinely isolated in samples from nondiseased groups. In aggressive and/or chronic periodontitis, however, it forms detrimental associations with *A. actinomycetemcomitans* and *P. gingivalis* and can aggravate the periodontal disease. The most significant result of this study was the detection of *P. gingivalis* in clinical samples dogs and cats. *P. gingivalis* was absent in all the healthy samples and was detected only in diseased samples, suggesting that, *P. gingivalis* is a marker for the periodontal disease. Interestingly, *A. actinomycetemcomitans* could not be detected in healthy or suspected samples from dogs or cats. This might be due to the age and periodontal status; the study participant companion animals were young and had only early to moderate periodontal disease. The presence and function of *A. actinomycetemcomitans* have been described only in advanced stages of periodontitis.

This study emphasizes the role of periodontal diseases in companion animals and stresses on the importance of diagnostic tools such as qPCR for identifying markers of periodontal diseases. It is significant since periodontal diseases are infectious and can be cross-transmitted to accompanying humans. *P. gingivalis* is characterized by making very significant associations with co-habiting pathogens and hence understanding its inter-bacterial interactions or modes of communication is crucial to developing new therapeutic targets.

Bacteria living in a community need to communicate with each other on the cell-cell basis. These communications are very important to carry out functions like biofilm formation, secretion of virulence factors, conjugation, which are a potential threat to their host systems

and default requirement for their survival. These functions are carried out by aggregation and interaction of small secreted molecules called quorum sensing(QS) molecules (Cho et al., 2016). As discussed previously, *P. gingivalis* contains different types of quorum sensing systems such as LuxS/AI 2. *P. gingivalis* does not have an AHL sensing system; however, earlier studies have suggested a possible role for 1, 3 Cyclohexanediones (CHDs) as a signaling molecule. Diaklyresorcinols are encoded by *darABC, darA* is an aromatase-encoding gene, *darB* is 3-ketoacyl-ACP synthase III (KAS III) encoding and *darC* is acyl carrier protein (ACP)-encoding (Schoner *et al*., 2015). The genome of *P. gingivalis* encodes only for DarB. DarB in other bacteria has been shown to function in a LuxI and LuxR circuit. PGN_1373 as we found, shares homology with LuxR and thus we hypothesized that DarB formed a LuI /LuxR like circuitry with PGN_1373. PGN_1373 has been previously studied in *P.gingivalis* as being involved in community behavior and interaction between itself and *S.gordonii*.(Chawla *et al*., 2010). Studies suggested PGN_1373 to play an important role in multispecies biofilm formation. In the present study, we provide preliminary evidence for the existence of a DarB/PGN_1373 circuit for quorum sensing. We not only extracted the signaling molecule CHD but also identified its effect on regulating virulence in *P. gingivalis*. Though further studies are needed to identify clearly using advanced molecular methods the role of CHDs and PGN_1373, this study serves to inform such an investigation.

1,3- Cyclohexanedione belongs to a group of cyclic β-diketones. 1,3-cyclohexanedione forms have been shown to be more stable. This molecule can form inter and intramolecular tautomers. It exists as three tautomers 1) Dihydroresorcinol, 2) 3-hydroxycyclohex-2-enone and 3) 1,3-Cyclohexanedione (Calabrese et al., 2013; Fuchs *et al*., 2013).

In our study, we first extracted CHD from crude extracts of *P. gingivalis* followed by an HPLC analysis, to confirm the presence of CHD like molecule in *P. gingivalis*. The chromatogram of standard showed us the presence of CHD in *P. gingivalis.* The compound

was eluted in the HPLC as two peaks corresponding to 3-hydroxycyclohex-2-enone1 and 1,3-Cyclohexanedione. The chromatogram of the crude sample shows that peaks corresponded with a retention time of the standard. Hence, proving that the compounds present in the standard have been detected in the sample as well. Further, Liquid Chromatographic and Mass spectrometry (LC-MS) analyses are needed to identify this compound as 1,3 CHD.

Extracellular quorum sensing molecules are sensed by receptors to modulate downstream gene expression. LuxI/LuxR function in a feedback manner. LuxR is the cytoplasmic receptor as well and the extracellular AHL is the ligand. Without the ligand, the LuxR protein is unstable and is rapidly degraded. When AHL accumulates, it is bound by LuxR, and the LuxR-AHL complex activates its expression of downstream regulatory genes. LuxI/LuxR exhibit "positive feedback". When the circuit is active, the expression of luxI is also activated by AHL-bound LuxR, and autoinducer production is induced, and the surrounding environment is flooded with the signal molecule. This mechanism probably maintains synchrony as the population of cells switches from low to high cell density.

In this study, we demonstrated the effect of CHD as new QS system on Growth and Phenotypes and virulence genes of *P. gingivalis.* To rule out any negative effect of CHD on the growth of *P. gingivalis* we performed a growth assay and we observed that the exogenous addition of CHD doesn't have any effect on growth.

P. gingivalis produces many virulence factors and to study the effect of extracellular CHDs on biofilm formation, proteolysis and the expression of key virulence factors we performed phenotype assays. We performed a CHD dose-dependent analysis for biofilm formation, proteolysis assay, and gene expression. A significant difference between the ranges of 1-100µM. We observed an increase in biofilm formation at 10 µM whereas the acute phase virulence proteolysis showed the highest activity at 100 µM CHD. Biofilm formation in *P.*

gingivalis involves surface recognition using minor fimbriae and attachment(Petersen, Pecharki, & Scheie, 2004).

Proteases involve the destruction of host tissue, attachment to the host oral cavity and most importantly degradation of host proteins. Cysteine proteases consisting of Arginine gingipains (Rgp) and Lysine gingipain(Kgp) (Bourgeau, Lapointe, Peloquin, & Mayrand, 1992) are considered one of the important and primary virulent factors. In the absence of oxygen, proteases lyse the bacterial cells and are released into the surrounding agar resulting in the increased zone of clearances. In our study significant zone of clearance was observed at 100µM CHDs in the media as compared to the negative control. The lower concentrations didn't show any zone of clearances.

Further to understand the effect of extracellular CHDs on gene expression, we analyzed the expression of various genes in *P. gingivalis* exposed to differing concentrations of exogenous CHD. *P. gingivalis* produces many virulence factors which are very important for the bacterial survival and damage to the host(Romero-Lastra et al., 2017). An increase in transcript levels of biofilm-forming genes such as *PGN_1373*, *mfa1*, *PG0183*, and *galE* was observed at 10 µM of exogenous CHD. Whereas an increase in acute virulence factors such as *fimA, rgpA,* PG0181, and *hagA* was observed at 100 µM of exogenous CHD. Interestingly, the biofilm forming genes displayed a pattern of expression; A significant increase in transcript levels at 10µM followed by a decrease at 50µM followed by another increase at 100 µM. The increase at 100 µM was not as high as the one at 10 µM.

PGN_1373 has been discussed already in terms of its function in heterogeneous biofilm formation. The *galE* in *P. gingivalis* encodes for an enzyme responsible for galactose metabolism. *galE* is involved in the synthesis of Lipopolysaccharide (LPS)-O- antigen. GalE aids in colonization of *P. gingivalis* to host tissue and protects the bacteria from bactericidal

(Gerits, Verstraeten, & Michiels, 2017; Nakao, Senpuku, & Watanabe, 2006). Mfa1 is the minor fimbrial protein produced by *P.gingivalis* which allows *P. gingivalis* to form robust microcolonies and biofilm maturation.(Kuboniwa *et al*., 2009; Lin, Wu, & Xie, 2006; Nagano, Hasegawa, Yoshida, & Yoshimura, 2015). PGN_0183 is a hypothetical protein predicted to function similar to minor fimbriae *mfa1*. At elevated extracellular quorum sensing molecule, in this case, 10 µM, the cells respond by forming biofilm as confirmed by biofilm quantitation assay.

For genes, *fimA, rgpA,* PG0181, and *hagA* are postulated to be active during the acute phase of *P. gingivalis* infection. FimA is the major fimbrial protein produced by *P. gingivalis*. FimA is known to activate production of adhesins that mediate colonization and invasion of host periodontal tissues. (Kuboniwa et al., 2009; Xie, Kozlova, & Lamont, 2004). HagA is a haemagglutinin protein and RgpA is arginine protease produced by *P. gingivalis*. *rgpA* contains a C-terminal haemagglutination adhesion domain Hgp44 and Hgp15.Surprisingly, Hgp44 is also encoded by *hagA.* These two genes have been shown regulate haemagglutination and host-directed proteolytic activity. (Ito, Ishihara, Shoji, Nakayama, & Okuda, 2010). Taken collectively, the gene expression assays for virulence factors in *P. gingivalis* under the differing concentration of exogenous CHDs show that at 10 µM a biofilm response is activated and an acute phase response is activated at 100 µM. This may be explained by two phenomena. Under physiological conditions, the amount of AHLs in the extracellular environment is low, whereas the levels rise when biofilms start to form. Bacteria can only recognize non-self CHDs. CHDs like AHLs are hypothesized to freely move across the cell membrane. Upon addition of exogenous CHDs in low amounts, quorum-sensing receptors function as kinases. An artificial stimulus is perceived by the cell and this activates positive-feedback loop activating other quorum-sensing molecule secretion and hence activate biofilm formation (Rabin *et al.*, 2015). However, at higher concentrations, above the

threshold, QS receptors function as phosphatases. Phosphate flow in the signal transduction pathway is reversed, resulting in dephosphorylation and inactivation of LuxR (Ng & Bassler, 2009). Therefore, at excess CHD extracellularly, luxR mRNA is stabilized, and LuxR protein is produced which acts as both a transcriptional activator and a transcriptional repressor. In addition to the luciferase operon, LuxR regulates adhesion secretion and proteases.

The second explanation for the observed results is the dispersal phenomenon. At low or at-threshold extracellular CHDs, biofilm formation is initiated however at higher or excessive CHD concentration such as 50 µM or 100 µM, the response for cell dispersal is initiated and hence the acute phase genes are activated(Kostakioti, Hadjifrangiskou, & Hultgren, 2013).

These results are difficult to explain in absence of a DarB and PGN_1373 knockout mutant as the effect of endogenous CHD levels cannot be ruled out. Further experiments are needed to define the role of CHDs as quorum sensing molecules in *P. gingivalis*.

This study provides preliminary evidence for the presence of a non-AHL quorum sensing circuit in *P. gingivalis*. Synthetic QS mimicking compounds can either enhance or inhibit quorum-sensing behavior could have potential clinical applications(Borlee, Geske, Blackwell, & Handelsman, 2010; Palmer, Streng, & Blackwell, 2011). Bacterial interactions are complex and may involve yet unknown molecules. Understanding such novel quorum sensing molecules may be helpful in designing novel and better therapeutic targets.

7. Conclusion and Future work

P. gingivalis can be considered as a marker for periodontitis in domestic cats and dogs as it was found to be predominant in diseased animals. *P. gingivalis* genome contains *darB* gene which encodes for CHDs. These CHDs are proposed as new quorum sensing molecules. This study reports the preliminary findings for the presence of these molecules in this bacterium and, we studied the functional aspect of cyclohexanediones in *P. gingivalis*. We found that exogenous CHD did not affect the growth of *P. gingivalis* but it significantly affected the biofilm and virulence genes expression. Future research on *darB* knockout can add more significant and exciting results to these findings.

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