Polyphosphate-accumulating Bacteria: Potential Contributors to Mineral Dissolution in the Oral Cavity

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Abstract

The role of oral bacteria in the dissolution of dental enamel and dentin that can result in carious lesions has long been solely ascribed to metabolic acid production. However, other microbial processes may also influence tooth dissolution. Recently, bacteria that accumulate polyphosphate in marine sediments have been shown to dynamically influence the solubility of phosphatic minerals. Here we show, using microscopy and genomic databases, that dental plaque and caries lesions, all contain abundant polyphosphate-accumulating bacteria.

Using a culture of the model organism, *Lactobacillus rhamnosus,* a known polyphosphate-accumulating bacteria that is known to inhabit advanced caries lesions, we show that polyphosphate accumulation can lead to undersaturated conditions with respect to hydroxyapatite under some, but not all, oral cavity conditions. Samples of *L. rhamnosus* grown in various environmental conditions, including exposure to changing oxygenation conditions, input/removal of organics and trace nutrients, were collected over a course of 24 hours and stained with 4',6-diamidino-2-phenylindole (DAPI) to confirm/deny the presence of poly-p in the cells. A comparison of changes in extracellular inorganic phosphate between cultures grown under conditions that result in polyP accumulation vs conditions that did not, was used a a means of measuring the phosphate fluctuation that was likely contributed by intracellular phosphate accumulation.

We suggest, through an extrapolation from our model organism results, that polyphosphate-accumulating bacteria, which we observed to be ubiquitous in oral fluids,

have a similar influence on the solubility of minerals that comprise the tooth structure. These results suggest that the generation of undersaturated conditions by polyphosphate-accumulating bacteria constitutes a new potential mechanism of tooth dissolution that may augment the effects of metabolic acid production.

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Introduction:

Dental caries is becoming an increasingly serious public health issue among the general public and a challenging endeavor within the dental community[1]. Early childhood caries, in particular, can have negative impacts on overall quality of life and oral health[2,3]. A complete mechanistic understanding of dental decay remains elusive due to the complexity and diversity of the microbial communities that populate oral biofilms and the dynamic microenvironmental conditions of the oral cavity[4–7]. It has long been believed that the colonization and proliferation of aciduric/acidogenic bacteria in dental plaque is the primary etiology for the initiation and development of carious lesions[5,6,8,9]. However, the localized ionic saturation state of oral fluids with respect to the thermodynamic solubility product of dental mineral phases: principally controlled by the concentration of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions, also influences mineral solubility and the likelihood of enamel dissolution at the tooth/plaque interface or within existing caries lesions[10,11]. Ion exchange between salivary fluids and the tooth surface has widely been accepted as a "chemical" pathway by which Ca^{2+} and PO_4^{3-} is modulated by human-induced, dietary, and non-microbial factors in the oral cavity[12]. Here, we provide evidence in support of the novel hypothesis that certain oral bacteria may play a considerable role in dynamically modulating the ion concentrations of PO_4^{3-}, and thus the saturation state/solubility of calcium phosphate minerals at the tooth/plaque interface, through intracellular polyphosphate (polyP) accumulation. The capacity of certain oral bacteria to accumulate substantial amounts of polyP, while maintaining an etiological relevance in carious environments (i.e. tolerating low pH), may affect the saturation state

of the fluids in contact with the tooth interface and result in dissolution and perhaps later remineralization of apatite group minerals.

Polyphosphate-accumulating bacteria (PAB) are microorganisms that, under specific environmental conditions, accumulate substantial intracellular inclusions of polyphosphate. Polyphosphates are linear polymers of orthophosphate residues linked by high energy phosphoanyhydride bonds. Polyphosphate has long been known to be associated with the ability of certain microbes to resist physical and chemical stressors, as well as provide an alternative source of energy under unfavorable or variable environmental conditions[13–16]. The metabolic processes of PAB have been extensively investigated in environmental systems such as enhanced-removal of phosphorus from wastewater and marine calcium phosphate mineral deposits that are thought to be mediated by polyphosphate-accumulating bacteria[17–20]. It has been demonstrated that PAB are capable of modulating the ionic constituents in equilibrium with apatite-group minerals in pore waters and subsequently altering the saturation state of the surrounding fluids resulting in microenvironments that are thermodynamically favorable for mineral precipitation[19,21–24]. The study of metabolic processes of PAB in these systems have achieved a new paradigm in our understanding of the modulation of PO_4^{3-} and Ca^{2+} activities and their relationship to the solubility of calcium phosphate minerals - but until now, these findings have not been applied to the oral environment where biofilms of bacteria also form biofilms in plaque and saliva that undergoes chemical exchange with the calcium phosphate minerals that comprise the inorganic portion of the vertebrate tooth.

Unrecognized factors such as dietary nutrient sources, continued organic acid exposure, and oxygenation conditions may initiate bacterial polyP uptake and reduce (or increase) localized concentrations of Ca^{2+} and PO_4^{3-}. The uptake and subsequent release of inclusions of polyP may significantly alter the saturation chemistry of the fluids surrounding the tooth surface by shifting the chemical equilibrium and result in dissolution of apatite-group minerals in the form of dental caries or perhaps the precipitation of apatite-group minerals as calculus (mineralized plaque).

The oral microbiome is a dynamic and diverse community that develops under a wide variety of environmental conditions. Although oral biofilm research has spanned over a hundred years, little work has been done to investigate the role microbes may play as dynamic mediators of ion concentrations. The current and widely accepted model of the caries process is based on the ability of cariogenic plaque microbiota to establish and thrive in low pH environments in which the metabolic production of mixed acids contributes to enamel demineralization[5,6,8,9]. Although localized acid production in cariogenic biofilms undoubtedly impacts mineral solubility, biological influence on chemical saturation of Ca^{2+} and PO_4^{3-} may present an additional component to the development and rapid progression of carious lesions. In order to address several different facets of the hypothesis that PAB may be affecting localized chemical saturation in the oral cavity, we employed the analysis of genomic databases, the direct clinical examination of plaque, saliva, and dentinal lesions, and we developed a defined *in vitro* single-species model of *L. rhamnosus* to assess and examine polyphosphate metabolisms

under constrained environmental conditions and ascertain how these metabolisms might contribute to PO_4^{3-} modulation and mineral dissolution in the oral cavity.

Materials & Methods:

Genomic Identification of Candidate Isolates:

Candidate isolates of oral bacteria that are known polyphosphate accumulators and/or possess the genetic potential to accumulate polyphosphate intracellularly (e.g., ppk1, ppk2, ppx) are listed in Table 1. These organisms were identified by searching the literature and the metadata of genomic databases for oral isolates to include DOE's Integrated Microbial Genome (IMG/M)[25] and the Human Oral Microbiome Database (HOMD)[26]. Our gene search focused on genomes publically available in IMG/M. Annotated genes and gene motifs (pfam, COG, KOG) were detected using the "all functions" search and when available, peer-reviewed studies were utilized in the interpretation of genetic potential. For a broader perspective of the clades found commonly in the oral microbiome, we include analyses of all genomes available within these clades. *Lactobacillus rhamnosus*, an organism that is known to accumulate polyphosphate in environments outside the oral cavity, and is also often associated with the progression of dental caries[27,28], was selected to be employed in a single species model in order to understand how polyP metabolism may relate to its development and survival under carious conditions as well as its potential contribution to altering mineral saturation conditions with respect to hydroxyapatite in dental enamel and dentin.

In Vitro Culturing of Lactobacillus rhamnosus:

Lactobacillus rhamnosus ATCC 7469 DSM 20021, obtained from the USDA Agricultural Research Station Culture Collection was used in our single species model to assess polyphosphate metabolic potential in a caries-associated organism. Specifically, growing *L. rhamnosus* under conditions that allow it to accumulate polyphosphate vs. those that do not, allowed us to quantify PO_4^{3-} uptake specific to polyphosphate that we can then use to make theoretical predictions about the possible magnitude of saturation state changes that fluctuations in intracellular polyP accumulation may have on oral saturation state chemistry. Two semi-defined mediums, designed to mimic a commercial *Lactobacillus* growth medium (BD Difco Lactobacilli MRS Broth), were developed containing the following (g/L): 20 D-(+)-Glucose Monohydrate, 99%; 10 peptone type 1; 5 yeast extract; 5 sodium acetate; 0.1 $MgSO_4$; 2 K_2HPO_4; 0.05 $MnSO_4 \bullet H_2O$; 1mL Tween 80. The second semi-defined medium, designated "Mn-" contained the same chemical proportions of the medium described above ("standard Mn+") , with the exception of $MnSO_4 \bullet H_2O$, which was removed from the medium all together. Triplicate cultures of *L. rhamnosus* (starting inoculum adjusted to 0.2 OD) were cultivated in an aerobic environment for 24 hours at 37°C with orbital shaking at 90 rpm. One milliliter of culture was collected from each replicate every three hours, optical density readings were collected and samples were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was transferred to a separate centrifuge tube for further chemical analysis and the cell pellet was resuspended in one milliliter of 50% ethanol for fluorescence microscopic examination after staining for polyphosphate (as described below). After

testing for its suitability to preserve polyphosphate granules (as opposed to other chemical fixatives like 4% paraformaldehyde), ethanol fixation was used to keep cellular structures intact and metabolisms inert. All samples were stored at -20°C until analyses were conducted.

Plaque and Dentin Sampling/Collection:

Plaque samples were collected from male or female children between ages 4-18 years old who satisfied one of the following inclusion criteria: (1) oral health with an absence of dental caries or hardened dental plaque, (2) dental caries or recent history of dental caries, (3) hardened dental plaque with an absence of dental caries. The process of collecting the dental plaque followed Institutional Review Board (IRB) procedures at the University of Minnesota (IRB#1507M75441). Plaque samples consisted of two separate samples collected from the anterior and posterior dentition using a sterile dental scaler. Two samples were taken from 30 patients for a total of 60 samples.

Prior to the appointment, subjects were expected to have fasted one hour prior to sampling as well as refrained from brushing their teeth the morning of sampling. Each sample was placed into separate test tubes containing 1 mL of 50% ethanol. The test tubes were immediately placed into ice for transport and stored at -20° C for future microscopic analysis. Three dentin samples were collected from extracted teeth that were to be discarded as pathologic waste. Once the teeth were extracted, gross debris consisting of heme, remnants of PDL/gingival attachments, and any granulomatous tissue was removed chairside with 2x2 cotton gauze. Dentin was extracted from each of the carious teeth with sterile hand instrumentation under a Biosafety Cabinet Class II/Type

A2. The dentin samples were immediately stained for microscopic visualization and the extracted teeth were placed into individual tubes containing 50% ethanol.

Polyphosphate Identification via Fluorescence Microscopy:

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain used to detect various cellular macromolecules in living or dead cells. While DAPI binds to both polyp and DNA, the corresponding complexes, polyP-DAPI complex and the DNA-DAPI complex have a distinct emission spectrum (461-nm and 525-nm) when excited by 360-nm light. To resolve the poly-P-DAPI complex, custom band-pass filters (Chroma), were employed (DNA-DAPI excitation/emission (nm) 345/455 and polyp-DAPI excitation/emission (nm) 415/550).This emission wavelength shift results in the emission of a distinct yellow color that can be used to differentiate the polyP-DAPI complex from the DNA-DAPI complex[29]. Nine samples of *L. rhamnosus*, collected every three hours over the course of a 24 hour period, as well as 60 plaque and three dentin samples, were collected and fixed for staining. Each ethanol fixed sample was placed in a designated well on a Teflon-printed microscope slide and allowed to air-dry until the cells were adhered to the slide. Eight microliters of 5 µg/mL DAPI was pipetted onto each sample containing well and left to incubate in the dark for 30 minutes in a hybridization chamber. After incubation, the slide was rinsed and air-dried prior to adding Vectashield (H-1000, Vector Laboratories) and a coverslip for microscopic visualization. Microscopic images were taken with an Olympus BX61 fluorescence microscope equipped with an XM10 CCD camera and cellSens Dimensions Imaging Software (Version 1.13).

Confocal Spectral Imaging:

Confocal spectral imaging was employed using a Nikon A1 spectral confocal microscope. The system is mounted on a Nikon Ti2000E inverted fluorescence microscope with DIC optics. NIS elements imaging software was employed for acquisition and analysis. Ethanol-fixed oral biofilm samples were stained with DAPI and analyzed with a standard polyp emission wavelength (433.6 nm/bandwidth 109.7 nm) and DNA emission wavelength (461.0 nm/bandwidth 98.0 nm).

Inorganic Phosphate Quantification:

An inorganic phosphate quantification method from Hansen and Koroleff (1999) was adapted to assess the influence of polyphosphate accumulation on the P_i concentration in the extracellular medium in our model organism, *L. rhamnosus*. In order to quantify intracellular polyphosphate accumulation, the supernatant of our *L. rhamnosus* cultures were analyzed spectrophotometrically. In the presence of inorganic phosphate, two reagents, ascorbic acid and a mixed reagent composed of ammonium heptamolybdate tetrahydrate $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, potassium antimony tartrate $K(SbO)C_4H_4O_6$, and sulfuric acid, yield a phosphomolybdate heteropoly acid complex, resulting in the appearance of a blueish solution. The intensity of the blue complex is directly proportional to the amount of inorganic phosphate present in the sample.

In order to account for the amount of phosphate that would have been utilized from the media for purposes other than polyphosphate accumulation, our semi-defined medium was modified to inhibit cellular polyphosphate accumulation while maintaining similar cell densities to those of the polyphosphate-accumulating culture. Cell counts of the

triplicate replicates were used to normalize the slightly different cell densities between the two culture types when calculating phosphate change/cell.

Geochemical Analysis and Saturation Calculations:

To assess the potential impact cellular polyphosphate accumulation/release has on enamel mineral solubility (i.e. hydroxyapatite), we employed WEB-PHREEQ: Aqueous Geochemical Modeling[30] (version 2) to evaluate chemical saturation state fluctuations in response to increasing and decreasing phosphate concentrations. A range of salivary phosphate and calcium concentrations (mmol/L) reported in the literature[31] were used as a series of arbitrary starting values in determining chemical saturation of hydroxyapatite in response to fluctuating pH and phosphate concentrations. Net polyphosphate accumulation (0.9075 mmol/L), as previously determined from our *L. rhamnosus* model, was subtracted and added to literature reported phosphate values to assess saturation index fluctuations during our maximum observed cellular polyphosphate accumulation and release.

Results:

Genome and literature survey:

Through literature and database reviews we were able to identify candidate PAB that inhabit the dental environment. The main enzymes responsible for synthesizing polyP and subsequently hydrolyzing polyP in bacteria are polyphosphate kinase (ppk1, ppk2) and exopolyphosphatase (ppx) respectively[15,27,32]. We included in our study lesser known and studied genes known for hydrolyzing polyP when supported by published *in situ* studies, (Table 1). In general, the genetic potential to accumulate polyphosphate was

found broadly across the oral microbiome. However, it was notably absent within the *Streptococci* (positive for 27 non-oral isolated out of 2337 genomes) to include the genome of *S. sorbrinus* SL-1 ATCC 33478 which was reported to accumulate polyP[33]. However, other caries associated clades more strongly demonstrated the genetic potential to accumulate polyphosphate, such as *Propionibacterium* (n = 159/162), *Lactobacillus* (n = 491/685), *Rothia* (n=11/11), *Actinomyces* (n=38/38), and *Bifidobacterium* (274/274).

Polyphosphate Visualization via Fluorescence Microscopy:

Our preliminary results demonstrate that plaque (Figure 1a), dentinal lesions (Figure 1c), and our model organism, *L. rhamnosus* (Figure 2), contain abundant intracellular polyphosphate inclusions that can be visualized with DAPI. Figure 1b illustrates our capability to resolve polyP inclusions using spectral-scanning confocal microscopy. Binding of polyP to DAPI, shifts its peak emission wavelength from 475 to 525 nm (excitation at 360 nm), resulting in the DAPI-polyphosphate complex to appear yellow and inclusions can be observed as discrete yellow spheres within the cell (Figure 1b)[34]. We found that the dental plaque samples of all 30 patients (60 samples in total) contained polyphosphate inclusion bodies in a morphologically diverse, and spatially heterogeneous oral biofilms. Staining of dentinal lesions, from three extracted teeth, also revealed abundant polyphosphate inclusion bodies. The bacterial morphotypes in the dental plaque samples include long filamentous organisms as well as small cocci and bacillus shaped microbiota. However, the morphologic diversity of the dentin samples appears to be less than that of the dental plaque, primarily consisting of small cocci and bacillus shaped organisms with a distinct absence of filamentous bacteria.

Polyphosphate Accumulation in Lactobacillus rhamnosus:

Lactobacillus rhamnosus is one of several caries-associated organisms that we have identified to obtain the genes necessary for polyphosphate accumulation and hydrolysis and because of this genetic potential, as well as its relevance in the dental community, has been selected as a model organism to manipulate and observe polyphosphate metabolisms in response to nutritional limitations and dynamic environmental conditions[27]. Using our semi-defined growth media, we cultured *L. rhamnosus* capable of accumulating polyphosphate within a 24 hour period. Polyphosphate accumulation was always observed under high cell density (OD >2). In *Lactobacillus* cultures, as cell density increases, pH decreases, and polyphosphate accumulation was initiated under moderate to low pH conditions (less than 5.5 and greater than 3.5). Through a series of experiments that vary specific nutrients in our semi-defined media, we determined that the relative concentration of manganese (Mn^{2+}) plays an important and complex role in the regulation of bacterial polyP metabolism. Standard MRS Broth concentrations of manganese (0.05 g/l) result in substantial intracellular polyP accumulation, while the depletion and/or omission of manganese yields cells devoid of polyP inclusion bodies. In *L. rhamnosus*, treatments that excluded the 0.05 g/l manganese ("Mn-") reduced polyP accumulation and overall cell density, while maintaining growth rates comparable to that of the "standard Mn+" media, suggesting manganese is essential to initiate an appropriate stress response when environmental conditions become unfavorable

Colorimetric Inorganic Phosphate Quantification:

By developing a media that enables us to grow *L. rhamnosus* under conditions that allow for polyP accumulation versus conditions that do not, we were able to spectrophotometrically quantify the relative concentration of phosphorus from our media that is being incorporated as intracellular polyP inclusions. Using an ascorbic acid assay, modified by Hansen and Koroleff (1999), we compared the supernatant of our *L. rhamnosus* cultures to that of a known set of phosphate standards to quantify the depletion of total inorganic phosphate at various periods of incubation.

Since bacteria use phosphate for a variety of different purposes other than polyphosphate accumulation, it is important to take into consideration the quantity of phosphorus necessary for cell growth. Figure 3 illustrates inorganic phosphate change, over the course of 24 hours, between our "Mn-" Mn^{2+} medium (negative polyP accumulation) and "standard Mn+" Mn^{2+} medium (positive polyP accumulation). *L. rhamnosus* grown under conditions that allow for polyP accumulation ("standard Mn+")show a decrease in media PO_4^{3-} concentrations of approximately 1.3 mmol/L after 18 hours incubation. *L. rhamnosus* grown in "Mn-" media, i.e. Mn^{2+} limited conditions, that prevent polyP accumulation, show a decrease of approximately 0.38 mmol/L PO_4^{3-} after 18 hours incubation. We make the assumption that, most, if not all, of the difference in the total PO_4^{3-} concentration results from P_i uptake and storage in the cells as the intracellular polyP granules that we observe by DAPI staining. Since the cell densities between the two mediums were similar, with the "Mn-" culture being slightly lower, the concentration of phosphate was adjusted to reflect equal cell density among the two

mediums, assuming polyphosphate accumulation would be comparable amongst individual cells. Using this assumption, we calculate a maximum net change of phosphate, via polyphosphate accumulation, in *L. rhamnosus* to be approximately 0.9075 mmol/L after 18 hours incubation. If polyP-accumulating bacteria in plaque or dentinal lesions are accumulating similar amounts of polyP as our model organism, *L. rhamnosus*, is capable of accumulating, then it is very likely that oral biofilms are capable of influencing the saturation chemistry of the saliva/mineral interface through the metabolism of polyphosphate. To assess the potential impact polyphosphate accumulation and/or release may have on mineral solubility, we used the geochemical modeling program, WEB-PHREEQ, in order to calculate changes that would result in supersaturated or undersaturated conditions with respect to the apatite group mineral, hydroxyapatite.

Chemical Saturation in Response to Polyphosphate Accumulation and Release:

One of the primary factors in demineralization and remineralization of hydroxyapatite in dental enamel or dentin, is the saturation state of saliva with respect to its primary ionic constituents, calcium and phosphate[35]. Despite the fact that saliva is generally supersaturated with these components[35,36], some individuals experience extensive mineral dissolution while others accumulate dental calculus (mineralized plaque). The saturation of hydroxyapatite is characterized by its solubility product (Ksp) and the concentrations of its components ions (IAP)[37]. The saturation state of the mineral (Ω) is in equilibrium when the quotient of IAP/Ksp=1, meaning that the system has the same rate of dissolution and precipitation. When $\Omega > 1$, the solution is supersaturated and

precipitation is favored. When $\Omega < 1$, the solution is undersaturated and mineral dissolution is favored.

Figure 4 illustrates mineral saturation state vs. pH for three different salivary concentrations of calcium and phosphate reported by Larsen et al. (1999). Using our calculated concentration of accumulated polyphosphate, from our ascorbate assay, we adjusted the salivary phosphate concentrations by subtracting the calculated value of 0.9075 mmol/L phosphate, from three reported literature values ranging from 12.6 to 2 mmol/L phosphate. When salivary calcium and phosphate concentrations are high (i.e. 4.2 and 12.6 mmol/L respectively), the impact on saturation state resulting from our assumed phosphate drawdown of 0.9075 mmol/L is minimal. However, when calcium and phosphate concentrations in the saliva are relatively low (i.e. 1.1 and 2.0 mmol/L respectively), a drawdown of 0.9075 mmol/L phosphate can have a sizable effect on mineral solubility. If this magnitude of change were to occur in saliva at a pH of 5.85 (assuming a stable calcium concentration of 1.0 mmol/L and an initial phosphate concentration of 2.0 mmol/L) then the system would go from saturated with respect to hydroxyapatite ($\Omega=\sim1$) in which neither precipitation nor dissolution are favored, to undersaturated ($\Omega=\sim0.48$), a condition that thermodynamically favors mineral dissolution.

Discussion:

Cariogenic activity of oral biofilms is a dynamic and multi-factorial disease whose etiology is largely based on the ability of the bacterial community to produce acid and survive in decreasing pH conditions[4]. Tooth enamel, primarily composed of hydroxyapatite ($Ca_5(PO_4)_3(OH)$), is highly susceptible to demineralization from prolonged exposure to organic acids, by-products of bacterial carbohydrate fermentation[8]. *Streptococcus mutans* was established early on as a key player in the caries process, and remains a focus of investigation due to its acidogenic and aciduric properties[4,38]. However, recent studies have shown that, although *S. mutans* is an early colonizer of dentinal lesions, *it* is part of a larger consortia of cariogenic bacteria that thrive in low pH conditions as a result of frequent carbohydrate exposure[5,8,9]. Recent developments in community characterization using 16S rRNA gene based amplicon sequencing and metagenomics has made it possible to identify and study the microbiota associated with cariogenic plaque. Along with *S. mutans*, cariogenic plaque is comprised of a community of diverse microbial species including *Rothia, Actinomyces, Bifidobacterium spp., Lactobacilli*, and other non-mutans streptococci[4,5,39–41]. Among the microbiota identified as key players in the development of carious lesions, several clades possess the genetic potential to accumulate polyphosphate. In most of the caries associated clades the capacity to accumulate polyphosphate has been demonstrated. Whether strains of *Streptococci*, in particular *S. mutans*, has the capacity to accumulate polyphosphate by a yet to identified genetic pathway remains to be explored. Regardless, the capacity to accumulate and release polyphosphate appears in many cases to be highly variable within

a clade down to the species level and may contribute to the phenotypic variability within caries microbiomes resulting in the modulation of disease progression.

Our preliminary DAPI staining of 60 plaque, three carious dentin, and 60 saliva samples demonstrates that PAB are ubiquitous in the oral cavity (n=123/123). Even though many of the organisms we observed containing accumulations of polyP in the oral cavity may not be associated with the development and progression of dental caries (i.e. certain plaque and salivary microbiota), the mere presence of heterogeneous dense assemblages of PAB in the oral community conceivably introduces a new paradigm in the realm of dental disease and oral microbial ecology. PAB present in our clinical samples of dentinal lesions provide intriguing evidence that polyphosphate accumulation may play a role in P_i modulation between bacteria and dental enamel. Since we were unable to identify which species of PAB were present within these dentinal lesions, we utilized *in vitro* culturing of *L. rhamnosus* to determine if polyP accumulation may have an effect on the saturation state of the surrounding fluids in an environment where PAB are well established.

Lactobacillus is a major genus of lactic acid bacteria (LAB) that inhabits human mucosal surfaces and lowers environmental pH via production of lactic acid during carbohydrate fermentation[27,42]. *L. rhamnosus* is a gram-positive, facultative anaerobe that produces, as well as thrives in, acidic environments (pH < 5) and also possesses the genetic potential to accumulate and hydrolyze intracellular polyphosphate[27]. Due to its association with the progression of dental caries, as well as its capability of accumulating considerable quantities of intracellular polyP, *L. rhamnosus* is a realistic model organism

for studying polyphosphate metabolic processes potentially associated with fluctuating PO_4^{3-} concentrations in the oral cavity.

Preliminary PO_4^{3-} quantification results demonstrates that *L. rhamnosus* accumulates clinically relevant concentrations of polyphosphate that could result in changes in saturation state to the surrounding oral environment in situations where PO_4^{3-} concentrations are depleted at the tooth/biofilm interface. *L. rhamnosus* grown under conditions that allow for polyP accumulation ("standard Mn+") show a decrease in media PO_4^{3-} concentrations of approximately 1.3 mmol/L after 18 hours incubation. *L. rhamnosus* grown under "Mn-" Mn^{2+} conditions, that prevent polyP accumulation, show a decrease of approximately 0.38 mmol/L PO_4^{3-} after 18 hours incubation. We suspect the difference in the total PO_4^{3-} concentration results from P_i uptake and storage in the cells as the intracellular polyP accumulation that we observe in that culture via DAPI-staining and microscopy. Initially thought to serve as an alternative energy reservoir, the presence of intracellular polyP has been directly linked to physiological processes such as mobility, biofilm development, quorum sensing, and response to various environmental stressors[15,43]. In a scenario where nutrient availability is limited, pH is relatively acidic, and oxygen levels are depleted, bacteria such as *L. rhamnosus* may exist as opportunists that establish themselves in an exclusive ecological niche in which their polyphosphate metabolisms may provide a competitive edge amongst other oral microbiota. Carious lesions that had initially demineralized from exposure to mixed organic acids may become even more susceptible to mineral loss as aciduric/acidogenic PAB, such as *L. rhamnosus*, establish themselves in the vicinity of dissolution. As tooth enamel

demineralizes, PAB may increase dissolution by further disrupting the chemical balance of Ca^{2+} and PO_4^{3-} by accumulating P_i from the dissolved enamel thus creating a run-away demineralization environment (Figure 5).

The sequestration of PO_4^{3-} by PAB has the potential to alter the chemical conditions of the oral environment that promote mineral dissolution under certain conditions in the mouth, leading to dental decay. Alternatively, the concentrated release of PO_4^{3-} from PAB could lead to the precipitation of dental calculus (mineralized dental plaque) under a different set of oral microenvironmental conditions. These ions can also be incorporated into various other phases of apatite such as fluorapatite ($Ca_5(PO_4)_3F$) and carbonate-hydroxyapatite ($Ca_5(PO_4,CO_3)_3(OH)$). These substitutions are common in the oral cavity and vary from individual-to-individual, as well as from tooth-to-tooth. Mineral solubility may increase or decrease depending on the substitutions in the lattice structure[37]. In order to assess the magnitude of the impact PAB metabolisms may have on the saturation chemistry of the saliva/mineral interface, we need to develop a comprehensive understanding of PAB in the oral environment and how this group of bacteria responds to the dynamic ecosystem of the oral cavity. The conditions in which PAB accumulate and hydrolyze polyP in oral biofilms have yet to be identified but are likely associated with the ability of certain microbiota to respond and adapt to nutrient and physio-chemical stressors. Identification and clinical assessments of PAB in oral biofilms will aid us in understanding their potential influence on saturation chemistry and mineral solubility in the oral environment as well as aid in our ability to treat oral diseases that remain poorly understood.

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Figures:

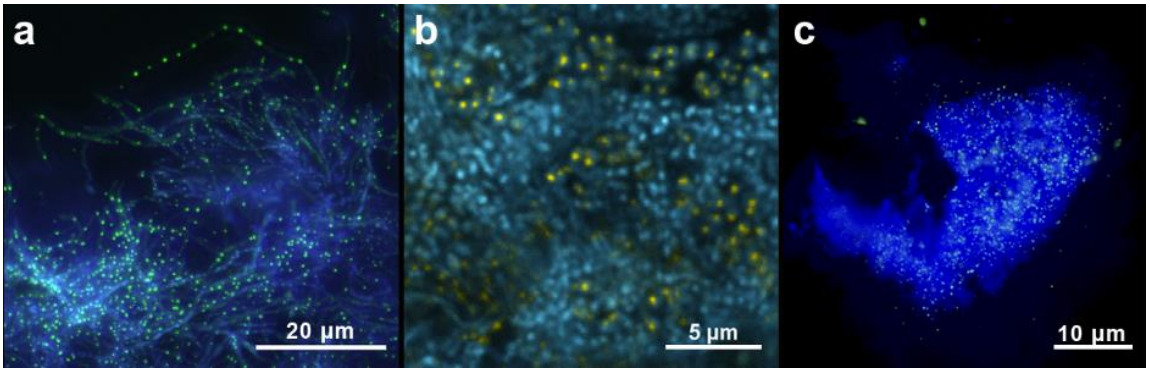

Figure 1: Preliminary staining of clinical samples with DAPI (4',6-diamidino-2-phenylindole). Fluorescence microscopic examination demonstrates that plaque (a,b) and dentinal lesions (c) contain abundant, morphologically diverse, and spatially heterogeneous bacteria that accumulate polyP that can be stained with DAPI (yellow dots).

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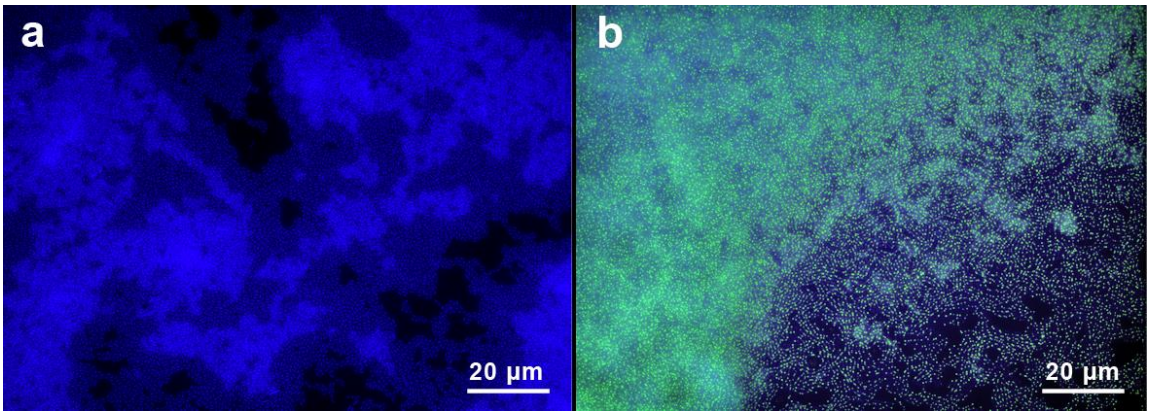

Figure 2: Polyphosphate accumulation in *L. rhamnosus* in response to nutritional limitations. (a) *L. rhamnosus* cultured in "Mn-"growth media for 21 hours exhibited no accumulation of polyP. (b)While *L. rhamnosus* cultured in "standard Mn+" growth media for 21 hours were replete with inclusions of polyphosphate (yellow spheres).

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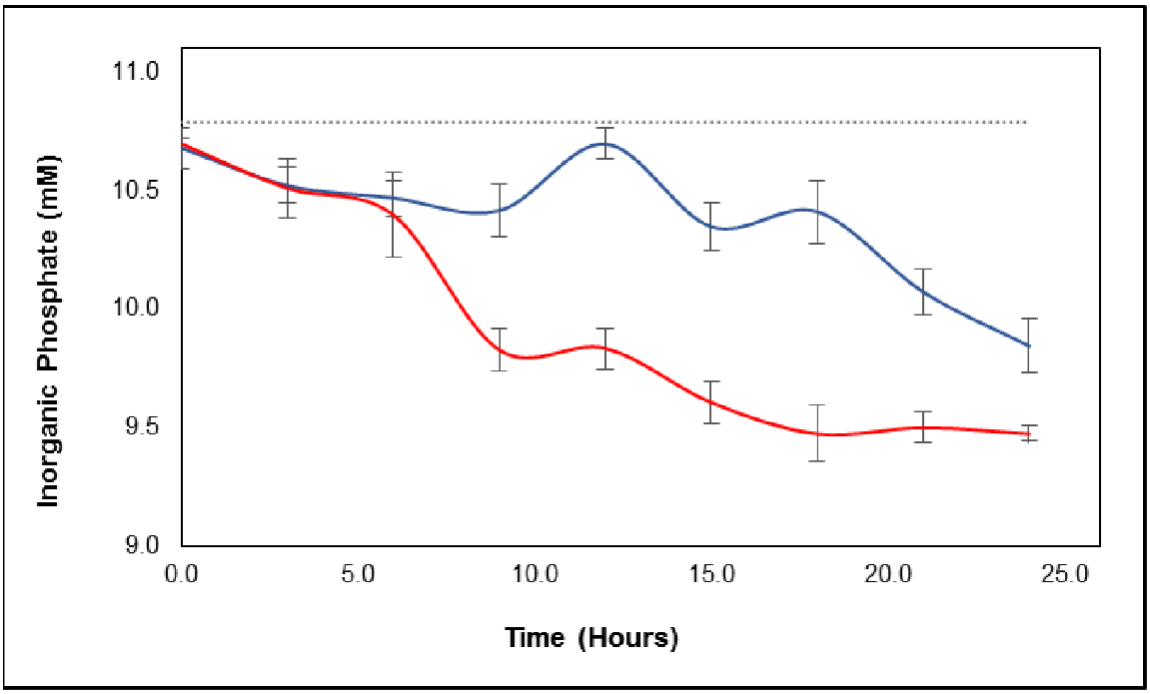

Figure 3: Inorganic Phosphate Quantification using the oral isolate *L. rhamnosus*. Preliminary results growing *L. rhamnosus* under conditions that allow for polyP accumulation (red curve) show a decrease in media P_i of approximately 1.3 mmol/L at 18 hours. L. rhamnosus grown under "Mn-" Mn^{2+} conditions that prevent polyP accumulation (blue curve) represents a decrease in media P_i of approximately 0.4 mmol/L. Initial media P_i concentration (dashed line) is approximately 10.8 mmol/L.

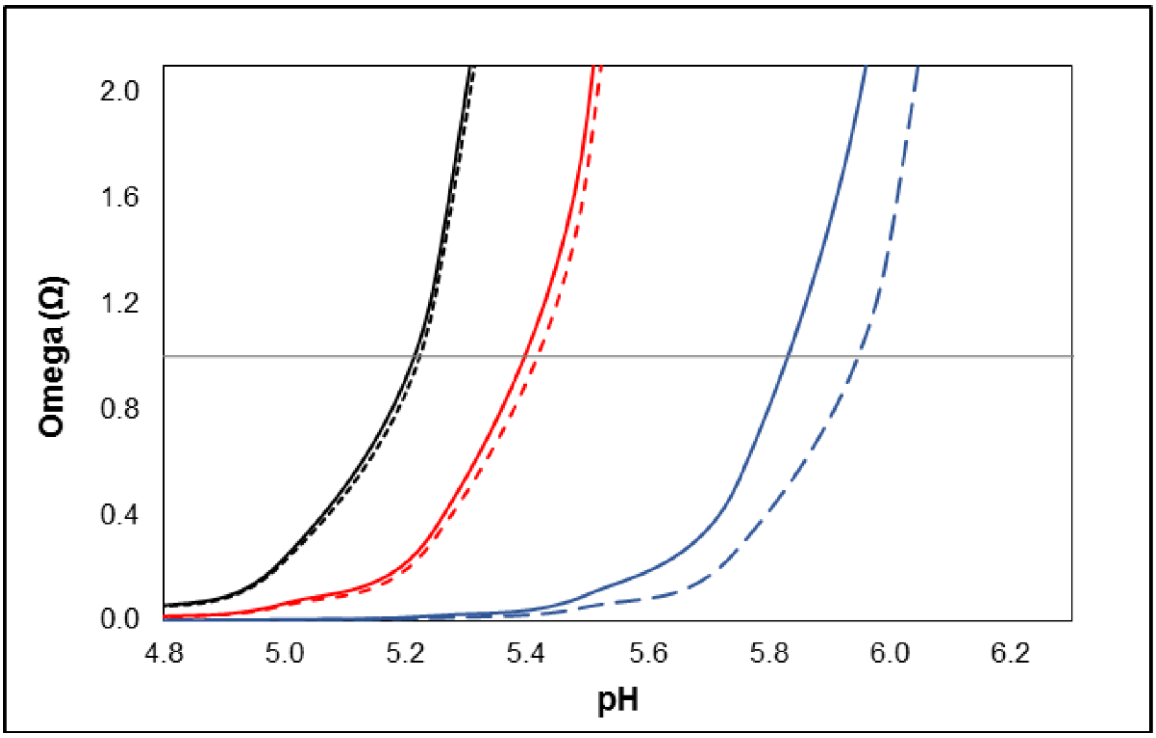

Figure 4: Theoretical shift in relative saturation of salivary fluids in response to bacterial polyphosphate accumulation. Solid lines represent literature derived salivary concentrations of PO_4^{3-} and Ca^{2+}. High PO_4^{3-} and Ca^{2+} values of 12.6 mmol/L and 4.2 mmol/L, respectively (black curve), medium PO_4^{3-} and Ca^{2+} values of 7.3 mmol/L and 2.65 mmol/L respectively (red curve), and low PO_4^{3-} and Ca^{2+} values of 2.0 mmol/L and 1.1 mmol/L respectively (blue curve). Corresponding dashed lines represent a shift in relative saturation in response to polyphosphate accumulation of approximately 0.9075 mmol/L PO_4^{3-}, as determined by our single species growth model of *L. rhamnosus*. Relative saturation equilibrium (Ω=1) is represented by the solid horizontal line.

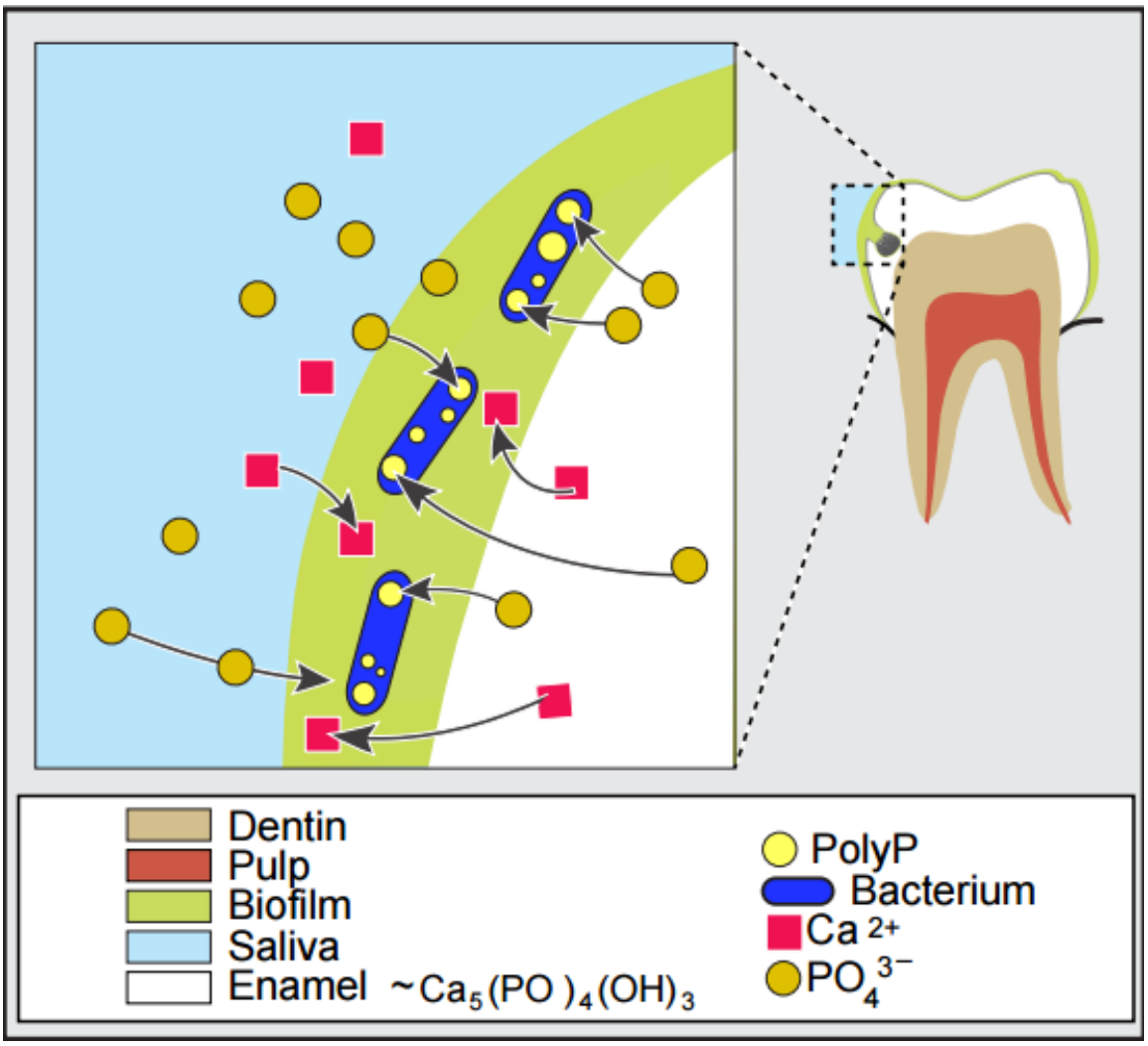

Figure 5: Our results suggest that Polyphosphate accumulating bacteria taking up inorganic phosphate to synthesize poly-P potentially resulting in undersaturated conditions that lead to mineral dissolution and caries progression Polyphosphate-accumulating bacteria may also acquire PO_4^{3-} from acid-induced dissolution of the enamel.

Appendix:

Annotated Genetic Potential: ppk1 + ppx/surE/GppA phosphatase and/or ppk1/2 + ppgk			
Legend: D = Demonstrated in clade (see references), otherwise not determined. + = Potentially positive for polyp accumulation - = Likely negative for polyp accumulation			
Phylum	**Genus**		**Genome Percentage**
Actinobacteria	+	*Actinobaculum*	n=5/7
		Actinomyces	n=38/68 (D)
		Alloscardovia	n=3/3
		Atopobium	n=1/17
		Bifidobacterium	n=274/274 (D)
		Corynebacterium	n=246/246 (D)
		Gardnerella	n=39/39
		Olsenella	n=7/11
		Parascardovia	n=3/3
		Propionibacterium	n= 159/162 (D)
		Rothia	n= 11/11 (D)
		Scardovia	n=2/2
	-	*Aggregatibacter*	n=0/34
Bacteroides	+	*Capnocytophaga*	n=30/30
		Prevotella	n=18/165
		Porphyromonas	n=70/70 (D)
		Tannerella	n=11/13
Betaproteobacteria	+	*Kingella*	n=48/48
		Lautropia	n=1/1
		Neisseria	n= 394/395 (D)
Chloroflexi	+	Unclassified Bacterium	n=6/31
Epsilonproteobacteria	+	*Campylobacter*	n = 510/544 (D)
Firmicutes	+	*Dialister*	n=4/4
		Enterococcus	n=7/801

Firmicutes Ctd.	**+**	*Fructobacillus*	n=1/4
		Johnsonella	n=1/1
		Lachnospiraceae	n=48/77
		Lactobacillus	n=491/685 (D)
		Lysinibacillus	n=43/45
		Megasphaera	n=16/16
		Mogibacterium	n=2/2
		Oribacterium	n=1/13
		Peptoniphilus	n=12/17
		Selenomonas	n=7/36
		Staphylococcus	n=394/5268
		Veillonella	n=23/30
	-	*Abiotrophia*	n=0/1
		Bulleidia	n=0/1
		Centipeda	n=0/1
		Eubacterium	n=0/56
		Filifactor	n=0/1
		Gemella	n=0/8
		Parvimonas	n=0/5
		Peptoanaerobacter	n=0/4
		Shuttleworthia	n=0/2
		Solobacterium	n=0/2
		Streptococcus	n=27/2337
Fusobacteria	**-**	*Fusobacterium*	n=2/88
		Leptotrichia	n=0/14
Gammaproteobacteria	**+**	*Endozoicomonas*	n=8/8
		Enterobacter	n=276/278 (D)
		Klebsiella	n=865/870
	-	*Haemophilus*	n=0/144
Spirochetes	**+**	*Treponema*	n=23/69

Table 1: Genus level associated polyphosphate accumulating genetic potential[32,33,44–52].