ROLE OF NOVEL QUORUM SENSING MOLECULES (DKPS-DIKETOPIPERAZINES) AS ACTIVATORS OF BACTERIAL VIRULENCE AND HOST RESPONSE

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ABSTRACT

Alex Gillone: Role of Novel Quorum sensing molecules (DKPs-Diketopiperazines) as activators of bacterial virulence and host response.
(Under the direction of Steven Offenbacher)

Objectives: The aim of this project was to establish the functional role of novel quorum sensing molecules (Diketopiperazines – DKPs) on activation of bacterial virulence properties and the potential effects on host cells as activators of the innate immune response. Methods: The effect of DKPs on the growth and virulence properties of the periodontal pathogen, Porphyromonas gingivalis (P.g.) A7436 strain was examined. Secondarily, the effect of DKPs on human monocyte (THP-1) viability, growth and cytokine production upon lipopolysaccharide (LPS) stimulation was determined. THP-1 cells were collected, counted and the cell lysate was evaluated for Interleukin 1β (IL-1β) mRNA expression. Results: Our results demonstrate that DKPs minimally affect the growth of P. g. DKP alone did not significantly alter THP-1 viability (p=0.20), indicating it was not toxic to the cells. However, analysis of the IL-1β mRNA expression indicates that DKP inhibited the inflammatory response of LPS-stimulated THP-1 cells. Conclusions: We have confirmed that DKPs minimally affect the growth of P. g. and cyclo(Leu-Pro) appears to slow the replication of THP-1 cells. Cyclo(Leu-Pro) seems to inhibit the expression of IL-1β in THP-1 cells.
To my family and Acela, I couldn’t have done this without your continued love and support.

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LIST OF ABBREVIATIONS

AHL  N-acyl homoserine lactones
BGI  Biofilm-gingival interface
CSI  Conserved signature indels
CNS  Central Nervous System
DKP  Diketopiperazine
E.c.  *Escherichia Coli*
e.g.  *exempli gratia*
FISH  Fluorescence in situ hybridization
Gly  Glycine
h  Hour
His  Histidine
i.e.  *id est*
IL  Interleukin
INF  Interferon
LPS  Lipopolysaccharide
MCP  Monocyte chemoattractant protein
MeOH  Methanol
MMPs  Matrix metalloproteinases
nmol  nanomole
NF-Kb  nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf  Nuclear factor-like
NRPS  Nonribosomal peptide synthetases
NUG  Necrotizing Ulcerative Gingivitis
OD   Optical Density
OTU  Operational Taxonomic unit
PCR  Polymerase Chain Reaction
P.g.  *Porphyromonas gingivalis*
Phe  Phenylalanine
p-NA p-nitroaniline
Pro  Proline
QS   *Quorum sensing*
ROS  Reactive oxygen species
rRNA Ribosomal RNA
S. aeureus  *Staphylococcus aureus*
THP-1 Human acute monocytic leukemia cell line
TRH  Thyrotropin-releasing hormone
TNF  Tumor Necrosis Factor
Tpr  thiol protease
Tyr  Tyrosine
Val  Valine
VRE  Vancomycin-resistant enterococci
CHAPTER 1: REVIEW OF SYNERGISTETES, FRETIBACTERIUM FASTIDIOSUM, CYCLODIPEPTIDES AND PERIODONTITIS

Introduction

Over the last two years evidence has been quickly mounting to implicate Synergistetes in the pathogenesis and progression of periodontal disease. Synergistetes have been identified as components of the oral microbiome. Synergistetes is a recently identified bacterial phylum that comprises one of the 13 different phyla identified in the human microbiome database from the oral cavity. Synergistetes was once defined as a rare phylum in the oral biofilm based on analysis utilizing 16s rRNA, with only 0.1-2% frequency. However, estimations based on fluorescence in situ hybridization (FISH) have suggested that Synergistetes may account for as much as 3.2-11.3% of the microbiota within deep pockets. Synergistetes are now not only considered a dominant phylum in the subgingival plaque, but their direct contact with host immune cells demonstrated by FISH suggests an important role in host-biofilm interactions.

This concept is further supported by the novel observation that Synergistetes species are strongly associated with the production of novel quorum sensing (QS) molecules that have become identified by state-of-the-art metabolomic analyses of saliva. These QS molecules are known to stimulate the expression of genes that enhance the virulence of periodontal pathogens. Two new QS molecules have been identified which have never been ascribed to the oral microbiome. These two molecules are diketopiperazines (DKPs), which are cyclic dipeptides: specifically, cyclo(Leu-Pro) and cyclo(Phe-Pro). These DKP molecules are putative QS signaling molecules...
that activate bacteria and possibly eukaryotic cells and are known entities in marine, food (spoilage) and plant-associated microbiology\(^6{}^8\).

**Synergistetes and Fretibacterium fastidiosum**

The *Synergistetes* are a phylogenetic cluster of Gram-negative anaerobes related to *Synergistes jonesii*, sufficiently distinct from all other phyla to be considered a distinct phylum or Division. They have been demonstrated in several environmental ecosystems: human, animal, terrestrial and ocean habitats\(^9\). *Synergistes jonesii* was the first species belonging to this phylum that was isolated in 1992\(^10\). It was found in culture from a goat rumen and was able to degrade the toxic compound, 3-hydroxy-4(IH)-pyridone, that is produced in the rumen from mimosine. Degradation of 3-hydroxy-4(IH)-pyridone by these ruminal microbes is critical for protection of animals from leucaena toxicity. The isolates showed not to ferment carbohydrates, but were able to use arginine and histidine as substrates for growth. The name *Synergistes* for the genus was chosen to highlight their role of co-workers with the animal\(^10\). There is confusion in the literature about the name of this new phylum, in fact in 1998\(^11\), the candidate phylum “*Synergistes*” was first described in a phylogenetic study upon the global diversity of bacteria. However, this name was illegitimate due to its prior use for a genus\(^10\) and for this reason Jumas-Bilak and colleagues in 2009 suggested the name “*Synergistetes*”\(^12\). Furthermore, most oral *Synergistetes* phylotypes were originally assigned/misclassified to the *Flexistipes* or *Deferrribacteres* groups (phylum *Diferrribacteres*) and *Selenomonas* genus, *Syntrophomonadaceae* family (*Firmicutes* phylum)\(^12{}^17\), but further phylogenetic re-analysis placed them in the *Synergistetes* group\(^12,18\).

In the late 90s-early 2000s early *Synergistetes* 16S rDNA sequences were found in molecular inventories of petroleum reservoirs\(^19,20\), pollution removal anaerobic digestors\(^21{}^25\),
termite hindguts, pig intestinal tract, human subgingival ecosystems and dental caries. Godon et al. in 2005, explored the Synergistetes in 93 anaerobic environments (soils, digestors, 49 animal guts, feces and the human oral cavity – subgingival plaque). They showed that from 16S rDNA gene-targeted PCR assays, this group appeared to be present in 90% of the anaerobic microbial ecosystems analyzed and also that there was a strong link between 16S rDNA sequences and given ecosystems. Indeed, Synergistetes 16S rDNA sequences from animal sources (termites, guinea pigs, pigs, birds, subgingival plaque, etc.) formed clustered phylogenetic groups. Synergistetes groups were also associated either with anaerobic digestors and soils or with thermophilic conditions. Synergistetes sequences were not found in human feces. Their results showed the wide diversity of the Synergistetes division as well as the specific ecological niche of each 16S rDNA sequence. Although Synergistetes were present in the majority of anaerobic ecosystems, this group belonged to a rare category since their frequency was generally below 1%. They concluded that the function of Synergistetes in anaerobic ecosystems remains unknown. However, Synergistetes seem to be anaerobic amino-acid degraders, suggesting that they may be involved in the turnover of amino acids in natural anaerobic ecosystems. The results (presence in 90% of the anaerobic ecosystems) reported by Godon are likely to be overestimated since their group-specific primers cross-reacted with other taxa. Horz et al. in 2006, described the first characterized human isolates of the division Synergistetes. They searched the R. M. Alden human clinical culture collection (Santa Monica, Calif.) for Synergistetes. They reported five slow growing and biochemically inert clinical isolates cultured from peritoneal fluid and two isolates from soft-tissue infections (sacral wound and diabetic-foot) that together constitute three separate evolutionary lineages within the phylogenetic radiation of the division Synergistetes. One of these clusters was formed by the
peritoneal isolates and had an 85% similarity to *Synergistes jonesii* (cluster I). The isolates from soft-tissue infections, on the other hand, formed two distinct lineages moderately related to each other with a similarity of approximately 78% (cluster II). In addition, by using a newly designed 16S rRNA gene-based PCR assay with intended target specificity for *Synergistetes*, they found that the dominant phylotype from a human fecal sample was nearly identical to that of the strains obtained from peritonitis. On the contrary, sequence types detected in periodontal pockets, from patients with chronic and aggressive periodontitis, formed a separate cluster (III), that shared a similarity of only 80% with the soft-tissue isolates. The proportion of *Synergistetes* relative to the total microbiota was only 0.04% in these oral samples. In conclusion, their findings suggested a high diversity of medically important *Synergistetes* clades, which apparently are unique to individual ecological niches in the human body, and a possible pathogenic role for these microorganisms.

In a review Vartoukian and co-workers described *Synergistetes* as a widely distributed phylum in nature, found in the human mouth, human gut and soft tissue infections. Generally, they represent only a minor constituent of the bacterial community in each habitat. They also hypothesized that these microorganisms have evolved to adapt to each habitat, and therefore exhibit a wide range of physiological and biochemical characteristics, although all cultivable taxa so far studied have the ability to degrade amino acids. They speculated them to be present in polymicrobial infections where anaerobes predominate and free amino acids are available. From an ecological standpoint, they supposed that *Synergistetes* likely play an intermediate role in the consortia found in these infections, such as periodontal disease, using amino acids made available from the breakdown of proteins and peptides by other organisms and, in turn, providing
short-chain fatty acids and sulphate for terminal degraders such as the methanogens and sulphate-reducing bacteria.\(^9\)

In 2007 Jumas-Bilak et al. reported *Jonquetella anthropi* as the first characterized species of this new phylum that has been isolated in man. This microorganism was described as an anaerobic, Gram-negative rod with fastidious growth recovered in cultures from a peritoneal fluid sample, wounds, cysts and abscesses.\(^31\) In 2009 another group\(^32\) isolated a new species, *Pyramidobacter piscolens*, from the human oral cavity (odontogenic abscess), that is closely related to *Jonquetella anthropi*. The genus *Pyramidobacter* comprises strains that are anaerobic, non-motile and asaccharolytic bacilli.\(^32\) The same year Jumas-Bilak and co-workers proposed that the genera *Aminiphilus*, *Aminobacterium*, *Aminomonas*, *Anaerobaculum*, *Dethiosulfovibrio*, *Jonquetella*, *Synergistes*, *Thermanaerovibrio* and *Thermovirga* should be assembled in the same high-level taxon, the phylum *Synergistetes*, on the basis of 16S rRNA gene phylogeny.\(^12\) Another genus, *Cloacibacillus*, belonging to the phylum *Synergistetes*, was derived from a municipal wastewater treatment plant and was described by Ganesan et al. in 2008.\(^33\)

Grice et al. in 2009 reported the presence of *Synergistetes* on the normal human skin of the umbilicus.\(^34\) Marchandin et al. in 2010\(^35\) demonstrated for the first time, using a culture independent-approach, the presence of *Jonquetella anthropi* and *Pyramidobacter piscolens* in vaginal microflora. They also proposed a classification of *Synergistetes* into five deeply branched clades named SYN-A to SYN-E, with an analysis based on larger data compared to Horz et al.\(^30\) Only the subgroups SYN-A and SYN-B contained clones and isolates of human origin while SYN-C, SYN-D and SYN-E contained environmental species and clones. They concluded that the detection of human *Synergistetes* at different sites, in independent studies under both pathological and physiological conditions, suggested that bacteria of this phylum
were not occasional contaminants, but that they are part of the commensal human microflora and may act as opportunistic pathogens.

In 2015 Domingo et al. proposed *Cloacibacillus evryensis* and *Cloacibacillus porcorum* as potential human pathogens associated with bacteremia. The same year Jumas-Bilak and co-workers described *Rarimicrobium hominis*, a species belonging to the fifth genus in the phylum *Synergistetes* that includes human clinical isolates from gynecological material.

Despite the habitat diversity that characterizes this phylum, isolates bear a physiological resemblance to one another in that they are all strictly anaerobic, neutrophilic Gram-negative rods or vibrios that ferment amino acids. In fact, Hugenholtz et al. in 2009 reported that *Synergistetes* have the highest average proportion of amino acid transport and metabolism genes of any bacterial phylum. They also reported that *Synergistetes* have the genes that are normally used for LPS biosynthesis but they appear to lack genes for the TolAQR-Pal complex that is responsible for assembly and maintenance of the outer membrane. Unfortunately, not many other common characteristics for all the species of this phylum are known. Bhandari et al. in 2012, reported the identification of 32 conserved signature indels (CSIs), i.e. insertions/deletions in the DNA of these microorganisms, in widely distributed proteins such as DNA-directed RNA polymerase $\beta$ subunit (RpoB), DNA-directed RNA polymerase $\beta'$ subunit (RpoC), UvrD/REP helicase (UvrD), DNA gyrase A subunit (GyrA), DNA polymerase I (PolA), DNA polymerase III $\alpha$ subunit (PolC), S-adenosylmethytransferase MraW (MraW), Nicotinate nucleotide adenyllyltransferase (NadD), Orotate phosphoribosyltransferase (PyrE), 30S ribosomal protein S1 (RpsA), Ribosomal protein S8 (RpsH), Cell division protein FtsA (FtsA), DNA repair protein RadA (RadA), etc., including a large >300 amino acid insert within the RpoC, that are present in various *Synergistetes* species, but except for isolated bacteria, these CSIs are not found in the
protein homologues from any other organisms. As CSIs are present in most or all Synergistetes and absent in other taxonomic groups, they provide strong evidence that species of the Synergistetes phylum constitute a monophyletic group that is distinct from all other prokaryotic taxa. Among these CSIs, 22 are specifically present in *Jonquetella* and *Pyramidobacter* indicating a close association between these two species. Additionally, they demonstrated that several CSIs were commonly shared by *Synergistetes* and some species from other bacterial phyla suggesting potential cases of lateral gene transfers.

*Synergistetes* are found in the human mouth where they appear to be more numerous in dental and gingival disease than health. Human oral *Synergistetes* can be divided into two main groups: cluster B, which comprises *Jonquetella anthropi* and *Pyramidobacter piscolens*, and cluster A, which comprises 22 other taxa (such as *Fretibacterium fastidiosum*) for which one cultivable representative is available.

*Synergistetes* are now not only considered a dominant phylum in the subgingival plaque, but their direct contact with host immune cells demonstrated by FISH suggests an important role in host-biofilm interactions. FISH analysis also revealed that uncultivable oral *Synergistetes* cells were large curved bacilli that are significantly more abundant and have increased diversity in the subgingival plaque of patients with periodontal disease as compared to healthy patients. In addition, only cluster A was detected in subgingival plaque samples, using FISH analysis, and *Synergistetes* OTU 4.2 was found in significantly more subjects with periodontitis than controls and was more abundant in subgingival plaque at diseased sites than at healthy sites in subjects with periodontitis or healthy controls. Park and colleagues demonstrated the presence of 4 dominant phyla, in a human periodontitis population from Korea, including *Bacteroidetes*, *Fusobacteria*, *Synergistetes*, and *Spirochaetes*. Similarly a previous report has shown that
Spirochaetes, Synergistetes, and Bacteroidetes were identified as the dominant phyla in periodontitis patients 47. More importantly, using HOMIM methods, patients with refractory periodontitis (based on mean attachment loss (AL) and/or >3 sites with AL ≥2.5 mm after SRP, surgery and systemically administered amoxicillin and metronidazole (within one year post-therapy) have been distinguished from those with treatable severe periodontitis and healthy patients by significantly higher frequency of specific pathogens, including Synergistetes species cluster II, that belong to the Synergistetes phylum, in subgingival plaque 48.

Baumgartner et al. 2012 49, in a Chinese population, demonstrated that Synergistetes cluster A bacteria, but not cluster B bacteria or Jonquetella anthropi, are more strongly associated with Necrotizing Ulcerative Gingivitis (NUG) than with gingivitis (9.4 fold higher numbers). Therefore, the mounting evidence points to the central importance of Synergistetes as an important phylum in the context of periodontal disease and in poor treatment outcomes. Synergistetes cluster A, but not cluster B, bacteria were found at higher prevalence, numbers and proportions in saliva from patients with periodontitis (generalized aggressive and chronic periodontitis), than non-periodontitis subjects in a Turkish population. The proportion of Synergistetes cluster A tended to be higher in generalized aggressive periodontitis patients than chronic periodontitis patients 50. Moreover, a positive correlation was revealed between the levels of Synergistetes cluster A and all clinical measurements, including probing pocket depth, clinical attachment loss, plaque index and bleeding on probing scores. Accordingly, a recent study demonstrated that subgingival plaque from Chinese patients with periodontitis exhibits higher numbers and more diverse operational taxonomic units of Synergistetes cluster A, compared to periodontitis-free subjects 45. The low prevalence and levels of Synergistetes cluster B, including Jonquetella anthropi, in all these studies may indicate that these taxa may not have
a crucial etiological role in periodontal diseases\textsuperscript{49, 50}. In periodontitis patients Marchesan \textit{et al.} in 2015 showed that the microbial community comprising \textit{Synergistetes} was highly associated with clinical parameters for periodontitis (high probing depth and high bleeding on probing); they also hypothesized that since \textit{Synergistetes} were associated with trans-4-hydroxyproline, a marker of collagen metabolism, these microorganisms could be associated in the destruction of soft tissue and bone\textsuperscript{5}.

Conversely to the above-mentioned studies Yu \textit{et al.} failed to show that \textit{Synergistetes} are more abundant and more readily detected within periodontitis and peri-implantitis lesions compared to healthy peri-implant and periodontal sites in a Chinese population. They were unable to establish concrete differences/similarities between \textit{Synergistetes} communities present within diseased sites compared to healthy sites; or between periodontal/subgingival niches compared to peri-implant/sub-mucosal niches. Taken together, their results suggest a more nuanced relationship between \textit{Synergistetes} taxa and polymicrobial infectious diseases of the periodontium\textsuperscript{51}. One of the reasons this study did not found differences could that this population comprised only patient with a history of periodontitis and considered healthy and diseased sites within the same patient, so colonization of the healthy sites could not be excluded. Accordingly, da Silva \textit{et al.} in 2014, using a Sanger sequencing-based approach, found higher levels of \textit{Synergistetes} taxa within healthy implant sites compared to peri-implantitis sites\textsuperscript{52}. Another group, Belibasakis \textit{et al.}, used FISH probes specific for \textit{Synergistetes} cluster A and B cells and epifluorescence microscopy, to study their distributions within biofilms sampled from healthy and diseased peri-implant sites. They noted that the prevalence and numbers of \textit{Synergistetes} cluster A were significantly higher within diseased peri-implant niches compared to non-diseased sites. Moreover, the levels of \textit{Synergistetes} cluster A in biofilms significantly
correlated not only with probing pocket depth, but also with bone loss, suppuration, and bleeding on probing, potentially indicating their stronger association with the severity of peri-implantitis than Spirochetes. In conclusion there has been an association of Synergistetes with peri-implantitis, but it is still less well established than with periodontitis.

Besides periodontal and peri-implant disease, the presence of Synergistetes has been demonstrated in man during endodontic infections and in dental caries. Siqueira and Rôças in 2007, using a 16S rRNA gene-based group-specific heminested PCR protocol, demonstrated that Synergistetes phylotypes are present in the microbiota from primary endodontic infections and suggested a possible role in causation of apical periodontitis. Considering that virtually all cultivated Synergistetes strains degrade amino acids in anaerobic ecosystems, these bacteria may be favored by the environmental conditions in necrotic root canals. The oral clones W028, BA121/P4G_18 P1, W090, BH017 and E3_33 were found in endodontic infection. The same Brazilian group found Synergistetes also in cases of persistent endodontic infections (root-filled teeth) associated with asymptomatic chronic periradicular lesions. Vianna et al. showed that the quantity of Synergistetes in primary endodontic infections with radiographic evidence of apical periodontitis was clearly within the range of the other analyzed pathogens (Prevotella intermedia, Porphyromonas gingivalis, Treponema), suggesting their clinical relevance in endodontic infections. In 2014 do Cabo Fernandes et al., through FISH analysis, showed the presence of Synergistetes cluster A in the apical tissue fluid of both an apical periodontitis and previously root-filled with apical periodontitis group of patients, but not in the irreversible pulpitis group. Synergistetes cluster B was not detected in any of the groups. They concluded that Synergistetes cluster A may be involved in the pathogenesis of apical periodontitis.
Fretibacterium fastidiosum (or strain SGP1T) has been identified as a key periodontal disease-associated species. Park and colleagues, through pyrosequencing analysis of subgingival microbiota associated with distinct periodontal conditions in a Korean population, found that F. fastidiosum, Fretibacterium genus and other unclassified Synergistetes are highly increased in periodontitis subjects. Among the taxa assigned by Abusleme to the “core” periodontitis-associated microbiome Fretibacterium fastidiosum and other Fretibacterium species were also included. Furthermore, You and colleagues showed that Fretibacterium fastidiosum is more prevalent in subgingival plaque samples from patient with periodontitis than periodontitis-free controls. Lourenço et al., in 2014, using HOMIM, noted that absence of Fretibacterium spp., Fusobacterium naviforme/Fusobacterium nucleatum ss vincentii and Granulicatella adiacens/Granulicatella elegans was associated with a higher risk for aggressive periodontitis in relation to chronic periodontitis. This finding will need to be confirmed in the future with other studies. Fretibacterium fastidiosum is not only associated only with periodontal disease, in fact it was found both before and after chemo-mechanical preparation of the root canals of teeth with endodontic-periodontal lesions as well as of single-rooted teeth with apical periodontitis.

Although Synergistetes have been traditionally classified as non-cultivable, Vartoukian and colleagues in 2010 were able to cultivate this new strain after isolation from the human subgingival plaque of a deep periodontal pocket by means of colony-hybridization-directed enrichment and co-culture with other oral bacteria.

The name Fretibacterium fastidiosum was given to underline both the dependence of this organism on co-culture for good growth and its nutritional requirements. Indeed, for good growth the strain SGP1T or Fretibacterium fastidiosum was dependent on co-culture with, or
extracts from, selected other oral bacteria (such as *Fusobacterium nucleatum*). Independent culture of SGP1T on blood agar resulted in a varying amount of growth, ranging from no growth to a biomass equivalent to approximately a quarter of that resulting from co-culture with the feeder strain \(^{44}\). More recent studies from the same group confirmed these results, implying a possible dependence of this species on helper strains to form a viable biofilm. The relationship between helper and recipient strain could be the result of a dependence on the helper for the provision of specific chemical factors, such as siderophores, because they have lost the ability to produce them \(^{64}\).

SGP1T cells were described as obligate anaerobic, motile, Gram-negative, curved bacilli and asaccharolytic. Their size was 1.0–1.56 µm in width and 2.0–13.0 µm in length or elongated in older cultures \(^{44}\). Ultrastructural analysis using transmission electron microscopy showed a distinctive internal structure comprising micro-compartment organelles \(^{43}\). After 21 days incubation on blood agar plates streaked with *Fusobacterium nucleatum*, colonies were approximately 0.5 mm in diameter with a circular, high convex central, smooth, off-white, opaque central region surrounded by a highly irregular, semi-translucent periphery, though some variations were also observed. After 3 months and numerous subcultures a second and distinct colonial morphology bigger in size (1.3 mm in diameter) and phenotypically different was observed. The optimal growth temperature was 37 °C (with minimal growth at 25 °C and 42 °C) and optimal pH of 6-7. Major amounts of acetic acid and moderate amounts of propionic acid were produced as end products of metabolism in peptone-yeast extract-glucose broth supplemented with a filtered cell sonicate of *Fusobacterium nucleatum*. Hydrogen sulphide was produced and gelatin was weakly hydrolyzed. Enzyme profiles generated with the Rapid ID 32A panel tests gave positive results for leucyl glycine arylamidase and glycine arylamidase. Nitrate
was not reduced and neither catalase nor indole were produced. A predominance of myristic, palmitic and stearic cellular fatty acids was found. Antibiotic susceptibility included amoxicillin, ampicillin, metronidazole and penicillin; the colonies were moderately susceptible to erythromycin; and resistant to ciprofloxacin and other antibiotics.  

**Cyclodipeptides**

Cyclodipeptides [also known as 2,5-diketopiperazines; 2,5 dioxopiperazines; cyclic dipeptides; or dipeptide anhydrides] are among the simplest naturally occurring peptide derivatives. They are considered relatively stable and inert molecules with a peculiar heterocyclic, chiral, rigid and functionalized structure that responsible for their unique biological properties. They are derived from the head-to-tail cyclization of two linear α amino acids to form a lactam. In fact, these compounds show a common scaffold, easily obtained by standard synthesis procedures, that favors structural diversity as a function of substituent side chains particularly oriented. Favorable pharmacodynamic and pharmacokinetic characteristics are acquired by the compounds through these properties. In addition, the core scaffold of DKPs makes these molecules resistant to proteolysis and enables them to cross the intestinal wall and even the blood–brain barrier. Generally, biosynthesis of cyclodipeptides can be achieved in three different ways. The first one is by a dedicated nonribosomal peptide synthetase (NRPS), a multimodular enzyme complex; an alternative second route that also uses NRPS but produces truncated side products during the synthesis of larger peptides, or by a novel type of synthetases named cyclopeptide synthases that use aminoacyl-tRNAs.

The first cyclic dipeptide, cyclo(Gly-Gly), was synthesized in 1888 by Curtius and Gloebel. The focus on this group of compounds in nature started between the late 1800s and early 1900s, when many simple diketopiperazines such as cyclo(Gly-Gly) were synthesized in
order to examine their interesting physicochemical properties and biological roles. Another early report comes from Abderhaden and Komm in 1924. Recently these compounds attracted attention due to their various therapeutic possibilities.

Most cyclopeptides are isolated from natural sources, as secondary functional metabolites or side products of terminal peptide cleavage, but because of their structural simplicity they also could be easily synthetized. In later years, some of the cyclic dipeptides have emerged as by-products of food processing under certain chemical, thermal and storage conditions. They also have been derived from proteins, polypeptide hydrolysates, fermentation broths and cultures of yeast, lichens, and fungi. Many cyclic dipeptides are endogenous to members of animal and plant kingdoms; these include cyclo(Pro-Leu), cyclo(Pro-Val), cyclo(Pro-Phe), cyclo(Ala-Leu), cyclo(Pro-Tyr), cyclo(Pro-Trp), cyclo(Pro-Gly) and cyclo(His-Pro).

A well-defined group of DPKs are those that derive from marine organisms. In the review from 2010 by Huang et al., a total of 124 DKPs molecules were described, covering the literature up to December 2008. They reported that some of these compounds have been found to possess various bioactivities including cytotoxicity, antibacterial, antifungal, antifouling, plant-growth regulatory, and other activities. They also described the marine sources and distribution of these molecules, 76% from marine microorganisms, 19% from sponges, 1% from sea stars and tunicates (ascidians) and 3% from red algae. The same group in 2014 published an update on the ninety 2,5-diketopiperazines from marine organisms, reported from 2009 to the first half-year of 2014.

Although the six cyclic dipeptides-cyclo(His-Pro), cyclo(Leu-Gly), cyclo(Tyr-Arg), cyclo(Asp-Pro), cyclo(Pro-Phe) and cyclo(Pro-Gly) exhibit interesting physiological and/or pharmacological activities in mammals, only two of these, cyclo(His-Pro) and cyclo(Pro-Gly), to
the best of our knowledge, have been shown to be endogenous to mammals. On the contrary, cyclo(Leu-Gly), cyclo(Tyr-Arg), and cyclo(Asp-Pro) are structurally related to endogenous peptides Pro-Leu-Gly-NHs (melanocyte-stimulating hormone release inhibiting factor), Tyr-Arg (kyotorphin), and Val-Pro-Asp-Pro-Arg (enterostatin), respectively, which may serve as precursor peptides.65,73

Cyclo(His-Pro), due to its structural similarity, has been regarded as having a precursor-product relationship with thyrotropin-releasing hormone (TRH).65,66,68 Cyclo(His-Pro) has been found in various human fluid compartments, such as central nervous system (CNS), pancreas, milk, blood, cerebrospinal fluid, semen, urine and amniotic fluid. In the CNS, Cyclo (His-Pro) appears to modify the sedative effects of ethanol (through mediation of the GABA iotropic receptor), ketamine and pentobarbital, and also to play an important role in the perception of pain stimuli, hypothermia and anorectic effects.65,66 Moreover it was described to have antioxidant and TRH-regulatory activities66,67 and to exert an anti-inflammatory effect in vivo in the CNS by counteracting LPS-induced reactive gliosis.74

Diketopiperazines have been proposed as a new family of signaling compounds, used in QS, that is a mechanism of cell-to-cell communication via secreted small molecules, used by bacteria.68 Furthermore, several reports have prompted the proposal that DKP could represent a new class of QS signals and potentially even interspecies and interkingdom signals.65,76-78 Some studies reported that DKPs from a range of Gram-negative bacteria were able to modulate LuxR, TraR, or LasR activity in AHL-sensitive biosensor strains, in which the LuxR-type proteins are overproduced, previously considered specific for N-acyl homoserine lactones (AHL).79-82 Campbell et al. 2009 challenged this concept and failed to demonstrate the same results and a direct interaction with LuxR-type proteins.79
In general, many DKPs show interesting biological activities such as: alteration of cardiovascular (heart rate, cardiac output) and blood-clotting functions, antiviral (e.g., the gliotoxins and sporidesmins), phytotoxic [e.g., cyclo(Pro-Tyr)], antibacterial (e.g., bicyclomycin), antifungal, antiprion, antiprotozoal, antitumor and antihyperglycemic properties, inhibition of glycosidase, inhibition of plasminogen activator inhibitor-1, inhibition against aflatoxin production, affinities for calcium channels, opioid, GABAergic, serotonergic 5-HT$_{1A}$ and oxytocin receptors$^{65,66,70}$.

Semon in 2010 hypothesized that cyclodipeptides from food (such as malt, cocoa and beer) and intestinal yeast cyclodipeptides synthetized by *Candida albicans* may play a role, throughout fetal development and postnatal development, in causing psychiatric disorders. This hypothesis is based on cancer research, where cyclic dipeptides such as cyclo(Pro-Phe) have been found to activate the pathways of apoptosis and to cause programmed cell death. Activation of such pathways is also thought to be central in causing the neurodevelopmental abnormalities seen in disorders such as schizophrenia, autistic disorder, and perhaps Alzheimer’s disease$^{83}$. Conversely Bellezza *et al.* in 2014 argue that, because of its capacity to regulate inflammation via glial cells and induce a protective response in neuronal cells, cyclo(His-Pro) has potential therapeutic utility in an array of neuroinflammatory diseases$^{68}$.

According to the literature and within the limits of the available investigations, we hypothesize that **DKPs could act as QS molecules in the oral biofilm inhibiting *P.g.* growth and gingipain production as well as modify cytokine production by THP-1 cells.**

One of the basic science goals of this study is to try to understand the role of these molecules in oral biology. Unraveling the pathways of cell-to-cell and cell-to-host communication could lead to improved knowledge of the oral biofilm and its difference from
other ecosystems. In addition, understanding their mechanism of action may lead to new treatment modalities.

**Statements of purpose, hypothesis and specific Aims:**

The role of these cyclo dipeptides in the oral ecosystem has not been explored. Previous studies, from other ecosystems, define them as QS molecules. We seek to establish the functional role of these molecules on activation of bacterial virulence properties and the potential effects on host cells. Two specific aims are developed to test our hypothesis that these molecules act as QS molecules within the oral biofilm.

**Aim 1: To assess the role of DKPs on the growth of periodontal pathogens and on the expression of virulence genes of key pathogens (e.g., *P. g.* expression of gingipain).**

We will grow *P.g.* strain A7436 and we will treat it with different concentrations of DKPs. Growth curves will be recorded and analyzed. Supernatant will be collected at the early stationary phase in order to assess gingipain activity through the use of a colorimetric assay.

**Aim 2: Measure the effect of DKPs on the LPS-mediated inflammatory cytokine response in a human monocytic cell line (THP-1).**

We will growth THP-1 monocytic cells and we will challenge them with varied concentrations of LPS\(^{84,85}\) and DKPs in order to assess the effect on cell viability after 24 hours. The cell lysate will be collected and IL-1β production will be assessed through qRT-PCR.
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CHAPTER 2: ROLE OF NOVEL QUORUM SENSING MOLECULES (DKPS-DIKETOPIPERAZINES) AS ACTIVATORS OF BACTERIAL VIRULENCE AND HOST RESPONSE

Introduction

Over the last few years evidence has been mounting quickly to implicate Synergistetes in the pathogenesis and progression of periodontal disease \(^1\). Synergistetes have been identified as components of the oral microbiome. Synergistetes is a recently identified bacterial phylum that represents one of the 13 different phyla identified in the human oral microbiome database from the oral cavity \(^2\). Synergistetes was once defined as a rare phylum in the oral biofilm based on analysis utilizing 16s rRNA, with only 0.1-2% frequency \(^3\). However, estimations based on FISH have suggested that Synergistetes may account for as much as 3.2-11.3% of the microbiota within deep pockets \(^4\). Synergistetes are now not only considered an important phylum in the subgingival plaque, but their direct contact with host immune cells demonstrated by FISH suggests an important role in host-biofilm interactions \(^5\). This concept is further supported by the novel observation that Synergistetes species are strongly associated with the production of novel QS molecules that have become identified by state-of-the-art metabolomic analyses of saliva. These QS molecules are known to enhance the overall expression of genes that enhance the virulence of pathogenic bacteria. Precisely, two new QS molecules have been identified, which have been ascribed only recently to the oral microbiome. These two molecules are DKPs which are cyclic dipeptides: specifically, cyclo(Leu-Pro) and cyclo(Phe-Pro) \(^6\). Not long ago, another metabolomic cohort study of HIV infected patients reported the presence of cyclo(Leu-Pro) in
the oral metabolome of the healthy control group (uninfected patients). This metabolite was shown to be paired with *Fusobacteriaceae Fusobacterium*.

These DKP molecules are putative QS signaling molecules that activate bacteria and possibly eukaryotic cells and are known entities in marine biology, food (spoilage) microbiology and likely bacteria swarming. Fuqua and Winans introduced the term QS to describe a cell-to-cell communication signaling pathway, based on population density, used by bacteria to detect changes in their environment and consequently to apply specific strategies that allow adaptation to environmental stress in space and time. This mechanism is regulated by small, diffusible signal molecules known as autoinducers, and their associated protein receptors. There are two main groups of signal molecules involved in bacterial QS; one is the peptide derivatives typically used by Gram-positive bacteria, while fatty acid derivatives are exploited by the Gram-negative bacteria. *Quorum sensing* in Gram-negative bacteria was first characterized in *Vibrio fischeri*, a luminous symbiotic species that provides its marine eukaryotic host with light, and is under the control of AHL signals and the LuxR-type family of cytoplasmic receptors. The AHL ligands are most frequently generated by LuxI-type synthases, and their local concentration correlates with cell density and other environmental factors. Above a threshold level (i.e., the *quorum* level) the AHLs bind to the LuxR-type receptors and activate the transcription/expression of target genes required for bacterial adaptation. The functional behaviors influenced by this cell-to-cell signaling include biofilm/extracellular matrix formation, bioluminescence, conjugation, pigment production, sporulation, secretion of virulence factors, adaptation/survival in hostile environments, etc. Considering that many of these pathways play central roles in both pathogenesis, symbiosis and host-bacteria communication, there is substantial interest in the development of ligands that can block or mimic native autoinducer
signals to attenuate QS and thus can be used as novel therapeutic strategies for the control of the human microbiome \(^{14, 21, 23, 24}\). Certain 2,5-diketopiperazines have been isolated from bacteria and reported to activate or inhibit LuxR-type proteins \(^{25-27}\).

*Porphyromonas gingivalis* is a recognized periodontal pathogen and is described as belonging to the red complex, a group of bacteria associated with severe forms of periodontal disease \(^{28}\). Moreover, significant negative correlations were found, in a study on primary endodontic infections, between *Synergistetes* and *P. gingivalis*. Since both species have a proteolytic capacity, and because the small amount of available carbohydrates in an infected root canal is quickly depleted, substrate (i.e. proteins) competition among these groups might be the most plausible reason \(^{29}\). For all these reasons we decided to study the effect of DKPs on *P. gingivalis* growth and virulence factor production. Virulence factors are molecules expressed and secreted by pathogens (bacteria, viruses, fungi, and protozoa) that enable them to replicate and disseminate within a host in part by subverting or eluding host defenses \(^{21}\). Gene expression of tpr proteases (thiol protease), a known virulence factor of *P. gingivalis*, has been shown to be regulated by peptide nutrients *in vitro*. Addition of the dipeptide phenylalanyl-phenylalanine to the growth medium resulted in a 10-fold decrease in tpr expression, underlying the importance of phenylalanine-containing peptides as a controlling factor \(^{30}\). Gingipain proteases are the major virulence factor produced by the periodontopathogenic bacterium *P. gingivalis* and thus will be the focus of our study \(^{31}\).

Macrophages are major sources of many cytokines (TNFs, interleukins, chemokines) that are involved in immune response, inflammation, hematopoiesis and many other homeostatic processes \(^{32}\). Upon stimulation/binding by micro-organisms, exogenous molecules, or endogenous factors including cytokines; macrophages can synthesize *de novo* and release, within
a few hours, a large variety of cytokines, such as IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-α, IFN-γ, MCP-1, MCP-3, MIF, M-CSF et al. Macrophages, central cells of the innate immune system, recognize invasive microbial pathogens and for this reason are of paramount importance in periodontal disease. Monocytic cell lines, such as THP-1 cells, have been shown to produce several cytokines in response to stimulation by LPS, either from E.coli and P.gingivalis. In mammals, DKPs such as Cyclo(Hys-Pro) have been shown to act on glial cells, the resident macrophages of the CNS, to control a conceptually homologous behavioral switch between homeostatic and inflammatory modes, as well as inducing a protective state in neuronal cells, with implications for the control of neurodegenerative disease. Considering this action of DKPs on macrophage cells in the CNS and since Synergistetes were found in close contact with immune system cells in the oral biofilm, we are interested in exploring the effect of these molecules on monocytic cells.

Thus, in this study we are aiming to establish the functional role of these novel QS molecules on activation of bacterial virulence properties and their potential effects on host cells. Firstly, we assessed the role of DKPs on growth of a specific periodontal pathogen, P. gingivalis and on its expression of virulence genes (gingipain). Secondly, we measured the effect of DKPs on the LPS-mediated inflammatory cytokine response in a human monocytic cell line (THP-1).

Materials and Methods

Porphyromonas gingivalis experiments

Periodontal pathogen P. gingivalis A7436 was cultivated in Wilkins-Chalgren (WC) broth (Oxoid LTD, Basingstoke, Hampshire, England) under anaerobic conditions at 37°C. P. gingivalis strain A7436 was incubated with variable concentrations of cyclo(Leu-Pro) and cyclo(Phe-Pro) (Bachem AG, Bubendorf Switzerland) for 26 hours (late exponential phase). The
DKP concentrations were 1000 ng/ml, 100 ng/ml and 10 ng/ml for both cyclopeptides. Cyclo(Leu-Pro) and cyclo(Phe-Pro) were initially dissolved in methanol (Fisher Scientific, Fair Lawn, New Jersey) (50 mg/ml in MeOH), following the company instructions. A final concentration of 0.002% MeOH was reached for each cyclopeptide. *Porphyromonas gingivalis* not treated with cyclopeptides and treated with the vehicle alone (0.002% MeOH) were used as negative controls. The OD was assessed with the use of a spectrophotometer equipped with cuvette port reader (Spectra Max M2, Molecular Devices, Sunnyvale, CA), at a wavelength (λ) of 660nm at different time points (0, 16, 23, 26 hours) until the stationary phase was reached. During the early stationary phase 1 ml of supernatant was collected from each sample and was stored at -80°C in order to evaluate gingipain activity.

The activity of the two cyclopeptides against on bacterial gingipain production was evaluated by a Trypsin colorimetric assay (Abcam, ab102531, Cambridge, MA, USA) using a synthetic substrate and the supernatant collected from the samples. In Abcam’s Trypsin Activity Assay Kit (Colorimetric), trypsin cleaves a substrate to generate p-nitroaniline (p-NA) which is detected at λ=405 nm. The substrates were dispensed into the wells of a 96-well microtiter plate. Fifty microliters of bacterial supernatant from each sample were added to the substrate and incubated at 25ºC for 4 hours. Using the 2mM p-NA standard, a standard curve dilution was prepared. Adsorption at a wavelength of 405 nm was determined by the microtiter plate reader (Spectra Max M2) at 0, and 4 hours. Background was corrected by subtracting the value derived from the zero trypsin standard from all sample readings. The concentration of p-NA in nmol was calculated comparing the 405 nm readings for each sample to the standard curve.
**THP-1 Experiment**

Human monocytic cells, THP-1[THP-1] (ATTC® TIB-202tm) were cultivated in RPMI-1640 (ATTC® 30-2001tm) with 10% fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA), and 0.05mM Beta Mercaptoethanol (Sigma-Aldrich) at 5% CO2 and 37°C. 500,000 cells were plated in each well, and were treated for 24 hours with a combination of different concentrations of LPS (0, 300 and 3000 ng/mL) from *E. coli* 0111:B4 (cat # L2630, Sigma-Aldrich Co) and cyclo(Leu-Pro) (0, 10, 100 ng/ml) (Bachem AG, Bubendorf, Switzerland). Cells were collected and counted with an automated cell counter (Cellometer™ AutoT4, Nexcelom Bioscience LLC, Lawrence, MA, USA) and cell lysate was evaluated for IL-1β mRNA expression, with results normalized to housekeeping GAPDH.

**RNA isolation and quality control**

Cells were lysed with RIPA buffer (R0278 from Sigma-Aldrich) and homegenized with a QIAshredder homogenizer (Qiagen, Valencia, CA, USA). RNA was isolated from cell lysate using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technology, Montchanin, DE, USA).

**Quantitative Real-time PCR and IL-1β expression analysis**

For each sample, a volume of 500 ng of RNA in a total volume of 20ul was used to generate complementary DNA (cDNA) through reverse transcription reactions using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, now ThermoFisher Scientific USA). Quantitative real-time PCR (qRT-PCR) was performed with 1.25µL of synthesized cDNA, 7.5µL of Taqman® Universal PCR Master Mix, 0.75µL of Taqman® Gene Expression Assay Mix for IL-1β gene and 5.5µL of dH2O using StepOnePlus Real Time PCR system.
(Applied Biosystems). The mRNA expression levels were normalized using GAPDH as the housekeeping gene.

**Statistics**

The data analysis for this paper was generated using SAS 9.4 software. Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. Statistical significance was set at $p<0.05$. Mean and standard errors were determined to describe the data. ANOVA and ANCOVA modeling were performed using SAS Proc GLM to calculate p-values. ANCOVA was used to assess *P. gingivalis* growth curves at different time points as well as trypsin-like activity. ANOVA was utilized to evaluate THP-1 cell viability and number of cells/ml, after logarithmic transformation, since the baseline number of cells (500000 cells per well) was equal in each group. ANOVA was used to study IL-β mRNA fold change (IL-β/GAPDH).

**Results**

*Porphyromonas gingivalis* growth curves

Our results demonstrate that both DKPs can minimally decrease the growth of *P. gingivalis*, with cyclo(Leu-Pro) having a slightly higher inhibitory effect when compared to cyclo(Phe-Pro) (Figures 1-2). The difference, in growth of *P. gingivalis*, between the vehicle and cyclo(Leu-Pro) at 1000 ng/ml and 10 ng/ml was statistically significant at 26 hours, with higher inhibition in the test groups (ANCOVA testing each timepoint adjusting for baseline and concentration, $p\leq0.05$). The biological significance of this finding could be limited. The presence at the experimental concentrations of both DKPs seemed not to have a significant effect on the growth of *P. gingivalis*. 
Gingipain activity

Trypsin-like activity of *P. gingivalis* strain A7436 treated with differing concentrations of DKPs, cyclo(Leu-Pro) and cyclo(Phe-Pro), after 4 hours of treatment is shown in Figure 3. The two cyclopeptides seemed not to enhance or inhibit the virulence factor production compared to the control (*P. gingivalis* alone). The vehicle showed higher but not statistically significant inhibition of this enzymatic activity (ANCOVA $p \leq 0.05$).

THP-1 cells viability and number

Our results demonstrated that Cyclo(Leu-Pro) had no statistically significant effect on cell viability at any of the concentrations tested both alone or upon LPS treatment (ANOVA $p \leq 0.05$), in fact the viability was between 81-97% for all the groups (Figure 4). Our results also showed that cyclo(Leu-Pro) alone did not significantly alter the number of cells ($p=0.2$) if compared to baseline (500000 cells per well), but it seemed to inhibit cell replication at higher doses. LPS significantly altered the cell number at 3000ng/mL when combined with DKP, as expected ($p=0.09$ and $p=0.018$, general linear models) (Figure 5). There was a non-significant trend for slightly lower cell numbers when DKPs were present, but it was not a large effect.

IL-1β change

Using real time PCR we observed that the IL-1β mRNA expression indicates that DKP at 10 ng/ml and 100 ng/ml inhibited the inflammatory response of THP-1 cells upon LPS [300 ng/ml] treatment (Figure 6) (ANOVA $p \leq 0.05$). When LPS was absent THP-1 cells, as expected, did not show a high IL-1β mRNA expression, but also in this case cyclo(Leu-Pro) showed to have an inhibitory effect (not statistically significant).
Discussion

Biofilms are structured microbial communities, where microorganisms are embedded in extracellular matrix, formed when bacteria adhere to inert or living surfaces. They are found in several chronic diseases such as periodontal disease\textsuperscript{37}. Within the biofilm, bacteria are protected against toxic agents and shear forces and can survive and grow in a hostile environment; they also can produce pathogenic factors. It has also been reported that microorganisms that grow in a biofilm are much more resistant to antibiotics than planktonic cells\textsuperscript{22}. Within, and in order to build this organized structure, bacteria communicate with each other, through a phenomenon known as QS, that is based on small, water-soluble molecules, which act as auto-inducers\textsuperscript{22,38}. A number of reports suggested that DKPs could comprise a novel family of signaling compounds, but some of them were conflicting. Their potential to act as auto-inducer antagonists, preventing bacterial biofilm formation has been described. However, to date the precise role played by DKPs in bacterial cell-to-cell communication still remains not fully established and speculative\textsuperscript{38-40}. Certain 2,5-diketopiperazines have been isolated from bacteria and reported to activate or inhibit LuxR-type proteins in AHL biosensor strains, albeit at significantly higher concentrations (up to $10^6$ times higher) than native lactones\textsuperscript{14,25-27,39,40}. Holden \textit{et al.} in 1999 showed that several Gram-negative bacteria produced and secreted cyclic dipeptides with the ability to activate and/or antagonize different LuxR-based QS systems. They reported that cyclo(L-Pro-L-Val) could activate violacein production in the mutant strain of \textit{Chromobacterium violaceum} and cyclo(Phe-Pro) could induce bioluminescence in \textit{E.coli}\textsuperscript{25}. Degrassi \textit{et al.} in 2002 reported that \textit{Pseudomonas putida WCS358} could produce and secrete four cyclic dipeptides, and some of these cyclic dipeptides interacted with the QS LuxI and LuxR homologues\textsuperscript{26}. Park \textit{et al.} in 2006 showed that cyclo(Phe-Pro) produced by \textit{V. cholera} inhibited the production of the virulence
factors, cholera toxin and toxin-coregulated pilus, by activating the expression of a LysR-family regulator, and thus down-regulating the expression of ToxRregulon. Also Bina et al. in 2013 confirmed these results. Moreover, Li et al. in 2011 showed that the human vaginal isolate Lactobacillus reuteri RC-14 produces cyclo(Phe-Pro) and cyclo(Tyr-Pro) that are able to interfere with the staphylococcal agr QS system, a key regulator of virulence genes, and repress the expression of toxic shock syndrome toxin-1 in Staphylococcus aureus MN8, a prototype of toxic shock syndrome S. aureus strains. These reports have prompted the proposal that DKPs represent a new class of QS signals and potentially even interspecies or interkingdom signals.

Campbell et al. in 2009 described and screened a library of synthetic DKPs, along with several previously reported natural DKPs, to determine the structural features necessary for LuxR-type protein activation and inhibition and probe their mechanisms of action. These DKPs, using lower concentrations than previous studies, were screened in bacterial reporter gene assays. In contrast to earlier reports, the native DKPs failed to exhibit either antagonistic or agonistic activities in these assays testing the same biosensor strains. However, non-natural halogenated cyclo(L-Pro-L-Phe) derivatives were capable of inhibiting luminescence in Vibrio fischeri. Interestingly, additional experiments revealed that these DKPs do not compete with the natural lactone signal, N-(3-oxo-hexanoyl)-L-homoserine lactone, to inhibit luminescence. Together, their data suggested that DKPs are not QS signals within the bacteria examined in their study. The authors concluded that although these compounds can influence QS-regulated outcomes, it seems that they do not do so through direct interaction with LuxR-type proteins and therefore their mechanisms of action and physiological relevance remain unknown.

Other studies on food microbiology seem to support the possible role of DKPs as QS molecules in food spoilage. Gu et al. in 2013 isolated and characterized four DKPs, that
function as QS signal molecules, from the extracellular metabolites of *Shewanella baltica*, the specific spoilage organism of *Pseudosciaena crocea* during 4 °C storage. By supplementation of four synthesized DKPs, the spoilage capability of *Shewanella baltica* was significantly enhanced.

Kim et al. in 2013 studied the effect of cyclo(L-Phe-L-Pro) on the expression of the total mRNA in *V. vulnificus*, using next-generation sequencing. They reported that the genes most highly induced by cyclo(L-Phe-L-Pro) comprised those associated with the transport and metabolism of inorganic molecules, particularly iron. The genes negatively regulated included those associated with energy production and conversion, as well as carbohydrate metabolism. Noticeably, numerous genes related with biofilm formation were modulated by cyclo(L-Phe-L-Pro). In our study we did not find an effect of cyclo(Phe-Pro) and cyclo(Leu-Pro) on the production of the virulence factor gingipain by *P. gingivalis*. Nevertheless, these molecules could be implicated in the enhancement or inhibition of other virulence factors produced by *P. gingivalis* or other periodontopathogens and this is worthy of further investigation.

We also showed minimal influence on *P. gingivalis* growth, but in the literature antibacterial action for DKPs containing of leucine-proline or phenylalanine-proline, considering the different stereoisomers, has been described. Organic extracts from cultures of the marine bacterium *Bacillus pumilis* furnished inhibitory fractions against *Mycobacterium marinum*, a genetically similar experimental model for *M. tuberculosis*. Among the active compounds isolated and identified was the diketopiperazine of leucine and proline. Cultures of two marine bacterial strains led to the first reported isolation, as natural products, of a series of DD-DKPs containing proline, such as cyclo (D-Pro-D-Leu), and established them as potent inhibitors of the pathogenic marine bacterium *Vibrio anguillarum*. Cyclo(L-Leu-L-Pro), also called gancidin
W, isolated from two *Streptomyces* species, was shown to inhibit the growth of vancomycin-resistant enterococci (VRE)-resistant *Enterococcus faecalis* strains and to have cytostatic activity against leukemic cell lines at the studied concentrations \(^{49,50}\). Cyclo(L-Leu-L-Pro), cyclo(L-Phe-L-Pro), cyclo(L-Leu-L-trans-4-OH-Pro), and cyclo(L-Phe-L-trans-4-OH-Pro) retrieved from the fermentation broth of an *Aspergillus fumigatus* isolate from soil were described as weakly antibacterial inhibiting the growth of *Staphylococcus aureus* and *Micrococcus luteus* \(^{51}\). Cyclo(L-Phe-L-Pro), from a *Psychrobacter* species showed a protective effect against *Vibrio vulnificus* and induced cytotoxicity in human intestinal epithelial cells \(^{52}\). Brack *et al.* in 2014 showed that several DKPs produced by *Bacillus pumilus* induced growth inhibition of pregrown bacteria, including gram-positive *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis*, and *A. citreus*. They also showed that the soil bacteria *Bacillus pumilis* produces cyclo(Leu-Pro) and cyclo(Phe-Pro) during bacteriolysis of *Arthrobacter citreus* \(^{53}\). One of the limitations in considering these studies for comparison is that different stereoisomers could have distinct activities, indeed some studies revealed that at least one D-amino acid was required for antibacterial activity \(^{54}\).

Future investigations could include studying the combination of the two DKPs on *P. gingivalis* growth. Indeed, Rhee in 2004 showed the combined synergistic effects of cyclo(L-leucyl-L-prolyl) and cyclo(L-phenylalanyl-L-prolyl) in inhibiting the growth of VRE and pathogenic yeasts (*Enterococcus faecium, Enterococcus faecalis* strains, *Escherichia coli, Staphylococcus aureus, Micrococcus luteus, Candida albicans* and *Cryptococcus neoformans*) \(^{55}\). Also Kumar *et al.* in 2012 described the synergistic inhibitory effects of DKPs cyclo-(L-Pro-L-Leu), cyclo-(D-Pro-L-Leu), and cyclo-(D-Pro-L-Tyr) in the growth of *Bacillus subtilis* MTCC 2756, *Staphylococcus aureus* MTCC 902, *Escherichia coli* MTCC 2622 and *Pseudomonas*
*aeruginosa* MTCC 2642. It is noteworthy that Gowrishankar et al. in 2014 reported the inhibitory efficacy of cyclo(L-leucyl-L-prolyl) from mangrove rhizosphere bacterium, *Bacillus amyloliquefaciens* (MMS-50) toward cariogenic properties of *Streptococcus mutans* in vitro. The same group reported on the combination of these and other DKPs with conventional antibiotics showing promising results.

In this investigation cyclo(Leu-Pro) did not show to be toxic to monocytic THP-1 cells, indeed it did not affect cell viability (Figure 4) but it demonstrated at higher concentrations to inhibit cell replication (Figure 5). On the contrary, two DKPs from marine fungi, from mud origin, Prenylcyclotryprostatin B and 9-hydroxyfumitremorgin C, isolated from *Aspergillus fumigatus*, were shown to be moderate inhibitors of human leukemic monocyte lymphoma (U937) cells.

We also confirmed that cyclo(Leu-Pro) inhibited monocytic THP-1 expression of IL-1β upon LPS stimulation. This preliminary result suggests that DKPs expressed by bacteria could be inhibiting a host response in order to survive. In support of these results Bellezza et al. in 2014 showed that cyclo(Hys-Pro) in the CNS, by acting on the glial cells (macrophage of the CNS), intervenes in the crosstalk between nuclear factor-like 2 (Nrf2) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling, enhancing antioxidant protection while depressing the proinflammatory response, thus resulting in neuronal protection. Conversely some studies have indicated that linear peptides stimulate production of cytokines in human monocytes, T cells, and rat spleen cells. Chen et al. in 2012 studied the effect of 5 natural DKPs, firstly isolated from the sponge *Callyspongia sp.*, on the release of four cytokines (interferon-γ (IFN-γ), pro-inflammatory (TNF-α), anti-inflammatory cytokine (interleukin-10, IL-10), and chemokine (monocyte chemoattractant protein-1, (MCP-1)) in murine macrophage-
like cell line J774A.1 after stimulation in vitro. Results suggested that these five DKPs, especially DKP 1 bearing 3-hydroxyl-L-proline (L-Hyp), might be useful as a mild macrophage cytokines stimulator. Kim et al. in 2015 showed that cyclo(Phe-Pro) suppressed the production of proinflammatory cytokines (TNF-α, IL-6, or IL-1β), nitric oxide, and reactive oxygen species in a LPS-stimulated monocyte/macrophage cell line and in bone marrow-derived macrophages. Specifically, cyclo(Phe-Pro) inhibited inhibitory kB (IkB) kinase (IKK) phosphorylation, IkBα degradation, and NF-kB translocation to the cell nucleus, indicating that this cyclopeptide affects the NF-kB pathway. Cyclo(Phe-Pro) did not show a significant level of cytotoxicity in the range of 0 to 10 mM.

An alternate hypothesis that could explain the association found between Synergistetes and cyclo(Leu-Pro) and cyclo(Phe-Pro) could be that these bacteria are the target of these molecules. Cyclopeptides may also cause damage to the host cells directly and this one could be another mechanism used by pathogenic bacteria. Indeed, gene expression analysis of mammalian cells (INT-407 cell, U2OS cells and Huh7) treated with cyclo(Phe-Pro) revealed that a subset of genes related to regulation of reactive oxygen species (ROS) scavenging and production is suppressed by the treatment with DKPs. It was found that cyclo(Phe-Pro) induces perturbation of the mitochondrial membrane, resulting in increased ROS, especially superoxide, production. This finding may help explain some of the roles these cyclopeptides have in the deep periodontal pocket.

This study has several limitations such as the high non-physiological concentrations, of cyclopeptides that were used as well as the effect on gingipain showed by the vehicle alone. Future directions could be to use lower concentrations of these cyclopeptides and to assess their effect on other periodontal bacteria and the expression of other inflammatory-related cytokines.
If DKPs will be shown to have a role in the oral biofilm and to enhance its formation and the production of virulence factors from the periodontal pathogens, they could represent a suitable target for new drugs that could result in a shift in the biofilm composition to create a more favorable, steady-state healthy environment.

In conclusion, we have confirmed that both DKPs, cyclo(Leu-Pro) and cyclo(Phe-Pro) have minimal effects on the growth of *P. gingivalis* and in the production of gingipain during early stationary phase. DKP cyclo(Leu-Pro) does not appear to be toxic to THP-1 cells upon LPS challenge but seems to inhibit cell replication in 24 hours treatment. Cyclo(Leu-Pro) showed inhibition of the expression of IL-1β, possibly suggesting that DKPs expressed by bacteria could be inhibiting a host response in order to survive. Further studies are needed to elucidate the role of these molecules in the oral biofilm and their association with *Synergistetes*.

If a role in QS is eventually confirmed, drugs directed toward these molecules could play a major role in controlling microbial gene expression related to human infection, possibly leading to prevention of periodontal infection and biofilm formation.
Cyclo(Phe-Pro) seems not to inhibit the growth of *Porphyromonas gingivalis* (Pg) compared to the vehicle alone. No statistically significant difference was found among the three different concentrations of cyclo(Phe-Pro) (ANCOVA, $p \leq 0.05$).
Cyclo(Leu-Pro) seems not to inhibit the growth of *Porphyromonas gingivalis* compared to the vehicle alone. No statistically significant difference was found among the three different concentrations of cyclo(Leu-Pro) (ANCOVA, \(p \leq 0.05\)).
Figure 3. Trypsin-like activity of *Porphyromonas gingivalis* A7436 treated with differing concentrations of diketopiperazines [cyclo(Leu-Pro) and cyclo(Phe-Pro)] after 4 hours of treatment.

The two cyclopeptides seemed not to enhance or inhibit this enzymatic activity if compared to the control (*P. gingivalis* alone). The vehicle showed higher but not statistically significant inhibition (ANCOVA, \( p \leq 0.05 \)).

Figure 4. Monocytic cell viability (%) upon LPS and DKP cyclo(Leu-Pro) treatments.

Cyclo(Leu-Pro) had no statistically significant effect on cell viability at any of the concentrations tested both alone or upon LPS treatment (ANOVA, \( p \leq 0.05 \)).
Figure 5. Monocytic cell growth/survival upon LPS and DKP cyclo (leu-pro) treatments.

Cyclo(Leu-Pro) had no statistically significant effect on cell numbers (p=0.2), but LPS treatment with cyclo(Leu-Pro) significantly diminished the cell number (p=0.01) (ANOVA, p≤0.05*). The yellow dashed line indicates the baseline number of cells (500000 cells/well).

Figure 6. Monocytic cell inflammatory response upon LPS and DKP cyclo (leu-pro) treatments.

Cyclo(Leu-Pro) greatly reduced the mRNA expression of IL-1β induced by LPS treatment of THP-1 cells (ANOVA, p≤0.05).
REFERENCES


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