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# The role of salivary antimicrobial peptides in shaping Streptococcus mutans ecology

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# THE ROLE OF SALIVARY ANTIMICROBIAL PEPTIDES IN SHAPING *STREPTOCOCCUS MUTANS* ECOLOGY

by

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An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Oral Science
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The University of Iowa

July 2010

Thesis Supervisor: Professor Jeffrey A. Banas

# ABSTRACT

Antimicrobial peptides are among the repertoire of host innate immune defenses. In mucosal immunity, the health-disease balance can be greatly modulated by the interplay between host immune factors and colonized microflora. Microbial ecology within dental plaque is constantly shaped by environmental factors present within the oral cavity. Several antimicrobial peptides are detected in saliva and their bactericidal activities against oral bacteria, including *Streptococcus mutans*, the primary etiologic agent of dental caries, have been clearly demonstrated. However, the role of these antimicrobial peptides in *S .mutans* ecology and host caries experience is not well-defined. We hypothesized that various strains of *S. mutans* possess different inherent susceptibility/resistance profiles to host salivary antimicrobial peptides and that host-specific quantities of these peptides may influence plaque colonization by particular *S. mutans* strains.

*S. mutans* strains from subjects with variable caries experience were tested for susceptibility to a panel of antimicrobial peptides, including HNP-1-3, HBD-2-3 and LL-37, revealing that the susceptibilities of *S. mutans* to these peptides were strain-specific. *S. mutans* strains from high caries subjects showed greater resistance to these peptides at varying concentrations than those from caries-free subjects. In addition, when combinations of these peptides were tested, they showed either additive or synergistic interaction against *S. mutans*.

Determinations of the salivary levels of these peptides showed that their concentrations were highly variable among subjects with no correlation to host caries experience. However, positive relationships between the salivary concentrations of HNP-

1-3 and MS in dental plaque were found. Additionally, the levels of a number of these peptides in saliva appeared to be positively correlated within an individual. An analysis of the salivary peptide concentrations and the susceptibility profiles of *S. mutans* strains showed that *S. mutans* strains obtained from subjects with higher concentrations of HNP-1-3 in saliva appeared to be more resistant to HNP-1.

Collectively, our findings showed that salivary antimicrobial peptides affect *S. mutans* ecology by restricting the overall growth of this bacterium within the oral cavity and that their activity may help select resistant strains of *S. mutans* to colonize within dental plaque. The relative ability of *S. mutans* to resist host salivary antimicrobial peptides may be considered a potential virulence factor for this species.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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To Mom and Dad

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# CHAPTER 1

# INTRODUCTION

## Dental caries

Dental caries is a complex disease process, affecting a large number of populations regardless of age, sex and cultural group. It is the most common chronic disease affecting children and adolescents worldwide[1]. In United States, a large percentage of the population enters late adolescence having previously experienced dental caries. The incidence of dental caries in the primary dentition of children aged 2–4 years has increased from 18.5% in 1988–1994 to 23.7% in 1999–2004. Among children aged 6–8 years, the incidence has increased among non-Hispanic black children from 49.4% to 56.1% and remained above 63% among Mexican American children. In non-Hispanic white children, the prevalence has remained unchanged at about 49%. Dental caries incidence in the permanent dentition has generally declined among adolescents aged 12–19 years but still remains involved in more than half of the population[2]. On the other hand, the frequency of dental caries among elderly people is increasing as more people are retaining their teeth throughout life. Therefore, despite advances in oral health prevention and treatment, dental caries continues to be a major public health problem.

Dental caries is a multifactorial process, resulting from the interplay of three main factors: the causative microorganisms, diet and host factors. The principal microbial cause of dental caries is a group of phenotypically similar bacteria, collectively called mutans streptococci (MS)[3]. In humans, MS consisting of two bacterial species, *Streptococcus mutans* and *Streptococcus sobrinus*, are the most prevalent cariogenic bacteria[4]. A large number of studies support the strong association between MS level and the incidence of carious lesions[5]. The presence of MS, both in dental plaque and saliva of young caries-free children, appears to be associated with an increase in caries risk[6]. Early

acquisition of MS is also a major risk factor of early childhood caries and the development of future caries[7].

Other bacteria also play a role in the dental caries process, though not to the same extent as MS. Lactobacilli are highly acidogenic and acid-tolerant. They however preferentially colonize the dorsum of tongue and do not adhere avidly to teeth[8]. Lactobacilli can be recovered from carious lesions, yet they are considered late colonizers, being favored by the pre-existing colonization of the MS[9,10]. Actinomyces have been shown to induce root surface caries in hamsters and gnotobiotic rats[11]. They are prevalent in the human mouth and are frequently found associated with both carious and sound root surfaces. However, they are not vigorously acidogenic or acid tolerant and their roles in root surface caries are rather variable[5].

*S. mutans* has been the most intensely investigated cariogenic bacterium in the literature, because of its strong correlation with dental caries and the relatively low presence without disease. *S. mutans* strains introduced into oral cavities of either gnotobiotic or specific germ-free animal models vigorously generate dental caries[12-16]. This bacterium, which is considered "a specialist" in causing dental caries, possesses multiple inherent virulence properties essential for its cariogenic potential.

## Virulence properties of *S. mutans*

Acidogenicity, acid tolerance, adhesion and biofilm formation are considered the main virulence properties associated with the cariogenicity of *S. mutans*[17,18]. *S. mutans* is capable of metabolizing a wide variety of sugars to lactic acids. The acids produced not only are responsible for tooth demineralization but also rapidly acidify the dental plaque environment around the bacterium. While *S. mutans* can adapt to the surrounding pH as low as pH 4.4, this vastly acidic condition is toxic to most other bacteria. As a result, this condition allows for the enrichment of this bacterium as well as other acid-tolerant bacteria, such as lactobacilli, which further contribute to the increase in acid production[19].

The adhesion of *S. mutans* to tooth surfaces is mediated by a two-step process, involving sucrose-independent and sucrose-dependent mechanisms. Sucrose-independent adhesion to salivary pellicle is believed to initiate the attachment process, whereas sucrose-dependent adhesion may enhance bacterial colonization to the tooth surface[17,18]. The *S. mutans* adhesin, termed SpaP, antigen I/II, PAc, or P1, is believed to modulate the initial sucrose-independent process and *S. mutans* aggregation via its interaction with salivary agglutinin, a high-molecular weight glycoprotein in human saliva. A *S. mutans* mutant lacking SpaP showed decreased adhesion to saliva-coated hydroxyapatite[20] and was less cariogenic in rats fed a 5% sucrose diet, compared to the wild-type strain[21].

*S. mutans* synthesizes and secretes three glucosyltransferase enzymes, encoded by *gtfB*, *gtfC* and *gtfD*. They function to metabolize sucrose into glucose and fructose and also utilize the glucose molecules to form water-soluble and water-insoluble polymers of glucans, called dextran and mutan, respectively. Dextran, is a primarily linear polymer with α-1,6-glycosidic linkages, whereas mutan is a branching polymer linked by α-1,3-glycosidic linkages. Both of these glucan polymers are believed to facilitate sucrose-dependent adhesion and biofilm formation of *S. mutans* on tooth surfaces via the action of glucan-binding domains of glucosyltransferase enzymes as well as three glucan-binding proteins, GbpA, GbpC and GbpD[17,18]. Water-insoluble glucan, however, may be particularly important in the development of smooth surface caries[22]. Studies of *S. mutans* mutants defective in *gtfB* and *gtfC* genes demonstrated reduced virulence in rats[23,24].

The ability of *S. mutans* to survive and catabolize its glycolytic activity under a very low pH provides this bacterium a distinct ecological advantage over other oral bacteria in the dental plaque community. This property of *S. mutans* is mediated by its acid-tolerance response, involving a $F_1F_0$-ATPase proton pump and changes in gene and protein expression. The membrane-bound ATPase is essential for maintaining a pH gradient across the cytoplasmic membrane at low pH[25-27]. In addition, the numerous changes in gene and protein expression have been observed in response to acid shock. Of

particularly importance is the increased expression of chaperone proteins as well as those involved in DNA repair mechanisms[28-30].

## Acquisition and transmission of mutans streptococci

Bacterial species can be detected in the oral cavities of infants soon after birth. The numbers and arrays of species gradually increase by the continued acquisition from the external environment. *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus oralis* are considered the pioneer viridians streptococci, being identified early in the oral cavities of neonates[31]. The timing of MS colonization has been of particular interest, since there is evidence that the early colonization of these bacteria in children is a major risk factor for the future development of dental caries[32,33]. Caufield *et al.*[34] followed infants from birth to 5 years to study the initial timing of MS acquisition. They introduced the concept of the "window of infectivity", suggesting that MS were acquired in infants during a discrete time period, inside which 75% of the cohort fell, defined by a mean age of 26 months and spanning between 19 and 33 months. Eruption of first and second primary molars was believed to be the key event determining this period. A strong correlation was noted between the total number of primary teeth surfaces and the cumulative probability of MS infection.

A number of studies have reported that MS could not be detected in predentate infants and only became detectable after the eruption of first primary molars[35-38]. This has led to the notion that MS require non-shedding surfaces for oral colonization. Recent studies, however, have defied this belief, showing that MS can colonize in the oral cavities of infants prior to tooth eruption[39-41]. Wan *et al.*[41] found that *S. mutans* was present in 30% of predentate 3-month infants and additionally its presence was associated with the development of Bohn's nodules. Tanner *et al*.[42] utilized a DNA probe assay to detect oral bacterial species and showed that *S. mutans* was detected in 70% of tongue scraping samples and 50% of plaque samples in children under 18 months. They

suggested that furrows of the tongue may be a potential ecological niche for a number of oral bacteria. Furthermore, several longitudinal studies have reported that *S. mutans* colonization increased with increasing age without any discrete window period[43-45]. Law and Seow[43] found that a large number of subjects were colonized by MS at later ages such as 37-72 months. Some of these data seem to suggest that there is no window of infectivity, merely individual variables that determine the time of acquisition.

Previous studies using bacteriocin typing, serotyping, plasmid DNA profiling or genotyping provide strong evidence that mothers are the primary source of MS in children, presumably due to their frequent and intimate contacts[34,46-49]. MS strains isolated from the majority of mother-child pairs showed similar phenotypic and genotypic profiles. The degree of this vertical transmission may be related to several factors, including the magnitude and the frequency of the inoculum, as well as the minimum infective dose[7]. The children whose mothers harbored high levels of MS in saliva acquired higher amounts of the bacteria and at younger ages, compared to those of mothers with low MS levels[50,51]. Berkowitz *et al.*[50] found that mothers with greater than $10^5$ CFU/ml of salivary MS had a 58% rate of transmission to their infants, whereas mothers with $10^3$ CFU/ml of MS transmitted the bacteria to their infants only 6% of the time. Alternatively, preventive measures to reduce the salivary levels of *S. mutans* in mothers help prevent or delay the colonization of *S. mutans* in their infants[52,53]. The frequency of matching genotypes between mother-child pairs decreases as the age of the child increases. It also varies among populations, which may suggest the role of cultural practice or socioeconomic status[54-56]. Although to a much lesser extent, the vertical transmission of MS from father to child has also been reported with a frequency as high as 32.6%[57].

A number of recent studies have revealed that in addition to vertical transmission, horizontal transmission of MS can also occur. Matching genotypes of *S. mutans* isolates can be observed among unrelated children of various ages ranging from 2 months to 6

years, attending the same nursery schools or kindergartens[56,58-60]. Furthermore, several studies found matching MS genotypes between spouses, suggesting that horizontal transmission can also occur in adults[49,57,61].

Following colonization, MS strains appear to persist with a relatively high degree of consistency for several years. The loss and gain of particular genotypes can also occur and may depend on the alteration in environmental stress factors, influencing the growth and survival of each bacterial strain[45].

## Genotypic diversity of *S. mutans*

Most people harbor MS in their oral cavities but not all acquire dental caries. This leads to the concept that these bacteria are genotypically diverse and possess variable virulence potentials. Some *S mutans* strains may be more virulent or in some way retain a significant advantage over others for effective colonization and infection of host tissue. The diversity of clinical strains may be generated from a single bacterial clone by a number of mutational events over many generations. The mutations that are beneficial to a particular clone may be accumulated and passed on vertically to offspring as previously mentioned.

Previous studies using DNA fingerprinting techniques indicated that clinical isolates of *S. mutans* from non-related individuals demonstrated unique genotypes[47,55,62,63]. The relationship between clonal diversity of *S. mutans* and dental caries experience has been reported with differing results. Several studies reported higher numbers of *S. mutans* or *S. sobrinus* genotypes in caries-active subjects, compared to those from caries-free subjects[62,64,65], whereas the inverse or no relationships were found in other studies[66,67]. As many as 7-8 MS genotypes can be found in a caries-active individual[62,68]. The interaction among strains with variable cariogenic potentials may further increase the risk of dental caries.

Several studies have investigated the differences between clinical isolates of *S. mutans* from caries-free and caries-active subjects with regards to a number of virulence properties. When compared with *S. mutans* isolated from caries-free subjects, isolates from caries-active subjects have been shown to synthesize higher amount of water-insoluble glucan which may translate to increased bacterial adhesion and biofilm formation on tooth surfaces[62,69]. Lembo *et al.*[67] demonstrated that caries-active subjects tend to harbor *S. mutans* genotypes that are highly efficient at biofilm formation and more resistant to acidic environments than caries-free subjects. An *in vivo* study using animal models revealed that *S. mutans* isolates from caries-active subjects had higher cariogenic potential than those form caries-free subjects[70]. However, no differences were reported between *S. mutans* isolates from both groups in the production of glucosyltransferases, adherence to glass surfaces, and acidogenicity[62,69].

In addition, Guo *et al*.[71] examined *S. mutans* isolated from dental plaque of caries-active subjects and found that two clonally distinct isolates from the same person could show significant differences in water-insoluble glucan synthesis, adhesion to hydroxyapatite and acid adaptation, the three recognized virulence traits of *S. mutans*. They also applied suppression subtractive hybridization to characterize the genetic differences between two isolates and found 27 genetic sequences in one strain that were lost in the other, involving genes with a wide variety of functions.

## Dental plaque ecology

Dental plaque is a complex biofilm comprising more than 600 different species. This network of various types of microbes are involved in different aspects of the caries process[4]. Microbial species within dental plaque may interact antagonistically or synergistically to maintain the microbial homeostasis in which each organism helps contribute to the organization of the biofilm community[72]. Individual microorganisms within a community have specific requirements for growth and survival. Microorganisms

entering the oral cavity first can influence the colonization of species arriving later. The early species have an ecological advantage that the later species need to overcome in order to compete sufficiently to establish colonization[73]. The physiological status of each oral bacterial species depends on environmental stress factors such as the lack of nutrients, the acidic pH, the competing bacteria and host salivary factors[74,75].

The composition of the resident bacteria is different in each habitat. The oral cavity consists of both shedding oral mucosal surfaces of which the continual desquamation helps reduce microbial load, and non-shedding tooth surfaces which provide distinct platforms for the adhesion and biofilm formation of a particular group of microbial species[76]. Dental plaque is formed by an ordered pattern of colonization by a variety of oral bacteria. As soon as the teeth erupt or are cleaned, tooth surfaces are coated by salivary pellicle, representing the conditioning film that directly influences the pattern of initial microbial colonization[76,77]. The early colonizers, primarily *S. sanguinis, S. mitis and S. oralis*, can adhere to the salivary pellicle by using both non-specific physicochemical interactions and specific molecular interactions between cell surface adhesins and receptors in the salivary pellicle[72,73]. Oral bacteria generally have multiple cell surface adhesins which can interact with host molecules as well as similar types of receptors on other bacteria. Following their growth, these bacteria modify the local microenvironment such that it is favorable to more fastidious bacteria. These later colonizers then bind to the earlier species and to the extracellular matrix within dental plaque to increase the diversity and form a complex biofilm[76]. The attached microbial species then multiply to form confluent growth and produce extracellular polymers, such as water-soluble and water-insoluble glucans and fructans. A relatively stable biofilm needs to develop some types of balance with its neighboring environment[74]. The stable biofilm of resident plaque microflora is of benefit to the host by acting as a barrier to later colonization by exogenous, often pathogenic species[77]. This microbial homeostasis when

disrupted can lead to imbalances in the resident microflora, resulting in the enrichment of certain pathogenic organisms that cause disease.

In the context of dental caries, frequent exposure to fermentable carbohydrates and subsequent low pH are believed to be the major factors altering dental plaque homeostasis. MS may be found naturally within dental plaque; however, they are poor competitor at neutral pH and are outnumbered by other resident microflora, presenting only as a small proportion of the total plaque community. The processes of demineralization and remineralization of tooth surfaces are then in equilibrium. At low pH, the microbial ecology within dental plaque shifts to favor the growth of more acid-tolerant and/or acidogenic bacteria, most importantly MS and lactobacilli. When dental plaque drops below the critical pH of approximately 5.5, the balance is tipped towards tooth demineralization. These bacteria can grow and produce high amounts of acid at low pH which further enhances the demineralization process. Therefore, the selection of pathogenic bacteria is directly coupled to the changes in the environment[72,76,78,79].

## Host susceptibility to dental caries

Other than microbial and dietary factors, host factors also contribute greatly to dental caries pathogenesis. This involves both individual life style and behavioral aspects, as well as host genetic factors. Among the risk factors for dental caries that involve individual behavioral aspects are oral hygiene performance, fluoride exposure, accessibility to dental care, socioeconomic status, and number of years of education. The role of host genetic predisposition to dental caries development has been investigated in several studies, however, due to the multifactorial nature of this disease, the specific allelic inheritance responsible for dental caries susceptibility has not been conclusively defined[80].

Early studies examining the caries prevalence within family members found that caries rates between siblings or between parents and their offspring were closely

correlated[81]. However, the strongest evidence for the role of genetically determined host factors in dental caries is derived from the monozygotic twin studies[82,83]. Borass *et al.*[82] studied a population of monozygotic twins reared apart to examine the genetic contribution to dental caries. They found that monozygotic twins showed statistically significant resemblance in caries incidence (teeth restored, surface restored, and caries index) as well as other dental parameters, such as teeth size, teeth present and malalignment. These results are in contrast to dizygotic twins reared apart who did not show within-pair resemblance in these parameters. The specific genetic factors that contributed to the similar caries incidence between monozygotic twins were not determined. However, several potential factors were speculated upon, such as salivary factors and microflora, time and sequence of tooth eruption, tooth morphology, arch shape and propensity to diet[82].

Several studies have investigated the association between specific HLA haplotypes and dental caries[84-89]. HLA-DR3 was found to be highly associated with increased enamel defects, and carious lesions, whereas HLA-DR5,7 appeared to be associated with the decreased frequency of enamel defects[84,85,88]. Several studies reported different findings with other HLA patterns. Lehner *et al.*[87] reported a significant relationship between HLA-DRw6,1,2,3 and caries experience, while de Vries *et al*.[86] found no such relationship with HLA-DRw6. Overall, these data suggest a potential relationship between certain immune complex genes and enamel defects that increase the risk of dental caries.

In addition, human saliva contains a number of protective factors against pathogenic microorganisms. The associations of various salivary factors and dental caries have been intensely investigated and are described below.

## Saliva and dental caries

The oral cavity is a unique environment consisting of both soft tissue and dental hard tissue, which are readily exposed to the external environment. These structures are constantly bathed with saliva and also serve as biological niches for a large assortment of oral microorganisms. Saliva is comprised of secretions from major salivary glands (the parotid, submandibular and sublingual glands) as well as minor salivary glands throughout the oral cavity, contributing to the majority of whole saliva. Also present in saliva are gingival crevicular fluid, oral microorganisms, sloughed epithelial cells, degenerating inflammatory cells, and diet-related components[90].

Saliva serves a variety of functions in the oral cavity. Not only does saliva help coat mucosa and exposed tooth surfaces, lubricate, moisten oral tissues, make possible oral functions, including speaking, chewing, swallowing, and provide digestive enzymes for starch hydrolysis, it also plays a vital role in the innate immune defense against potentially pathogenic microorganisms and modulates the demineralization and remineralization process of the tooth structure. Its role in dental caries protection is well-recognized and depends on both its physical properties and chemical composition[91-93].

Oral salivary clearance, by flushing and neutralizing effects, stands as one of the most important caries-protective functions of saliva. This function depends largely on the salivary flow rate. An increased salivary flow rate helps to effectively remove microorganisms from the oral cavity and also provides necessary immune as well as non-immune salivary factors[94]. Subjects with impaired salivary flow rates often show increased dental caries incidence[95]. However, it should be noted that the effect of the salivary flow rate also depends on the individual threshold limit which is variable within a population[94].

Saliva also possesses a buffering capacity, which is a measure of the ability of saliva to absorb an acidogenic challenge. This process involves several buffering systems, including bicarbonate, phosphate and protein buffering. The bicarbonate system

makes the biggest contribution to salivary buffering capacity during food ingestion. Its concentration in saliva is increased when saliva flow is increased. The inverse relationship, albeit weak, between buffering capacity and dental caries has been observed[93,96].

Salivary acinar cells secrete a number of proteins with a variety of functions into saliva. One group of salivary proteins, which includes statherin, acidic proline-rich proteins (PRPs), cystatin and histatin, is responsible for the inhibition of demineralization and promotion of remineralization, and thus help delay the loss of tooth structure. These proteins function collectively to maintain the supersaturated state of calcium and phosphate in saliva, by inhibiting the precipitation of calcium and phosphate crystals, together with binding free calcium that can adsorb to hydroxyapatite on tooth surfaces[91,93]. Seemingly contradictory to this well-established protective function, these proteins with their strong affinity to the tooth surface are found as components of salivary pellicle and mediate the adhesion of early bacterial colonizers on tooth surface. This observation coincides with the concept of amphifunctionality of several salivary proteins, which indicates that proteins may have several distinct functions, some of which benefit the host and others benefit bacteria[97]. Several studies attempting to correlate the levels of these proteins with dental caries have reported conflicting results[94,98-100] (Table 1-1).

Several proteins in saliva, when existing in the liquid phase, are involved in the aggregation and clearance of bacteria from the oral cavity. The most important of these are agglutinin and mucin. Agglutinin was originally considered the major protein for agglutination of *S. mutans* and various other bacteria[101]. Mucin constitutes 20-30% of unstimulated whole saliva[102]. The major salivary mucins are the high-molecular-weight form, MG1, and the low-molecular-weight form, MG2. MG2 is more efficient in promoting bacterial aggregation, whereas MG1 tightly adsorbs to the tooth surface, contributing to salivary pellicle. However, both these proteins, when found in salivary pellicle, tend to mediate initial bacterial adhesion to tooth surface[91]. It has been suggested

that variation in the aggregation and the adhesion properties of saliva may be the determining factor for the relative susceptibility to bacterial colonization in individuals[99,103].

The majority of salivary immunoglobulins are of the IgA subclass (>85%) and to a lesser extent the IgG subclass. Secretory IgA is synthesized by plasma cells within soft tissue and is taken up by salivary ductal cells to be secreted into saliva. IgG is derived primarily from gingival crevicular fluid. In contrast to their specific immune defense function in serum or tissue, the main function of salivary immunoglobulins is to inhibit bacterial adhesion and colonization by blocking surface structures involved in binding[102].

Also present in relatively low quantity in saliva is fluoride. Fluoride may be derived from many sources and its salivary concentration is increased in people from fluoridated areas. The benefit of fluoride in caries prevention is clearly established. Fluoride is able to diffuse into dental plaque and greatly reduce the rate of tooth demineralization and enhance the remineralization process[94]. In addition, fluoride in oral fluids may function by inhibiting metabolic enzymes in bacteria, reducing bacterial growth and proliferation[93].

Beside these major salivary components, saliva also contains a number of proteins that may be present in a lesser amount but exert distinct antimicrobial activity. Two of these proteins with unique enzymatic activity are salivary peroxidase and lysozyme. Peroxidase is produced by acinar cells. It interacts with thiocyanate and hydrogen peroxide, present in glandular secretions or produced by bacteria, to form hypothiocyanate and cyanosulfurous acid. These products oxidize bacterial sulfhydryl groups and inhibit glucose metabolism[93]. Peroxidase also functions to protect salivary proteins from bacterial degradation. Lysozyme is capable of hydrolyzing bacterial cell wall polysaccharide and makes bacteria more susceptible to hypo-osmotic conditions in saliva or other antimicrobial proteins[102]. Lactoferrin is a non-enzymatic antimicrobial

protein, which acts through its iron-binding capacity to sequester iron, an essential element for bacterial metabolism, from the oral environment[102].

Besides the essential roles for these proteins in the antimicrobial properties of saliva, recent research has focused on the diverse groups of small cationic antimicrobial peptides that are ubiquitous and highly conserved among various organisms. Their significance to oral health and dental caries is described in detail in the following section.

## Salivary antimicrobial peptides

Cationic antimicrobial peptides are diverse groups of small molecules, defined as peptides of 12-50 amino acids with a net positive charge of +2 or more due to their high basic amino acid contents[104,105]. They are produced by various organisms, including plants, insects, birds, crustaceans and mammals. These peptides have a broad range of antimicrobial activities against various microorganisms, including Gram-positive bacteria, Gram-negative bacteria, fungi, parasites and enveloped viruses[106].

In general, they have 50% or more hydrophobic amino acids and fold into molecules that have the charged and hydrophilic portions segregated from the hydrophobic portion. This amphipathic nature allows these peptides to interact electrostatically with anionic microbial membranes as a part of their mechanisms of action[107]. These peptides tend to work better on membranes that have a negatively charged surface with a large membrane electrical potential and no cholesterol. As a result, normal human cells are relatively resistant[107].

These cationic peptides are bactericidal. Their minimal inhibitory concentrations and minimal bactericidal concentrations coincide or differ by no more than two fold. They kill microorganisms very rapidly. It is believed that these peptides are not affected by the bacterial resistance mechanisms such as those of conventional antibiotics[106].

In humans, three main families of antimicrobial peptides are present in the saliva, namely: 1) β-sheet peptides with cysteines forming disulfide bonds (α-defensins-1-4

(HNP-1-4) and β-defensins-1-3 (HBD-1-3)), 2) α-helical peptides without cysteine (the cathelicidin, LL-37), and 3) extended peptides enriched with specific amino acids (histatins)[108]. Among these peptides, defensins and LL-37 represent the two major salivary antimicrobial peptides because they are produced from the greatest variety of sources within the oral cavity[109]. Among various defensins, their sequences, length and net positive charges are variable with no common pattern in the distribution of polar and hydrophobic residues. The α-defensins and β-defensins differ in their cysteine spacing and disulfide connectivity. However, the structural motif of their β-strands appears to be conserved[110].

## Alpha-defensins

The α-defensins are arginine-rich peptides with three disulfide bridges connecting cysteine residues 1-6, 2-4, and 3-5. In humans, six α-defensin molecules, HNP-1, HNP-2, HNP-3, HNP-4, HD-5, and HD-6, and five α-defensin genes have been identified, as HNP-2 is a truncated version of either HNP-1 or HNP-3[111-113]. HD-5 and HD-6 are enteric defensins and not present in the oral cavity. HNP-1-4 are made constitutively and predominantly stored in azurophilic granules of neutrophils[114]. Collectively, they are the most predominant proteins in neutrophils, constituting nearly 5-7% of total protein in these cells[105]. However, HNP-1-3 account for almost 99% of the total defensin content in neutrophils; HNP-4 concentrations are approximately 100-fold lower[115].

In the oral cavity, HNP-1-3 are present in saliva primarily from two sources, the neutrophils circulating through gingival crevicular fluid and the salivary glands[114,116]. Of these neutrophils are believed to be the more abundant source of these peptides in saliva. Levels of HNP-1-3 in saliva are markedly reduced in completely edentulous patients, most likely due to the absence of gingival crevices[117]. HNP-1-3 can be detected in quantities consistent with their antimicrobial function in gingival crevicular fluid and is of lower concentration in whole saliva[118].

## Beta-defensins

The β-defensins also contain six conserved cysteine residues but differ from α-defensins by the pairing of these cysteine residues (disulfide bridges 1-5, 2-4, 3-6). They are mainly expressed in epithelial tissues of various organs, including skin, mucosa of oral cavity, air ways, gastrointestinal tract, genitourinary tract, and kidney. Many β-defensin genes are identified, however, only 4 β-defensins (HBD-1-4) have been described in detail.

In the oral cavity, HBD-1-3 are expressed from various sources, including gingival epithelia, tongue, palate and buccal mucosae, salivary glands/ducts and saliva[119-122]. The expression of these peptides is increased in the upper portion of the stratified squamous epithelium and may be retained within the keratinized surface[123-125]. The expression is strongest at the gingival margin adjacent to the area of plaque formation on tooth surfaces and in inflamed sulcular epithelium[125].

The expression of HBD-2-3 in oral keratinocytes is inducible in response to bacterial lipopolysachharides (LPS), proinflammatory mediators, such as IL-1β, TNF-α, IFN-γ, and upon contact to bacteria or yeasts[120,126-128], whereas HBD-1 is constitutively expressed[128,129]. It is suggested that HBD-1 may play a role in preventing normal flora from becoming opportunistic pathogens, while HBD-2 and HBD-3 may be more effective against pathogens[130]. The expression of HBD-1-3 has also been observed in ductal cells but not in acini of salivary gland tissue and these peptides are detected at low concentrations in gingival crevicular fluid[118,122,128,131,132].

HBD-1 and HBD-2 are predominantly active against Gram-negative bacteria and yeasts[133], whereas HBD-3 is also active against Gram-positive bacteria, in addition to Gram-negative bacteria and yeasts[134]. HBD-4 is predominantly expressed in testis as well as gastric epithelium, and has not been detected in saliva

In humans, the genes for α-defensins and β-defensins are located in adjacent loci on chromosome 8p22-p23, suggesting an evolution from a common ancestral gene with several duplication events[135-137].

## LL-37

LL-37 is the only cathelicidin present in humans. Unlike defensins, LL-37 is stored in specific granules of neutrophils in an inactive form that requires processing by proteinase-3 to obtain the full antimicrobial activity[138,139]. The gene for LL-37 is located on chromosome 3. LL-37 is also expressed throughout the body, including the epithelial cells of skin, testis, gastrointestinal tract, and respiratory tract. In addition to neutrophils, LL-37 is produced by other leukocytes, such as monocytes, T cells, B cells and NK cells[140]. In the oral cavity, LL-37 is found in neutrophils, the epithelium of the gingiva, tongue, buccal mucosa and salivary acinar and ductal cells. LL-37 has a broad-spectrum of activity against various bacteria, fungi and virus[141-143].

The clinical importance of LL-37, together with HNP-1-3, is clearly shown in patients suffering from Morbus Kostmann syndrome, a severe recessive syndrome usually diagnosed in infancy. These patients lack LL-37 in saliva and neutrophils and have reduced HNP1-3 levels, leading to recurrent bacterial infection early in life and severe periodontitis[144,145]. Unless treated by cytokine GM-CSF early, all patients die from bacterial infections. This finding suggests that LL-37 and defensins are crucial in the defense against periodontopathic bacteria, especially *Aggregatibacter actinomycetemcomitans*, a Gram-negative bacterium associated with rapidly progressive periodontitis in young patients[130].

Besides their established role as antimicrobial agents, more recent studies have shown that antimicrobial and chemokine functions of these cationic peptides overlap considerably[146,147]. HNPs can act as chemoattractants for naïve CD4 T cells and immature dendritic cells, stimulate mast cell degranulation, regulate complement

activation and enhance macrophage phagocytosis[148-151]. HBD-1 and HBD-2 also attract dendritic cells and T cells and are able to bind receptors on dendritic cells, inducing their maturation[152]. In addition, HBD-2 can activate the release of histamine from mast cells[153]. Similar to defensins, several studies have revealed multiple roles for LL-37, including chemotactic activity for T-lymphocytes, monocytes and neutrophils, wound healing, angiogenesis and the binding of lipopolysaccharides (LPS), neutralizing its biologic activity[140]. These findings indicate that these antimicrobial peptides also function to promote adaptive immune responses against various antigenic stimuli.

## Histatins

Histatins are the major antimicrobial peptides in saliva, representing a family of at least 12 histidine-rich peptides. They are constitutively expressed from parotid and submandibular salivary ductal cells. In contrast to the more general expression pattern of defensins and LL-37, the production of histatins is not found in other oral tissues. The histatin genes are located on chromosome 4q13.

Histatins are primarily antifungal agents with a little effect on oral bacteria[130,154]. Proteolytic cleavage of histatin-1 and histatin-3 generates multiple other histatin molecules. Histatin-5 has the strongest fungicidal activity; therefore, most research has focused on this peptide[118]. Histatins appear to play a key role in the control of oral *Candida*[155].

## Mechanisms of action

Several mechanisms have been proposed to explain the bactericidal activity of cationic antimicrobial peptides, however, the interaction of these cationic peptides with the negatively charged bacterial membrane is regarded as an essential step in their actions[106]. These negatively charged targets include LPS in Gram-negative bacteria, teichoic acids in Gram-positive bacteria, and phospholipids (phosphatidylglycerol) in the inner membrane of both Gram-negative and Gram-positive bacteria[104]. Following this

electrostatic attraction, peptides must cross the outer membrane of Gram-negative bacteria or the thick cell wall of Gram-positive bacteria to approach the bacterial cytoplasmic membrane where they interact with the lipid bilayer and subsequently insert into the membrane and form transmembrane pores[106,156].

The mechanisms by which peptide insertion forms transmembrane pores in cell membranes have been studied using various peptides. Transmembrane pores appear to be created when peptides orient perpendicular to the membrane at a high peptide/lipid ratio. The optimal peptide/lipid ratio varies depending on the peptide and the physical condition and composition of the lipid bilayer[156-158]. Several models have been described for the process of transmembrane pore formation, including the barrel-stave model, the carpet model and the toroidal-pore model[156,158-161].

Although membrane insertion is considered crucial for the bactericidal activity of these cationic antimicrobial peptides, there is increasing evidence that several other mechanisms are also involved or may in fact represent the principal mechanism of action[162]. Several intracellular targets have been described. Following translocation into the cytoplasm, a number of peptides can alter cytoplasmic membrane septum formation, inhibit cell wall synthesis, inhibit protein synthesis, inhibit nucleic acid synthesis or inhibit enzymatic activity[156]. Lehrer *et al.*[163] reported a synchronous inhibition of DNA, RNA and protein synthesis following membrane permeabilization in *E. coli* treated with a bactericidal concentration of HNP-1. Histatins have been shown to kill *Candida albicans* without causing significant cell wall lysis[164]. They appear to induce the non-lytic loss of ATP from actively respiring cells, which can induce cell death. In addition, they are able to disrupt the cell cycle and lead to the generation of reactive oxygen species[165].

## Bacterial resistance to antimicrobial peptides

A number of studies have demonstrated several mechanisms by which bacterial pathogens develop resistance to host antimicrobial peptides. Antimicrobial peptide

resistance is considered a key virulence factor for various pathogens involved in various types of infection[166-168]. Since the ability of cationic antimicrobial peptides to bind and integrate into bacterial cell membranes is crucial for their action and depends largely on the net negative charge of bacterial surface, modification of the anionic membrane targets of several bacterial pathogens, resulting in the reduction of the net negative charge of the cell surface, has been shown to increase resistance to antimicrobial peptides. One of the well-defined bacterial cell targets is lipoteichoic acid that extends outward from the cytoplasmic membrane of Gram-positive bacteria. Disruption of the *dlt* operon, involved in the D-alanine modification of lipoteichoic acids, in a number of Gram-positive bacteria, resulted in the decreased bacterial resistance to antibiotics as well as cationic antimicrobial peptides, due to the increased net polyanionic charge in *dlt*-deficient mutants[169-174]. Similarly, an *mprF* mutant of *Staphylococcus aureus*, a mutant deficient in L-lysine modification of phosphatidylglycerol, showed increased resistance to defensins due to the lower net positive charge resulting from fewer free amino groups of lysine[175].

A number of other mechanisms have also been described in various other bacterial species. *Salmonella enterica* can modify lipid A, a portion of the LPS of Gram-negative bacteria, to alter its surface charge[176-179]. Several bacteria produce proteases capable of cleaving enzyme to cleave antimicrobial peptides[180-182]. Two proton-motive-force-dependent efflux pumps have also been implicated in the resistance to these peptides in *Neisseria gonorrheae*[183]. *Shigella* species were shown to resist LL-37 and HBD-1 by inhibiting the production of these peptides in human rectal epithelial cells[184]. Although *S. mutans* strains showed variable susceptibility patterns to a number of antimicrobial peptides present in saliva[185-187], their resistance mechanisms have not been characterized.

## Rationale

The basic mechanisms behind the development of dental caries are largely known. The disease is characterized by the actions of cariogenic bacteria which metabolize dietary substrates to organic acid destructive to the tooth surface. As the paradigm of dental caries development has shifted to highlight the importance of the dynamic relationship between host and oral microflora within the dental plaque community, dental caries is now considered a consequence of an alteration in the ecological balance between healthy and diseased states. This reflects the fundamental impact of environmental cues on microbial species within biofilms[72,76,79].

Nevertheless, MS bacteria, primarily *S. mutans*, are undoubtedly the principal causative agents of dental caries. These bacteria can be found in the oral cavities of predentate infants, and their levels are substantially increased following tooth eruption[34-36,40-42,44]. In addition, they are regularly found associated with carious lesions[5,6], though the presence of *S. mutans* is not always a reliable predictor of caries risk[4]. The fact that *S. mutans* clinical strains demonstrate marked genetic heterogeneity puts forward the notion that S. *mutans* clones are variable in their virulence potentials and some clones may be more virulent than others[4,47]. This may not only involve several long-recognized virulence factors, such as biofilm formation, acid-tolerance or acidogenicity, but also the ability of particular strains to better adapt and survive under unfavorable environmental conditions in the host, such as the lack of nutrients, excess oxygen, extreme pH or the actions of salivary defense factors.

In the oral cavity, a number of host factors are present in saliva that may potentially influence dental plaque ecology. The composition of the salivary pellicle may determine the ability of oral bacteria to colonize the tooth surface. Salivary flow and buffering capacity are crucial in oral clearance, flushing of microorganisms and maintaining optimal environmental pH. The presence of calcium, phosphate and fluoride influences the stability of the demineralization-remineralization process. Lastly, various

host innate and adaptive immune factors function to defend against the colonization of pathogenic organisms.

In this thesis, we investigated one facet of how host factors might shape the ecology of the dental plaque microflora, in particular the colonization of *S. mutans*. We hypothesized that various strains of *S. mutans* possess different inherent susceptibility/resistance profiles to host salivary antimicrobial peptides and that the host-specific quantity and distribution of these peptides may influence plaque colonization by particular *S. mutans* strains. The aims are directed towards determining the levels of correlation between *S. mutans* carriage, antimicrobial peptide susceptibilities, salivary antimicrobial peptide levels and caries experience.

Saliva and plaque samples used in this study were collected as a part of the Iowa Fluoride Study. The Iowa Fluoride Study (IFS) is an on-going NIH-funded study which has followed a large cohort of children since birth from eight eastern and central Iowa hospital post-partum wards to study the epidemiology of fluoride intake in relation to dental fluorosis, dental caries, dental growth and development. This has provided great resources for the study of microbiological as well as immunological aspects of dental caries in relation to variable host demographics, caries experience and other environmental exposures. The children in the IFS were generally representative of Iowa children. Inclusion criteria included that children were placed in the 'well baby newborn nursery area', that the families had plans to remain in Iowa for at least 3-4 years, and that they were willing to receive periodic questionnaires and screening examination for their children. Exclusion criteria were that the children had health challenges that placed them in the 'intensive care' or 'intermediate' nursery.

*S. mutans* isolates were recovered from plaque samples to preferentially reflect the clonal members most highly represented within individual dental biofilms. Sufficient plaque samples were available from IFS subjects of age 13. This study focused mainly on *S. mutans* to examine the strain variability in susceptibility to host salivary antimicrobial

peptides due to several reasons. The association of *S. mutans* and dental caries has been clearly established from a large number of studies both *in vitro* and *in vivo*. It is the most prevalent cariogenic bacterial species associated with carious lesions. The virulence properties of *S. mutans* are well-known is knowledge of its clonal diversity and the availability of its genetic information.

The salivary antimicrobial peptides chosen to be tested were based on the reports of their presence in saliva and commercial availability. In addition, preliminary experiments were performed to evaluate their activities against various *S. mutans* strains. Of all the peptides tested, three peptides, HBD-1, histatin-5 and lysozyme, did not show bactericidal activity against clinical strains of this bacterium at the peptide concentrations 20 μg/ml or below in our preliminary experiments and were therefore dropped from further experimental analyses.

With the experimental design of this thesis, we provide important information regarding the capacity of host innate immune factors, in the form of antimicrobial peptides in saliva, to influence the colonization of *S. mutans* as well as the caries process.

## Thesis overview

In this thesis, the potential roles of several salivary antimicrobial peptides in the colonization of *S. mutans* strains and their contributions to host caries experience were investigated.

In Chapter 2, the methodology used in the experiments in this thesis is described. This includes the selection of subjects, bacterial strains as well as experimental reagents. Statistical analyses of the data are also detailed.

In Chapter 3, we present the susceptibility profiles of distinct *S. mutans* clinical strains and correlate this information with caries experience of subjects. The relationships between the susceptibility profiles of S. *mutans* to each antimicrobial peptide and between these susceptibility profiles and MS levels in dental plaque are also presented. In

addition, we examined the antimicrobial effects of peptide combinations against a representative *S. mutans* strain. We found that *S. mutans* showed variable susceptibilities to different antimicrobial peptides. The susceptibility patterns were strain-specific and correlated with host caries experience. There were correlations among the susceptibility patterns of individual strains to certain peptides. No correlation between susceptibility patterns and MS levels in dental plaque was observed. Antimicrobial peptide combinations revealed additive and synergistic effects against *S. mutans*, suggesting that these peptides may work cooperatively within the oral environment.

In chapter 4, we describe the potential relationship between levels of antimicrobial peptides in saliva and host caries experience in attempt to clarify the contribution of these peptides to dental caries risk. We found that the salivary levels of antimicrobial peptides were highly variable and were not associated with caries experience in this subject group. However, high caries subjects had significantly greater levels of total protein in saliva than caries-free subjects. Within particular subjects, significant moderate relationships between the salivary levels of HNP-1-3 and LL-37 and between HBD-2 and HBD-3 were noted. In addition, there were weak increasing relationships between the salivary levels of HBD-3 and LL-37 and between HNP-1-3 and HBD-2. Moreover, an increasing relationship between levels of HNP-1-3 and *S. mutans* resistance to HNP-1 was noted.

In Chapter 5, the potential contributions of host salivary antimicrobial peptides in dental caries are discussed based on the findings in this study as well as previous studies in the literature. We also describe how this information adds to the current knowledge and paradigm of dental caries pathogenesis. In addition, potential future research to expand the current understanding of the interactions of host salivary antimicrobial peptides and dental caries pathogenesis are proposed.

**Table 1-1 List of studies on the associations between salivary factors and dental caries.**

| Salivary factors | Number of references | | |
|---|---|---|---|
| | Positive correlation | No association | Negative association |
| Statherin | 1[188] | - | 1[98] |
| Acidic proline-rich protein | Db-s variant - 1[99] | - | All – 3[98,189,190]<br>Non-Db variant - 1[99] |
| Cystatins | 2[188,191] | - | 1[189] |
| Histatin-1 | - | - | 1[98] |
| Agglutinin | 1[99] | 1[192] | - |
| Mucin | 1[193] | 1[194] | 1[190] |
| sIgA | 11[98,189,195-203] | 6[204-209] | 6[192,210-214] |
| Amylase | 3[98,189,215] | - | - |
| Thiocyanate/ hypothiocyanate | 1[216] | 3[204,217,218] | 1[192,205] |
| Peroxidase | - | 5[204,205,216-218] | - |
| Lysozyme | 1[216] | 4[192,204,205,219] | - |
| Lactoferrin | 4[98,189,202,216] | 2[192,205] | - |
| HNP-1-3 | - | - | 1[116] |
| HBD-3 | - | 1[116] | - |
| LL-37 | - | 1[116] | - |

A positive correlation indicates an increasing relationship between the level of the specified protein/peptide in saliva and host caries experience. A negative correlation indicates an inverse relationship between the two variables.

# CHAPTER 2

# MATERIALS AND METHODS

## Sample collection

Saliva and plaque samples were obtained from sixty children enrolled in the Iowa Fluoride Study. Subjects were randomly selected and were equally divided between caries-free and high caries groups. All children were 13 years old at the time of sampling. Subjects were designated high-caries when 3 or more carious lesions, with regard to the cavitated ($D_{2-3}$) or filled surfaces, were found. These criteria for dental caries did not differentiate between cavitated enamel ($D_2$) and dentin ($D_3$) lesions and have been previously reported[220]. Oral examination and sample collection were performed by trained calibrated investigators using a standardized protocol. Subjects were constrained from eating, drinking (other than plain water), chewing gum or brushing teeth within 60 minutes before sampling. Plaque samples were taken from pits and fissures of occlusal surfaces of second molars using sterile CytoSoft® Brush. Stimulated saliva was collected using Parafilm® wax. Samples were frozen at -80°C for later analysis.

## Isolation and biochemical characterization of *S. mutans*

Plaque samples were diluted in Trypticase Soy Broth supplemented with Yeast Extract (TSBYE) and plated on blood agar and Mitis-Salivarius-Kanamycin-Bacitracin (MSKB) agar for determination of total bacteria count and MS count, respectively. Ten colonies of presumed *S. mutans* from each MSKB plate were selected and subcultured onto subsequent agar plates to test the fermentation of mannitol, raffinose, sorbitol, salicin and the arginine hydrolysis. Bacterial colonies were selected at random; however, colonies with different morphologies were preferentially chosen to potentially increase the possibility of obtaining more diverse bacterial strains. Isolates were identified as *S. mutans* when they were positive for fermentation of these sugars and negative for arginine hydrolysis and further confirmed by Gram stain and negative catalase tests. Ten

*S. mutans* isolates per plaque sample from each subject were frozen in TSBYE with 10% glycerol and kept at -80$^{o}$C.

## DNA extraction

All DNA extractions from *S. mutans* isolates were performed using the MasterPure™ Gram Positive DNA extraction kit (Epicentre Biotechnologies, Madison, WI) with some modifications from the manufacturer's protocol. Briefly, *S. mutans* isolates were grown overnight in Todd-Hewitt Broth supplement with 5% Yeast Extract (THBYE) in $CO_2$ incubator at 37$^{o}$C. Overnight bacterial cultures were centrifuged and resuspended in TE Buffer. Ready-Lyse Lysozyme was added and resuspended bacteria were incubated at 37$^{o}$C for 1 hr, followed by 25 minutes incubation at 67$^{o}$C with proteinase K and Gram Positive Lysis solution and 1 hr incubation at 37$^{o}$C with RNase A. Samples were then cooled and protein was precipitated by MPC Protein Precipitation reagent. Following centrifugation, supernatants were recovered by isopropanol and DNA pellets were rinsed twice with 75% ethanol. DNA pellets were resuspended in TE buffer.

## Genotyping

Genotypic characterization of *S. mutans* isolates were performed by arbitrarily primed polymerase chain reaction (AP-PCR) using 2 commercially available, single-stranded 10-mer oligonucleotide primers, OPA-2 (5'-TGCCGAGCTG) and OPA-13 (5'-CAGCACCCAC). These two primers were shown to generate multiple discrete DNA fragments with appropriate lengths. PCR reactions were performed in a volume of 25 µl containing 2.5 µl of 10X reaction buffer, 7 mM $MgCl_2$, 1.25 units of Taq DNA Polymerase (Applied Biosystem, Foster City, CA), 200 mM of dNTP mix (Invitrogen, Calsbad, CA), 100 pmole of primer and 50 ng of template DNA. AP-PCR reactions were run for 45 cycles of 1 minute at 94$^{o}$C, 1 minute at 36$^{o}$C, and 2 minute at 72$^{o}$C. Template DNA from *S. mutans* laboratory strain ATCC25175 was used as a control in all reactions. The reaction in which template DNA was omitted was also used to exclude any possible

DNA contaminations. All PCR amplitypes were run on 1.5% agarose gel electrophoresis and subsequently stained with ethidium bromide.

Similarity coefficient and clustering analysis

AP-PCR fingerprinting patterns of each unique *S. mutans* genotype from all subjects were analyzed using the GelCompar II program (Applied Maths Inc., Austin, TX). AP-PCR gel images were digitalized into the program database and normalized according to manufacturer's instruction. Similarity coefficients were calculated by curve-based Pearson product-moment correlation. The unweighted pair group method using arithmetic averages (UPGMA) was used for clustering analysis of all strains. Dendrodrams were generated from the composite data set of both OPA2 and OPA13 fingerprinting profiles.

Antimicrobial susceptibility tests

Each unique *S. mutans* strain from AP-PCR genotyping was subjected to antimicrobial susceptibility tests with a panel of salivary antimicrobial peptides, which include HNP-1, HNP-2, HNP-3, HBD-2, HBD-3 and LL-37. The selection of antimicrobial peptides was based upon literature that reported their presence in saliva[116,118,221], and their commercial availability. Additionally, the antimicrobial activity of three other peptides, HBD-1, lysozyme and histatin-5, against sixteen *S. mutans* strains were also assessed in preliminary experiments. Data revealed no susceptibility of *S. mutans* strains to these peptides at concentrations 20 μg/ml or below; therefore, we omitted these three peptides from future experiments.

Antimicrobial susceptibility tests were performed using the alamarBlue® assay as previously described[222]. The active ingredient in this assay is resazurin, an oxidation-reduction indicator that detects the metabolic activity of viable cells. Upon cell growth, resazurin is reduced to resorufin, exhibiting both fluorescent and colorimetric change which corresponds to cellular metabolic activity. It has been shown in previous studies,

as well as in our preliminary data, that the results of alamarBlue® assays and viable count assays for colony forming units (CFU) are comparable for evaluating bacterial viability[223-230]. *S. mutans* strains were grown overnight in THBYE, centrifuged, and resuspended to$10^6$ CFU/ml in Müeller-Hinton broth (MHB) for the HNP-1-3 tests or resuspended to $10^8$ CFU/ml in 0.01 M sodium phosphate buffer (pH 7.4) for the HBD-2-3 and LL-37 tests. The choice to resuspend in MHB or sodium phosphate buffer and the selection of bacterial concentrations were based on conditions that optimized the activity of each particular peptide.

All peptides were tested at 3 different concentrations, 5 μg/ml, 1.5 μg/ml and 0.5 μg/ml, by incubating 90 μl of bacterial suspension with 10 μl of 10X stock of each peptide in BD Falcon™ black with clear bottom 96-well Microtest™ Optilux™ Plates. A growth control reaction was also set up using peptide diluent in place of each peptide. Following 2 hours incubation at 37°C, 10 μl of alamarBlue® reagent was added to each reaction and incubation continued for 4 hours. Fluorescent intensity was then measured at 560 nm and 590 nm using a fluorescent microplate reader (SpectraMax® M2e, Molecular Devices, Sunnyvale, CA). Assays were performed in duplicate. Percent viabilities of bacteria in each reaction were calculated using the following formula: Percent viability = (relative fluorescent units (RFU) of test peptide/relative fluorescent units of growth control) x 100.

Antimicrobial peptide combination analysis

To examine the combined effect of these salivary antimicrobial peptides in relation to the antimicrobial activity against *S. mutans*, the alamarBlue® assays were performed using serial dilutions of two antimicrobial peptides in a checkerboard assay format[231]. In addition, the alamarBlue® assays were modified to permit the combination analysis of all peptide pairs. *S. mutans* clinical strains were grown as previously described, centrifuged and resuspended to $10^6$ CFU/ml in 0.01 M sodium phosphate

buffer (pH 7.4). The concentrations of peptides used ranged from approximately twice the concentration that produced the 50% killing of bacteria (EC50) to five serial two-fold dilutions below this concentration. Serial dilutions of two peptides were mixed together so that each row and column of a microtiter plate contained a fixed concentration of one peptide and increasing concentrations of another, as well as a row and column in which the serial dilution of each peptide was present alone. The bacterial suspension was incubated with specified concentrations of peptides for 2 hours at 37$^{o}$C. Following incubation, 100 µl of MHB and 10 µl of alamarBlue® reagent were added to each reaction and incubation continued for 4 hours. Plates were read using the fluorescent microplate reader as previously described.

Fractional inhibitory concentration (FIC) of each peptide was calculated as the concentration that gave 50% killing when used in combination divided by the concentration that showed the same effect (EC50) when used alone[231,232]. The interaction of two peptides was assessed using the FIC index, calculated by adding up their individual FIC values from the most effective combination. An FIC index approximately equal to 1 indicates an additive interaction. An FIC index of less than 1 reveals synergistic interaction, whereas an index of more than 1 is indicative of an antagonistic interaction. Peptide interaction was also evaluated by plotting an isobologram using the FIC values of different peptide combinations. The x- and y-intercepts were plotted based on the FIC values of the two peptides when used alone. The straight line connecting these two intercepts is the additive line. When two peptides exhibit additive interaction, the isobol is straight. If the peptide combination is synergistic or antagonistic, the isobol will be concave or convex, respectively. The degree of concavity or convexity also corresponds to the degree of synergy or antagonism[233,234].

In addition, to test if there were interactions between our panel of antimicrobial peptides and lysozyme or histatin-5, both of which exhibited no bactericidal activity against *S. mutans* isolates, subinhibitory concentrations (10 µg/ml) of lysozyme and

histatin-5 were added to a series of serial dilutions of HNP-1, HNP-2, HNP-3, HBD-2, HBD-3 and LL-37. Percent viabilities were evaluated using the alamarBlue® assay as previously described.

## ELISA

Saliva samples from all subjects were thawed, cleared by centrifugation twice at 15,000 rpm for 10 minutes and used to measure the levels of antimicrobial peptides by ELISA assays as following

### HBD-2 and HBD-3

Sandwich ELISA assays for HBD-2 and HBD-3 were developed using recombinant peptide standards, capture and detection antibodies from Peprotech, Rocky Hill, NJ. Briefly, Nunc Maxisorp™ 96-well plates were coated with 100 µl of capture antibody overnight. The capture antibodies used were 2 µg/ml goat anti-HBD-2 and 3 µg/ml of rabbit anti-HBD-3 for HBD-2 and HBD-3 assays, respectively. Plates were blocked with 300 µl of 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hr and incubated with recombinant peptide standards and saliva samples for 1 hr. Standards and saliva samples were diluted in the sample diluent (0.05% Tween-20, 0.1% BSA, 300 mM $MgCl_2$ in PBS) and assayed in triplicate. Saliva samples were diluted 1:5 and 1:10 for HBD-2 and HBD-3 assays, respectively. Following sample incubation, plates were sequentially incubated with 100 µl of detection antibodies for 1 hr, avidin-horse radish peroxidase (HRP) conjugate for 30 minutes and 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) liquid substrate (Sigma-Aldrich, St. Louis, MO). Optical density readings were monitored at dual wavelengths of 405 nm and 650 nm for approximately 20 minutes or until the appropriate optical density at 405-650 nm for a standard series was reached. Detection antibodies used were biotinylated goat anti-HBD-2 and biotinylated rabbit anti-HBD-3 for HBD-2 and HBD-3 assays, respectively.

## HNP-1-3

Because HNP-1-3 are highly homologous, the ELISA assay for HNP-1-3 (Hycult Biotechnology, Uden, The Netherlands) was performed collectively, according to the manufacturer's instruction. Briefly, 96-well ELISA plates were incubated for 1 hr with a standard series and saliva samples. All saliva samples were diluted 1:1000 and assayed in duplicate. Plates then were sequentially incubated with tracer, streptavidin-peroxidase conjugate, tetramethylbenzidine (TMB) substrate and stop solution and optical density measured at 450 nm.

## LL-37

The ELISA assay for LL-37 (Hycult Biotechnology, Uden, The Netherlands) was performed according to the manufacturer's instruction with the exception that $MgCl_2$ was added to the wash/dilution buffer to a final concentration of 300 nM. Briefly, 96-well ELISA plates were incubated for 1 hr with a standard series and saliva samples. All saliva samples were diluted 1:5 and assayed in duplicate. Plates then were sequentially incubated with tracer, streptavidin-peroxidase conjugate, tetramethylbenzidine (TMB) substrate and stop solution and optical density measured at 450 nm.

## BCA assay

Total protein concentrations in saliva samples were measured using the BCA Protein assay kit (Pierce Biotechnology, Rockford, IL). Assays were performed according to the manufacturer's instruction using albumin as a protein standard. All saliva samples were diluted 1:2 in modified Dulbecco's PBS (pH 7.4) and assayed in triplicate. Briefly, the diluted standard series and saliva samples were added to Nunc Maxisorp™ 96-well plates, followed by working reagent and incubated at 37$^{o}$C for 30 minutes. Plates were then read at 562 nm to measure the optical density.

## Statistical analyses

Descriptive statistics were performed. Under each condition, a two-sample t-test was conducted to determine whether a statistically significant difference existed between subjects with no caries and high caries experience, or between males and females, with respect to the percent viability of *S. mutans* exposed to antimicrobial peptides, MS count and ratio of MS to the total bacteria count, *S. mutans* amplitypes and the salivary levels of each antimicrobial peptide. The nonparametric Wilcoxon rank-sum test was also performed if the assumption of normally distributed data became invalid by the Shapiro-Wilks test.

A Pearson correlation test and Spearman's rank correlation test were used to evaluate whether there was an apparent increasing or decreasing relationship between two variables under each condition. The following is an approximate guide for interpreting the strength of the relationship between two variables, based on the absolute value of the Pearson correlation coefficient:

i) $\sim 1$= perfect correlation,

ii) $\sim 0.8$=strong correlation,

iii) $\sim 0.5$=moderate correlation,

iv) $\sim 0.2$=weak correlation,

v) $\sim 0.00$=no correlation.

For all tests, the criterion for statistical significance was a p-value of $\leq 0.05$. SAS for Windows (v9.1, SAS Institute Inc, Cary, NC, USA) was used for the data analysis.

# CHAPTER 3

# SUSCEPTIBILITY OF *STREPTOCOCCUS MUTANS* CLINICAL STRAINS TO SALIVARY ANTIMICROBIAL PEPTIDES IN ASSOCIATION WITH HOST CARIES EXPERIENCE

## Introduction

*S. mutans*, the primary etiologic agent of dental caries, demonstrates a vast genotypic diversity among the population. Caufield *et al.*[4] noted that most, if not all MS strains isolated from unrelated individuals are genotypically different. This finding, together with the fact that the level of *S. mutans* in saliva does not stand alone as a reliable predictor of dental caries risk, suggests that different *S. mutans* clonal types may be variable in their virulence potentials. Several studies have investigated the differences in a number of virulence factors between *S. mutans* isolated from caries-free and caries-active subjects. Lembo *et al.*[67] found that caries-active subjects tended to harbor *S. mutans* genotypes that were highly efficient at biofilm formation and were more resistant to acidic environments than those from caries-free subjects. *S. mutans* isolates from caries-active subjects also demonstrated higher cariogenicity than those from caries-free subjects in animal models[70].

Besides these potential differences, the ability of bacteria to resist host defense factors is crucial for their survival and subsequent colonization within biofilms. Dental plaque represents an ecological niche for a large array of microbial species. The proportion of each microbial member in dental plaque may vary depending on the dynamics of the surrounding environment. As for dental caries, the changes in dental plaque pH are believed to be the primary factor driving the ecological shift of the bacterial population within the plaque from a primarily nonpathogenic resident microflora to a plaque enriched in aciduric, cariogenic bacterial species[72,76,79].

The significance of saliva in the prevention of dental caries is well-recognized. Apart from its physical cleansing and buffering capacity, saliva is also comprised of a number of host immune factors[91,93,235,236]. Recently, families of cationic antimicrobial peptides have been detected in saliva, including the α-defensins (HNP-1-3), the β-defensins (HBD-1-3), the cathelicidin LL-37 and histatins[122,237-239]. These peptides possess broad spectrum antimicrobial activity to various microorganisms. In the oral cavity, the defensins and LL-37 are potent against a number of bacterial species, while histatins are predominantly antifungal[185,186,238,240]. These peptides may work individually and/or synergistically against pathogens[222,232,241].

The roles of these cationic antimicrobial peptides in a range of infections have been intensely investigated. A recent *in vivo* study by Nizet *et al.*[242] showed that a Group A *Streptococcus* (GAS) mutant with increased resistance to CRAMP, the mouse homologue of LL-37, was more virulent and caused more severe skin infections in mice than the parental strain. Groisman *et al.*[243] also demonstrated that *Salmonella typhimurium* mutant strains with increased susceptibility to host antimicrobial peptides were attenuated in virulence *in vivo* when inoculated into mice. These data not only established the significance of host antimicrobial peptides in defense against infection *in vivo* but also suggested that the ability to resist host antimicrobial peptides is a virulence property of these species.

A handful of studies have investigated the susceptibility of *S. mutans*, primarily laboratory strains, to host antimicrobial peptides in saliva[185,187,244,245]. *S. mutans* showed variable susceptibility to these peptides and their susceptibility patterns appeared to be strain-specific. However, the potential contribution and association of the susceptibility profiles of this cariogenic bacterium and host caries experience has not been defined.

The objective of this study was to determine the susceptibility of *S. mutans* to a panel of salivary antimicrobial peptides, which included HNP-1-3, HBD-2-3 and LL-37, in relation to host caries experience. We showed that in general, *S. mutans* strains from

caries-free subjects were more susceptible to host antimicrobial peptides than those from high caries subjects. The differences in strain susceptibility to these peptides may influence the colonization of *S. mutans* strains in biofilms and potentially contribute to an individual's relative risk for dental caries.

## Results

### Subject demographics

Of the sixty subjects participating in this study, thirty-eight subjects were females and twenty-two were males. We initially tried to match the sex of subjects between the high caries and caries-free groups. However, we were not able to isolate *S. mutans* from approximately 30% of the original subjects, the majority of whom were caries-free subjects. Substitutes for those subjects were necessary and due to the limited number of samples available from high caries boys, the final breakdown of samples were from 20 girls/10 boys and 18 girls/12 boys in the high caries and caries-free groups, respectively. Fifty-eight subjects were Caucasian and one each were Hispanic and Black. All except two subjects were in their permanent dentitions. Subjects were healthy with no current health condition. One subject reported current antibiotics use. The majority of subjects (87%) brushed their teeth at least daily. These subjects had variable fluoride exposure and percent carbohydrate intake, ranging from 0.079-2.044 mg/day and 31.72-67.08%, respectively. Detailed demographics and dietary history by subject are shown in Appendices A and B, respectively.

### MS in dental plaque

To examine the relationship between the presence of MS in dental plaque and the caries experience of subjects, the levels of MS and total cultivable flora from plaque samples were enumerated. Since the amount of bacteria recovered from plaque samples

may depend on possible variations in dental plaque collection, the level of MS is presented as the ratio of MS to total plaque bacteria, in addition to the MS count alone.

Subjects showed highly variable MS counts within dental plaque. We found a statistically significant difference in the level of MS between high caries and caries-free groups, for both the straight MS count ($p=0.0391$) and the ratio of MS to total plaque bacteria ($p=0.0243$). The average MS recovered from high caries subjects was $1.56x10^6$ CFU/ml (ranging from $2.10x10^3$ to $1.22 \times 10^7$ CFU/ml), whereas caries-free subjects harbored an average of $5.42x10^5$ CFU/ml (ranging from $5.40x10^3$ to $4.25x10^6$ CFU/ml) (Figure 3-1A). Likewise, the average ratio of MS to total bacteria from dental plaque of high caries subjects was 9.622 (ranging from 0.073 to 24.458), also significantly greater than the average value of 5.580 (ranging from 0.106 to 27.333) of caries-free subjects (Figure 3-1B). No difference was observed between total plaque bacteria between both groups ($p>0.05$). When comparing MS levels between males and females, no significant differences in the mean *S. mutans* count and the mean ratio of *S. mutans* to total bacteria in dental plaque were observed.

## Genotyping of *S. mutans* clinical isolates

Ten *S. mutans* isolates were collected from plaque samples of each subject, giving a total of 600 isolates equally divided between caries-free and caries-active subjects. AP-PCR patterns generated from the DNA of these *S. mutans* isolates contained approximately 8-15 bands from each primer, representing fragments ranging from 0.3 to more than 1.5 kilobases in size. Overall, we found 74 distinct *S. mutans* amplitypes from 60 subjects. A single distinct amplitype was observed in 47 subjects (78.33%). Twelve subjects (20%) harbored two distinct amplitypes and one subject (1.67%) harbored three amplitypes. Of the 13 subjects harboring more than one *S. mutans* amplitype, only 2 subjects (15.38%) were caries-free whereas 11 subjects (84.62%) belonged to the high caries group (Table 3-1).

Based on the nonparametric Wilcoxon rank-sum test, there was strong evidence that the two groups differed in the number of *S. mutans* amplitypes per subject. The mean number of *S. mutans* amplitypes was significantly greater in subjects with high caries experience than what was recovered from caries-free subjects (p=0.0051). No differences in the numbers of distinct *S. mutans* amplitypes between males and females were observed (p=0.9316). This suggested that high caries subjects were likely to harbor a more diverse population of *S. mutans* than caries-free subjects. Overall, 42 genotypically distinct *S. mutans* isolates were recovered from the high caries group and 32 isolates were recovered from the caries-free group. The AP-PCR fingerprinting profiles of all *S. mutans* isolates are shown in Appendix C.

A dendrogram analysis further demonstrated the degree of genotypic variation among the 74 *S. mutans* strains obtained from all subjects (Figure 3-2). The percent similarities between each strain-pair based on the Pearson product-moment correlation ranged from 47% to 98.6%, indicating a large amount of genetic heterogeneity among the strains. Strains from either caries-free or high caries subjects did not appear to be consistently clustered in the dendrogram, indicating that the genetic variation of among *S. mutans* strains based on the AP-PCR with two random primers was inadequate to reflect their cariogenicity.

The majority of *S. mutans* amplitypes obtained from the same subjects tended to cluster together with a fairly high percent similarity, suggesting that they are closely related. These amplitypes likely were generated from mutational events in a single early established clone within a particular host, rather than by acquisition of new strains from the external environment. The dendrogram analysis of 27 *S. mutans* strains obtained from subjects harboring more than 1 distinct amplitype is shown in Figure 3-3.

## Susceptibility of *S. mutans* clinical strains to salivary antimicrobial peptides and host caries experience

Seventy four *S. mutans* clinical strains, from 60 subjects were analyzed for their susceptibilities to HNP-1, HNP-2, HNP-3, HBD-2, HBD-3, and LL-37. Of all peptides tested, HBD-3 and LL-37 showed the most potent bactericidal activity against *S. mutans* strains. Among the α-defensins, *S. mutans* strains were most susceptible to HNP-1, followed by HNP-2 and HNP-3, respectively.

To assess the relationship between the susceptibility of *S. mutans* strains and host caries experience, two-sample t-tests were used to compare the susceptibility profiles of *S. mutans* isolates from high caries and caries-free subjects. *S. mutans* strains were exposed to 3 different concentrations (5 μg/ml, 1.5 μg/ml and 0.5 μg/ml) of each peptide. Since some subjects possessed more than one amplitype and because the proportion of each amplitype was variable in these subjects, we calculated the average susceptibilities of *S. mutans* strains from each clinical group using two methods: the first used the percent viability of the predominant *S.mutans* amplitype from each subject to ensure equal weighting per subject, and the second method averaged the percent viabilities of all distinct amplitypes to account for all amplitypes that were isolated.

Comparison of the *S. mutans* susceptibilities to HNP-1 showed that strains from the two clinical groups differed in their average percent viabilities for the predominant strains at all three HNP-1 concentrations. *S. mutans* strains from caries-free subjects showed significantly greater susceptibility to HNP-1 than those from children with high caries experience at 5 ug/ml ($p=0.0093$), 1.5 μg/ml ($p=0.0366$), and 0.5 μg/ml ($p=0.0287$). Analyzing the average percent viability of all strains resulted in significant differences at HNP-1 concentrations of 5 μg/ml ($p=0.0148$) and 0.5 μg/ml ($p=0.0320$). The similar trend was also observed at 1.5 μg/ml HNP-1; however, this did not quite reach statistical significance ($p=0.0754$) (Figure 3-4 A and B).

*S. mutans* strains from high caries and caries-free subjects also showed significant differences in their susceptibility to HNP-2. Analyses of both the predominant strains and all strains revealed that the average percent viability of *S. mutans* exposed to 5 μg/ml HNP-2 was significantly greater when isolated from subjects with high caries experience than when isolated from children with no caries experience ($p=0.0116$ and 0.0222, respectively). No significant differences were observed at concentrations of 1.5 and 0.5 μg/ml HNP-2 (Figure 3-5A, B).

In contrast to results with HNP-1 and HNP-2, *S. mutans* isolates showed nearly 2-fold less susceptibility to HNP-3. At all three concentrations tested, no statistically significant differences in susceptibility to HNP-3 were detected between *S. mutans* strains from high caries and caries-free groups (Figure 3-6A, B).

Susceptibility tests with two β-defensins, HBD-2 and HBD-3 also showed significant differences in the percent viability between *S. mutans* strains from high caries and caries-free subjects. *S. mutans* strains from caries-free subjects showed significantly greater susceptibility to HBD-2 at 1.5 μg/ml and 0.5 μg/ml (Figure 3-7A, B) and HBD-3 at 0.5 μg/ml (Figure 3-8A, B) whether analyzing the predominant strains or all strains ($p<0.05$). Statistically significant differences between both groups were not observed at 5 μg/ml of HBD-2, nor at 1.5 μg/ml and 0.5 μg/ml of HBD-3.

Lastly, the tests of *S. mutans* strains against LL-37 revealed significant differences in susceptibility between isolates from high caries and caries-free subjects. *S. mutans* strains from caries-free subjects showed greater susceptibility to LL-37 at 1.5 μg/ml and 0.5 μg/ml, compared to those from high caries subjects (Figure 3-9A, B). Though the difference did not reach statistical significance, the same trend was also noted at 5 μ/ml LL-37 for both the analyses of predominant strains ($p=0.0586$) as well as all strains ($p=0.0556$).

No significant differences were observed between males and females in the susceptibility profiles of *S. mutans* strains to each antimicrobial peptide at all concentrations tested ($p>0.05$).

## Relationships between genotypic variations of *S. mutans* strains and the susceptibility profiles to antimicrobial peptides

We next asked if the differences in susceptibility patterns of *S. mutans* to salivary antimicrobial peptides were related to the genotypic variation among strains. To test this, Spearman's rank correlation analyses were performed to examine the correlations between the percent similarities of *S. mutans* amplitypes obtained from the subjects who harbored more than 1 unique amplitype, according to the clustering analysis and the pair differences in percent viability to each antimicrobial peptide at 5 ug/ml. Data showed no statistically significant correlations between the strain genotypic similarities, based on the AP-PCR pattern, and their susceptibilities to HNP-1 ($p=0.5075$), HNP-2 ($p=0.3278$), HNP-3 ($p=0.9095$), HBD-2 ($p=0.5159$), HBD-3 ($p=0.3212$) or LL-37 ($p=0.2714$).

## Relationships between susceptibility profiles of *S. mutans* to antimicrobial peptides

Our findings showed that all antimicrobial peptides tested were active against *S. mutans* strains in a dose-dependent manner. These peptides share several common properties. They are small, cationic and are believed to exert their antimicrobial activities on the bacterial cytoplasmic membrane. It was of interest to examine if *S. mutans* strains showed similar susceptibility patterns to these antimicrobial peptides. To determine this, the percent viabilities of *S. mutans* exposed to each peptide at 5 μg/ml was correlated using Pearson Correlation analysis.

The data showed significant correlations between the percent viability to HNP-1 and the percent viability to HNP-2 ($r=0.94$, strong correlation; $p<0.0001$), between the

percent viability to HNP-1 and the percent viability to HNP-3 (r=0.69, moderate correlation; p<0.0001), between the percent viability to HNP-2 and the percent viability to HNP-3 (r=0.78, moderate correlation; p<0.0001), between the percent viability to HBD-2 and the percent viability to HBD-3 (r=0.56, moderate correlation; p<0.0001), between the percent viability to HBD-2 and the percent viability to LL-37 (r=0.60, moderate correlation; p<0.0001), and between the percent viability to HBD-3 and the percent viability to LL-37 (r=0.53, moderate correlation; p<0.0001) (Figure 3-10A-F). Analyses between the three α-defensins and the β-defensins or LL-37 revealed no significant correlations (Table 3-2).

### Correlations between the susceptibility of *S. mutans* to antimicrobial peptides and MS in dental plaque

Pearson correlation analyses were also performed to investigate whether there were relationships between the susceptibility profiles of *S. mutans* strains to each antimicrobial peptide and the levels of MS in dental plaque. No statistically significant correlations were found in all analyses (p>0.05).

### Additive and synergistic activities of antimicrobial peptide combinations

We further examined the effect of various antimicrobial peptides in combination using the checkerboard assay. Synergy or antagonism was evaluated based on the deviation from the additive in isobolograms. As shown in the preceding sections, the antimicrobial activities of HNP-1-3, HBD-2-3, LL-37 were dose-dependent. The activity of each possible combination of these peptides was examined against a representative *S. mutans* clinical isolate.

Firstly, the combinations among the α-defensins and between the β-defensins (HNP-1/HNP-2, HNP-1/HNP-3, HNP-2/HNP-3, and HBD-2/HBD-3) all produced straight isobols, indicating additive interactions between these peptide pairs (Figure 3-11

A-C, M). Various combinations across different types of peptides showed either additive or synergistic interactions. The combinations of either HNP-1 or HNP-2 with HBD-3 produced additive interactions, while combining HNP-3 and HBD-3 showed synergistic effect (Figure 3-11 D-F). All three α-defensins combined with HBD-2 showed synergistic interactions (Figure 3-11 G-I). Also functioning synergistically were the combinations of each of HNP-1-3 and LL-37 (Figure 3-11 J-L), whereas LL-37 and β-defensins worked together additively (Figure 3-11 N, O). None of the combinations tested demonstrated antagonistic activity. The combined effects of these antimicrobial peptides were also represented mathematically as an FIC index shown in each figure.

The antimicrobial susceptibility tests examining the activities of lysozyme and histatin-5 against selected *S. mutans* clinical strains revealed no susceptibility to these substances at concentrations 20 μg/ml or below (data not shown). Lysozyme has been shown to hydrolyze the cell wall polysaccharide of Gram-positive bacteria and histatins are broad-spectrum antimicrobial peptides with predominantly antifungal activity. Previous studies reported the potentially increased antimicrobial activities of β-defensins and/or LL-37 in the presence of lysozyme[222,244]. To investigate this effect in *S. mutans*, we examined the bactericidal activity of these antimicrobial peptides in the presence of subinhibitory concentrations of lysozyme and histatin-5. The combinations of lysozyme or histatin-5 with HNP-1-3, HBD-2-3 or LL-37 showed no differences in *S. mutans* susceptibility compared to those of a single peptide working alone (Figure 3-12A-L). This indicated that no synergistic activity was detected when either lysozyme or histatin-5 was combined with other peptides at the ratio of peptides tested.

## Discussion

It is recognized that *S. mutans* is widely distributed in persons with dental caries, however, this bacterium also can be found in the caries-free population[246-249]. A few differences in virulence traits between *S. mutans* strains from caries-free and caries-active

individuals have been demonstrated, such as the magnitude of water-insoluble glucan synthesis and the degree of acid tolerance[62,67,69]. However, the interactions between *S. mutans* clinical strains and salivary factors in relation to host dental caries experience have not been examined. In this chapter, several findings were demonstrated. We showed for the first time that *S. mutans* strains from individuals with high caries experience showed greater resistance to host salivary antimicrobial peptides, compared to those obtained from caries-free individuals, suggesting that the ability of *S. mutans* to resist the antimicrobial activity of these peptides may be another factor that can help define the relative virulence of this bacterium.

Following testing of *S. mutans* strains with each peptide individually, LL-37 and HBD-3 appeared to be the most potent antimicrobial peptides against this bacterium, whereas HNP-3 was the least potent. In this study, we deliberately selected lower concentrations of antimicrobial peptides to assay in order to limit the amount (and expense) of peptides used and to better reflect physiological levels of these peptides in saliva. We observed significantly greater resistance of *S. mutans* strains from high caries subjects than those from caries-free subjects to HNP-1, HNP-2, HBD-2, HBD-3 and LL-37 at varying concentrations. Although the susceptibility profiles of *S. mutans* strains to the three HNPs were highly correlated, significant differences between the susceptibilities to HNP-3 between both groups were not observed at the three concentrations tested, potentially due to the overall lower antimicrobial activity of this peptide against *S. mutans*. The susceptibility profiles of *S. mutans* strains to HBD-2, HBD-3 and LL-37 were also correlated, suggesting that these peptides may act by similar mechanisms yet possess varying potentials for combating pathogens.

Based on our data, we speculate that *S. mutans* strains that are more resistant to host antimicrobial peptides may have an ecological advantage over the more susceptible strains for surviving within dental plaque and causing dental caries. We acknowledge that our results are based on the *in vitro* susceptibility testing of the monocultures of clinical

isolates of *S. mutans* with a single peptide, whereas under *in vivo* conditions, multiple factors must be taken into consideration, including the interactions between mixed populations of microbial species, the different quantities of each species, and the presence of other environmental factors such as available nutrients, pH, oxygen, as well as the actions of various other host defense factors in saliva. These multiple factors are likely to varied within each subject at any particular point in time, as biofilms constantly adjust to the dynamic equilibrium with the surrounding environmental factors. Longitudinal controlled studies are needed to potentially provide more insight into the interactions of all these components *in vivo* in relation to the dental caries risk. However, we believe that the differences in the inherent susceptibilities to salivary antimicrobial peptides among *S. mutans* strains shown in this study could support a biologically relevant role for host antimicrobial peptides in saliva for several reasons.

Within a mixed microbial population in dental plaque, a particular bacterial strain or species not only must resist host defense factors but also has to compete with other species for survival and successful colonization. It has long been observed that *S. mutans* compete relatively poorly with other non-pathogenic microorganisms at neutral pH[250]. Several studies have shown that *S. mutans* strains are more susceptible to host antimicrobial peptides than oral streptococci that constitute the major proportion in normal non-pathogenic plaque, such as *S. sanguinis S. mitis* and *S. oralis*[185-187,240]. This suggests that salivary antimicrobial peptides may play a role in maintaining the health of the oral cavity by limiting the growth of potentially pathogenic organisms such as *S. mutans* and allowing the more resistant non-pathogenic bacteria to establish colonization and dominate, thereby diminishing colonization by potentially pathogenic organisms. Therefore, differences in susceptibility profiles of *S. mutans* strains, either large or small, may be significant in providing the resistant strains with a more competitive advantage over susceptible strains. In particular, under conditions favorable for growth, such as the

increased presence of fermentable sugar and low pH, resistant *S. mutans* strains may better survive and populate leading to greater cariogenic potential.

In addition, the relative antimicrobial susceptibility of biofilm bacteria could be influenced by inter-species interactions. It has recently been shown that *S. gordonii* can inactivate competence-stimulating peptide (CSP) of *S. mutans*, antagonizing its quorum-sensing mechanisms[251]. Inactivation of CSP in turn leads to the diminished resistance of *S. mutans* to a variety of antimicrobial agents[252]. Therefore, the *in vivo* sensitivity of *S. mutans* to host antimicrobial peptides in saliva may be increased due to interactions with other plaque biofilm species.

Another consideration is that the numbers of bacteria used in the assays were controlled, based on the optimal assay conditions and may not be directly equivalent to those present *in vivo*. For example, to obtain a sufficient level of alamarBlue® reduction in sodium phosphate buffer for testing with HBD-2, HBD-3 and LL-37, we used $1x10^8$ CFU/ml of *S. mutans* in suspension, which is approximately 50-100-fold greater than that commonly used in other *in vitro* studies and also 10-$10^4$-fold higher than the MS levels detected in the dental plaque of subjects in this study. In addition, these antimicrobial peptides when present together acted additively as well as synergistically to increase their antimicrobial activity. Overall, although the actual antimicrobial activity of peptides *in vivo* may not easily be determined, it could be substantially higher than that demonstrated *in vitro*.

Lastly, to be able to cause disease, *S. mutans* must adhere to tooth surfaces and accumulate in sufficient numbers with dental plaque[253]. In the process of biofilm formation from planktonic phase cells, oral bacteria initially adhere to the tooth surface, as a dispersed monolayer. Relying primarily on the nutrients from the environment, adhered bacteria continue to grow to a point where their extent and mass are limited by characteristics of the local environment. Under *in vitro* studies, these stages occur during the first 12-20 hrs, whereas *in vivo* the period is more prolonged, varying from 18-48 hrs,

before the plaque numbers become relatively stable[254]. The time it takes for *S. mutans* to accumulate and form a critical mass within dental plaque may be another factor determining the effect of antimicrobial peptides *in vivo*. This longer timeframe relative to *in vitro* growth may allow the various antimicrobial peptides to act on microorganisms more efficiently as they are continuously produced from their sources.

In this study, we used the AP-PCR technique to genotype *S. mutans* isolates using two random primers. AP-PCR genotyping has been widely used to study the genetic differences among MS isolates with a good discrimination ability[62,255-258]. We found that the majority of subjects harbored only one unique *S. mutans* genotype in dental plaque. This could be due to the fact that we used site-specific plaque samples, not pooled plaque samples from multiple sites or saliva samples as a source of *S. mutans*. It is possible that each oral site represents a unique ecological niche that may benefit different strains of bacteria. However, even though the numbers of *S. mutans* genotypes recovered per subjects have been variable among studies, a number of these studies have found, similar to this study, only one unique *S. mutans* genotype in the majority of subjects, using either plaque or saliva samples as bacterial sources[56,63,255,259].

In accordance with previous studies[62,64], we also showed that caries-active subjects tended to harbor greater numbers of *S. mutans* genotypes than caries-free subjects. It has been proposed that environmental factors such as the availability of fermentable carbohydrate, the presence of inhibitory substances or competing bacteria may be responsible for the gain, loss or preservation of certain bacterial genotypes[45]. Regardless of the causes, the increased diversity of *S. mutans* may permit simultaneous interactions among different strains that may further increase the overall cariogenicity of this bacterial species[62].

Dendrogram analysis of AP-PCR fingerprint data from both primers revealed the genetic heterogeneity among *S. mutans* clinical strains. Strains from caries-free or high caries subjects did not form uniform clusters in the dendrogram. We also observed that *S.*

*mutans* strains derived from subjects with more than 1 unique genotype appeared to have highly similar fingerprint profiles. Strains from 9 out of 13 subjects showed only a few band differences in the AP-PCR profiles compared to other genotypes from the same subject. The similarity values of these strains were more than 90% and tended to cluster closely together in the dendrogram. Only two subjects harbored *S. mutans* strains with less than 80% similarity. The high similarity among strains from the same subjects suggests that mutation or genetic rearrangement of a single colonizing strain may be primarily responsible for the increased strain diversity, rather than the independent acquisition of unique strains from the external environment.

We also examined the relationships between the susceptibility profiles of *S. mutans* to each antimicrobial peptide and found that the profiles appeared to be significantly correlated among the three HNPs and among HBD-2, HBD-3 and LL-37. Pearson correlation coefficients indicated moderate to strong increasing relationships among *S. mutans* susceptibilities to the three HNPs. HNP-1-3 are highly homologous in their amino acid sequences and structures. Both mature HNP-1 and HNP-3 contain 30 amino acids and are identical except for the N-terminal amino acid, which is alanine for HNP-1 and aspartate for HNP-3. HNP-2 lacks this N- terminal amino acid and is believed to be formed by the degradation or processing of either HNP-1 or HNP-3. The amino acid sequences and structures of the β-defensins are more variable than those of HNP-1-3 with only the 6 cysteine and 1 glycine residues are conserved[260]. On the other hand, LL-37 is an α-helical peptide without cysteines, representing a distinct group of antimicrobial peptide with structural features clearly different from defensins. Nevertheless, Pearson correlation coefficients also indicated moderate increasing relationships between HBD-2-3 and LL-37.

The correlation analyses did not show significant relationships between the susceptibility profiles of *S. mutans* strains for HNP-1-3 and HBD-2-3/LL-37. It should be noted that the assays we used for detecting the susceptibility of *S. mutans* to these two

groups of peptides were slightly different. While we tested the activity of HNP-1-3 in MHB using $1x10^6$ CFU/ml of bacteria, the activity of HBD-2-3 and LL-37 were examined in sodium phosphate buffer with $1x10^8$ CFU/ml of bacteria. Therefore, we cannot rule out the possibility that these differences in assay conditions affected the correlation analyses of the susceptibility profiles between these two peptide groups. However, overall, we did observe significant correlations between antimicrobial peptides tested with similar assay conditions that showed moderate to strong increasing relationships, suggesting that these peptides may utilize similar mechanisms of antibacterial action. According to a number of previous studies, the interaction of these peptides with the bacterial cytoplasmic membrane appears to be the crucial step for their activities[107,156,261,262].

We further analyzed the relationships between the susceptibility profiles of *S. mutans* strains and the MS levels in the dental plaque samples from each subject. The correlation analyses did not show statistically significant correlations between the susceptibility profiles of *S. mutans* to any antimicrobial peptides tested and the MS count in plaque or the ratio of MS to total cultivable plaque flora. This may be due to the fact that MS levels are not strictly equivalent to the *S. mutans* measurement. While we limited the susceptibility testing to strains of *S. mutans*, MS levels can include *S. mutans* and *S. sobrinus*, the two common MS species found in human dental plaque. This may cause some discrepancies in the correlation analyses. In addition, in the antimicrobial susceptibility assays the susceptibility profiles of all *S. mutans* strains were compared using similar concentrations of antimicrobial peptides, whereas *in vivo* the levels of antimicrobial peptides in saliva were variable among subjects. This could represent another variable in the analyses, since the survival and growth of MS in dental plaque are likely influenced by the actual levels of antimicrobial peptides in host saliva at the particular moment of collection.

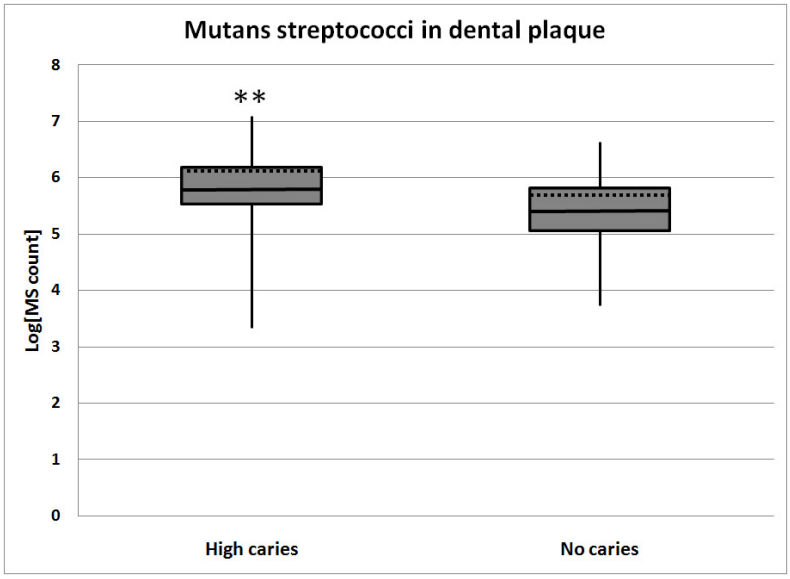
The interactions of antimicrobial peptides when used in combination were also examined. We found that any combination of HNP1-3, HBD-2-3 or LL-37 increased the antimicrobial activity against *S. mutans*. Combinations between the three α-defensins yielded additive interactions. Likewise, additive interactions were also observed in combinations of HBD-2, HBD-3 and LL-37. The latter findings were in agreement with a previous study in *E. coli* by Singh *et al.*[232], which reported the additive interactions between the β-defensins and LL-37. Also consistent with a previous study by Nagaoka *et al.*[241] in *E. coli* and *S. aureus*, we found that the combinations between HNP-1-3 and LL-37 were synergistic. Interactions between HNP-1-3 and HBD-2-3 have not been reported previously. In this study, we observed synergistic interactions when combining HNP-1-3 with HBD-2, and combining HNP-3 with HBD-3 whereas combinations between HNP-2-3 and HBD-3 appeared to be additive.

Evaluation of peptide pairing between either lysozyme or histatin-5 and HNP-1-3, HBD-2-3 or LL-37 did not reveal synergistic interactions. In contrast to our results, Singh *et al*.[232] using time-kill methods demonstrated that subinhibitory concentrations of LL-37 in combination with lysozyme, were synergistic against *E. coli*. This difference may be due to the fact that *S. mutans* clinical isolates did not show susceptibility to lysozyme, whereas *E.coli* in their study was susceptible to both lysozyme and LL-37. However, they noted the disagreement between the time-kill method and a checkerboard assay regarding the LL-37 and lysozyme combination; the time-kill method suggested synergism whereas the checkerboard method was consistent with an additive interaction. This suggests that the ratio of peptides used may be another variable that can influence the outcome of peptide interactions. In other words, whether the peptide pair shows a synergistic or additive interaction may depend on the relative amounts of each peptide in the combination[263]. Overall, these findings suggest that HNP-1-3, HBD-2-3 and LL-37 when present in the same environment can work additively or synergistically to further enhance their activity against microorganisms, in this particular case *S. mutans*.

Collectively, the experiments described in this chapter have corroborated that the increased MS levels in dental plaque as well as *S. mutans* genotypic diversity are associated with high caries experience, and have revealed that *S. mutans* strains from dental plaque are variably susceptible to host salivary antimicrobial peptides, and that *S. mutans* strains from high caries subjects have greater resistance to salivary antimicrobial peptides than those from caries-free subjects. In addition, it was determined that these peptides may exert their antimicrobial activity individually or in combination. Combining these peptides increased their activity against *S. mutans* in either an additive or synergistic manner. Thus, it can be concluded that the degree of resistance to host salivary antimicrobial peptides may represent a heretofore undescribed virulence factor for *S. mutans* that helps determine an individual's relative risk of caries.

**Figure 3-1 Enumeration of MS in dental plaque.** High caries subjects harbored significantly greater amounts of MS in dental plaque, compared to caries-free subjects. (A) MS count (p=0.0391). (B) the ratio of MS to total bacteria (p=0.0243). Box plots demonstrated median (solid line), mean (dotted line), range (error bar) and the interquartile range (box length), representing middle 50% of data.

A.




B.

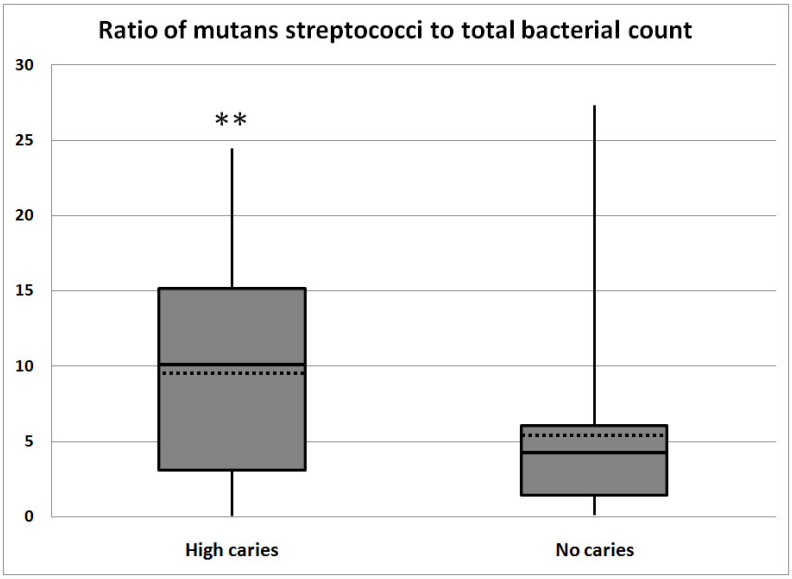
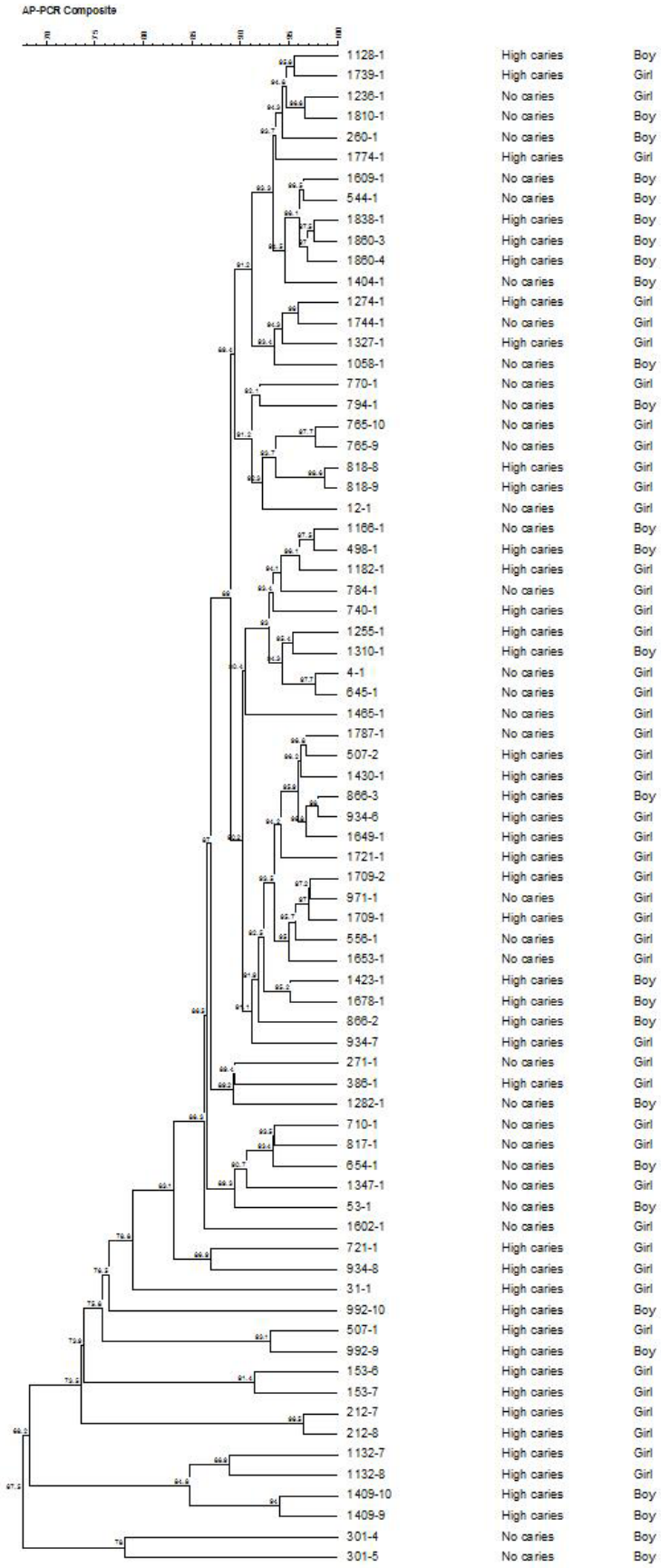


**Table 3-1 Numbers of subjects harboring 1-3 distinct *S. mutans* amplitypes.**

| Subjects | Sex | Numbers of distinct *S. mutans* amplitypes per subjects | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| All subjects | Girls (38) | 30 | 7 | 1 |
| | Boys (22) | 17 | 5 | 0 |
| | All (60) | 47 | 12 | 1 |
| High caries subjects | Girls (20) | 13 | 6 | 1 |
| | Boys (10) | 6 | 4 | 0 |
| | All (30) | 19 | 10 | 1 |
| Caries-free subjects | Girls (18) | 17 | 1 | 0 |
| | Boys (12) | 11 | 1 | 0 |
| | All (30) | 28 | 2 | 0 |

Subject distributions according to caries experience and sex are shown.

**Figure 3-2 Genetic relationships between the 74 *S. mutans* amplitypes obtained from 60 subjects.** The dendrogram was generated by UPGMA clustering analysis from the similarity matrices, based on Pearson product-moment correlation. Percent similarity values are shown. Amplitypes with the same number prior to the hyphen are from a single subject.


AP-PCR Composite
1128-1 High caries Boy
1739-1 High caries Girl
1236-1 No caries Girl
1810-1 No caries Boy
260-1 No caries Boy
1774-1 High caries Girl
1609-1 No caries Boy
544-1 No caries Boy
1838-1 High caries Boy
1860-3 High caries Boy
1860-4 High caries Boy
1404-1 No caries Boy
1274-1 High caries Girl
1744-1 No caries Girl
1327-1 High caries Girl
1058-1 No caries Boy
770-1 No caries Girl
794-1 No caries Boy
765-10 No caries Girl
765-9 No caries Girl
818-8 High caries Girl
818-9 High caries Girl
12-1 No caries Girl
1166-1 No caries Boy
498-1 High caries Boy
1182-1 High caries Girl
784-1 No caries Girl
740-1 High caries Girl
1255-1 High caries Girl
1310-1 High caries Boy
4-1 No caries Girl
645-1 No caries Girl
1465-1 No caries Girl
1787-1 No caries Girl
507-2 High caries Girl
1430-1 High caries Girl
866-3 High caries Boy
934-6 High caries Girl
1649-1 High caries Girl
1721-1 High caries Girl
1709-2 High caries Girl
971-1 No caries Girl
1709-1 High caries Girl
556-1 No caries Girl
1653-1 No caries Girl
1423-1 High caries Boy
1678-1 High caries Boy
866-2 High caries Boy
934-7 High caries Girl
271-1 No caries Girl
386-1 High caries Girl
1282-1 No caries Boy
710-1 No caries Girl
817-1 No caries Girl
654-1 No caries Boy
1347-1 No caries Girl
53-1 No caries Boy
1602-1 No caries Girl
721-1 High caries Girl
934-8 High caries Girl
31-1 High caries Girl
992-10 High caries Boy
507-1 High caries Girl
992-9 High caries Boy
153-6 High caries Girl
153-7 High caries Girl
212-7 High caries Girl
212-8 High caries Girl
1132-7 High caries Girl
1132-8 High caries Girl
1409-10 High caries Boy
1409-9 High caries Boy
301-4 No caries Boy
301-5 No caries Boy

.

**Figure 3-3 Genetic relationships between 27 *S. mutans* amplitypes obtained from 13 subjects who harbored more than 1 distinct *S. mutans* amplitype.** The dendrogram was generated by UPGMA clustering analysis from the similarity matrices, based on Pearson product-moment correlation. Percent similarity values are shown. Amplitypes with the same number prior to the hyphen are from a single subject.

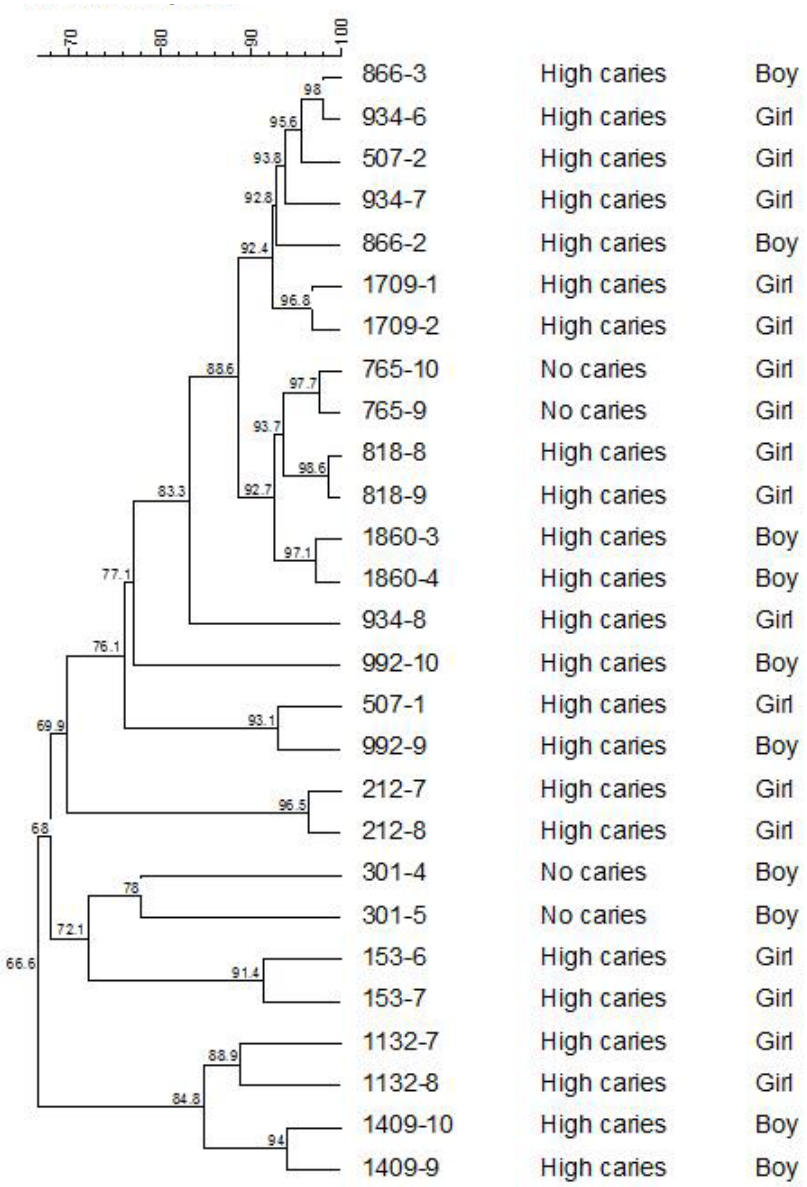
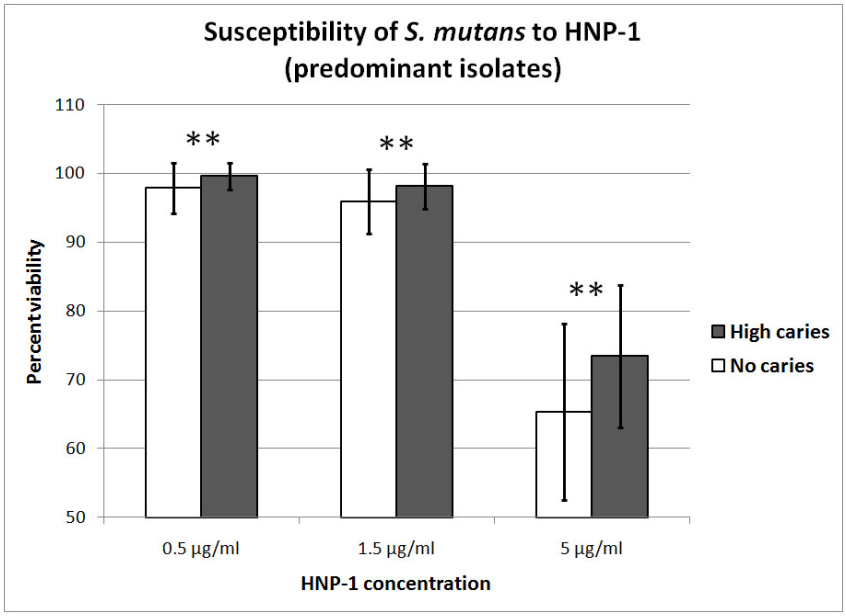


**Figure 3-4 Susceptibility of *S. mutans* to HNP-1 and host caries experience.** (A) Average percent viability based on the predominant amplitypes from each subject (B) Average percent viability of all amplitypes. Asterisks indicate statistically significant differences ($p<0.05$).

**A.**




**B.**

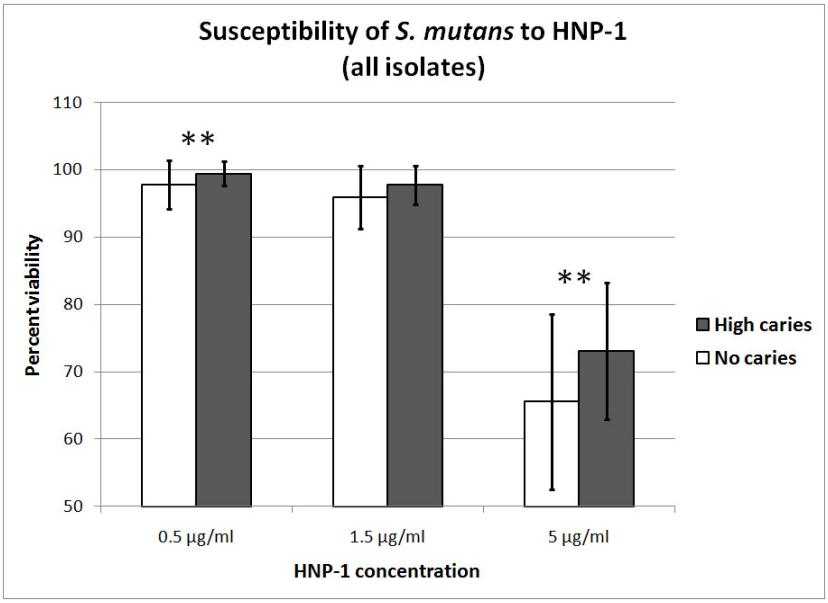
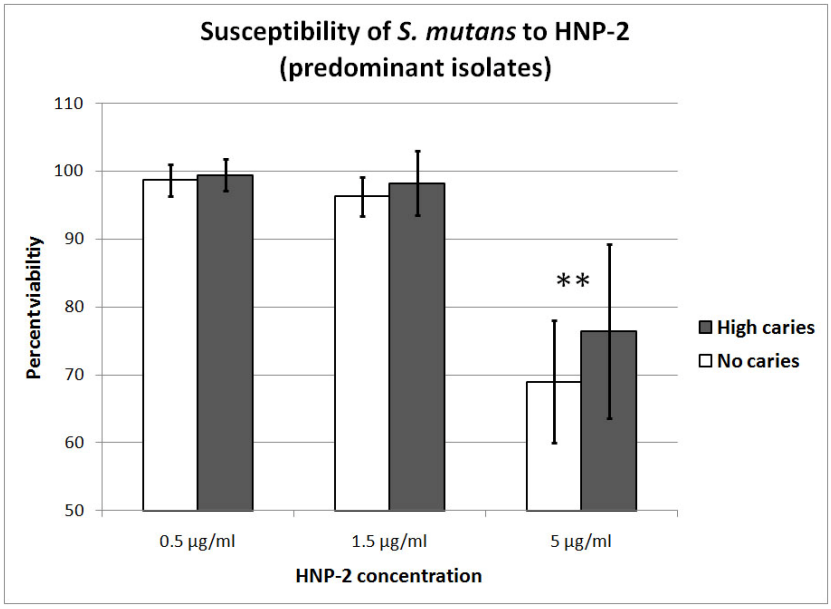


**Figure 3-5 Susceptibility of *S. mutans* to HNP-2 and host caries experience.** (A) Average percent viability based on the predominant amplitypes from each subject; (B) Average percent viability of all amplitypes. Asterisks indicate statistically significant differences ($p<0.05$).

**A.**




**B.**

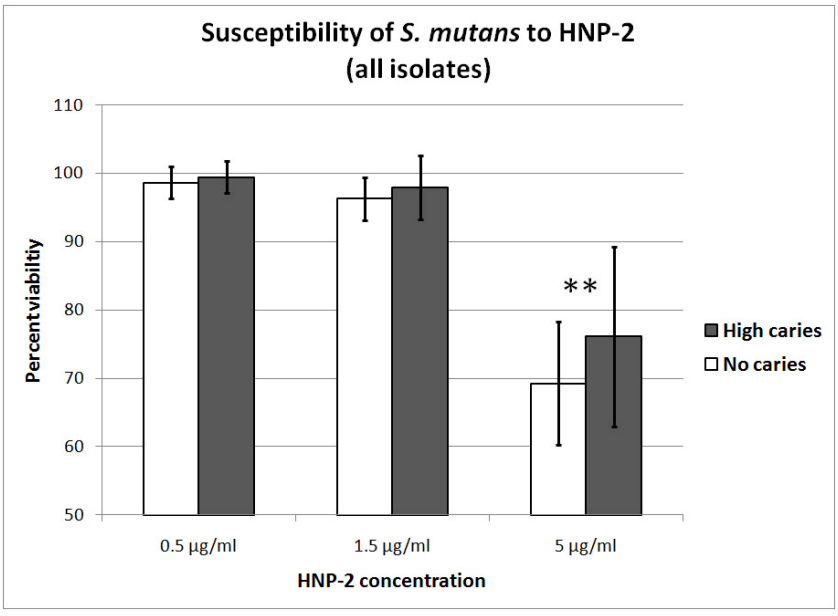
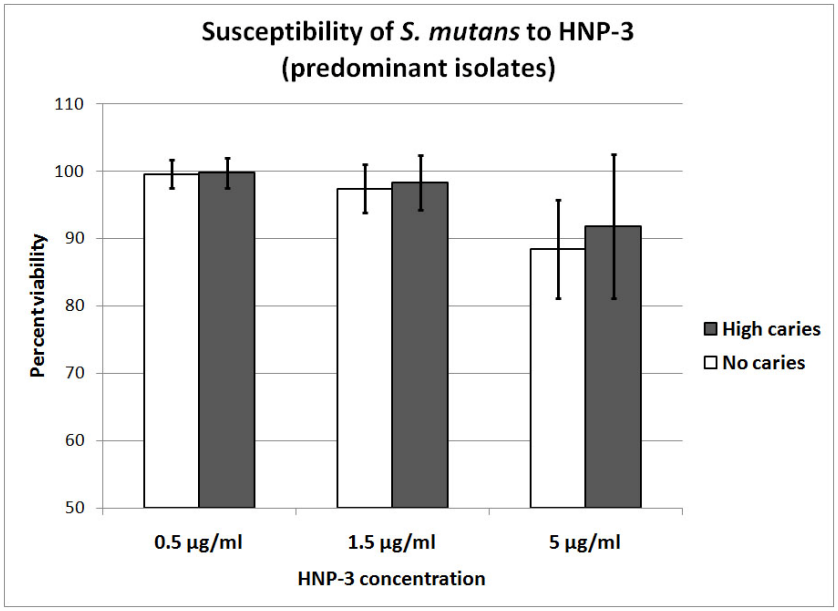


**Figure 3-6 Susceptibility of *S. mutans* to HNP-3 and host caries experience.** (A) Average percent viability based on the predominant amplitypes from each subject; (B) Average percent viability of all amplitypes. No statistically significant differences were found at any of the 3 HNPs concentrations tested.

**A.**




**B.**

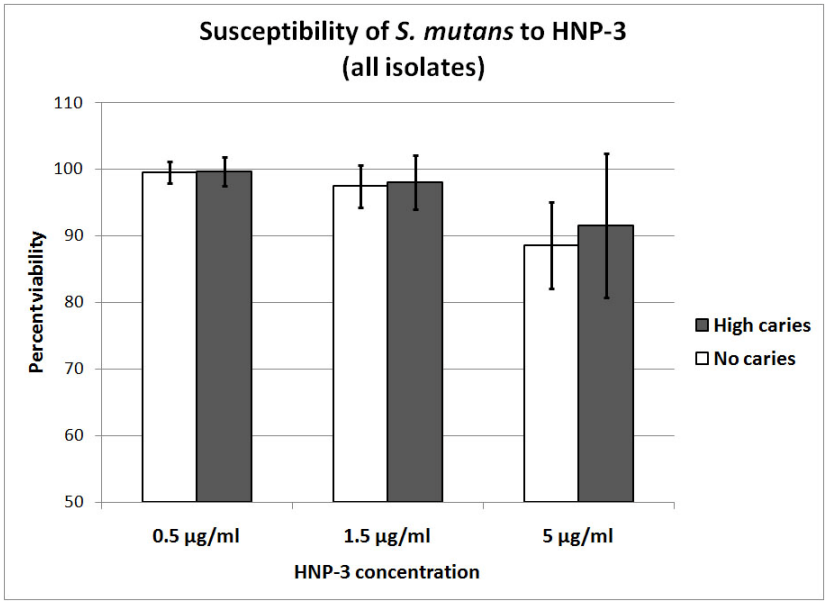
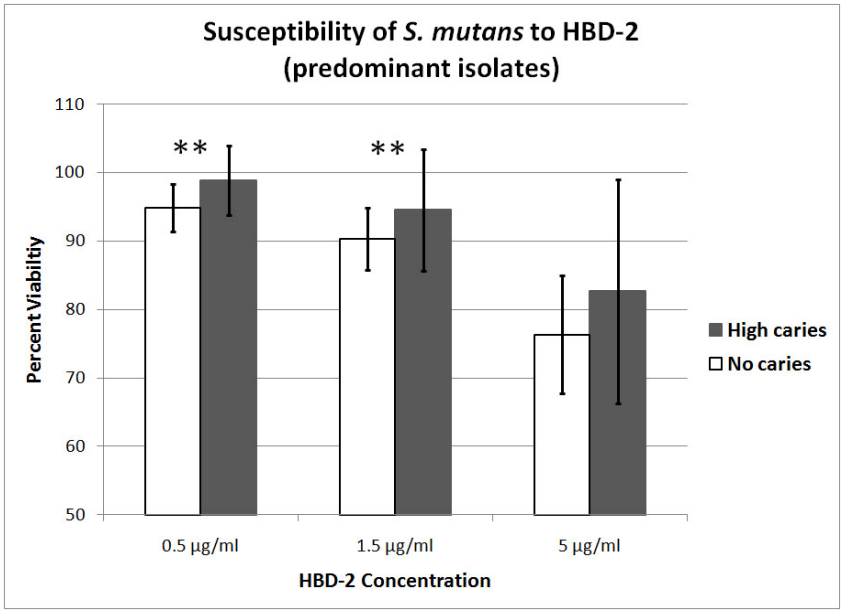


**Figure 3-7 Susceptibility of *S. mutans* to HBD-2 and host caries experience.** (A) Average percent viability based on the predominant amplitypes from each subject; (B) Average percent viability of all amplitypes. Asterisks indicate statistically significant differences ($p<0.05$).

**A.**




**B.**

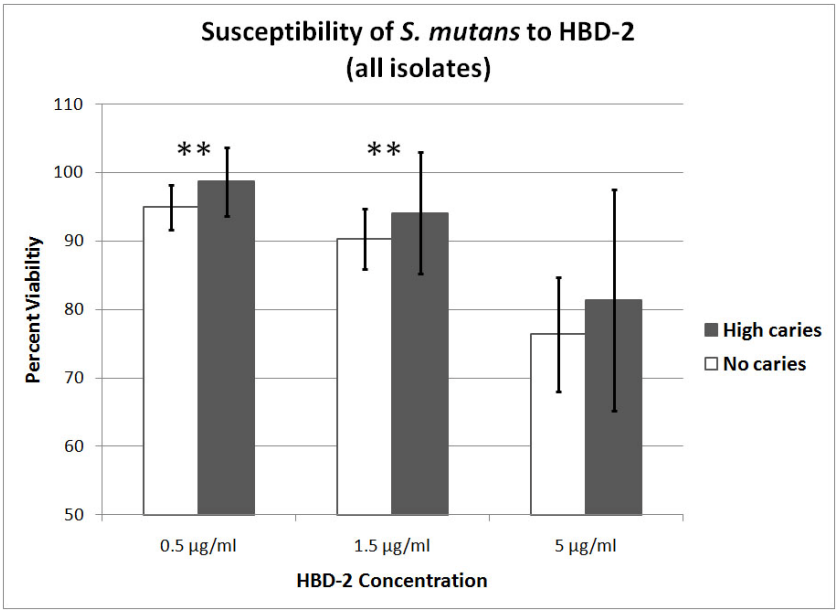
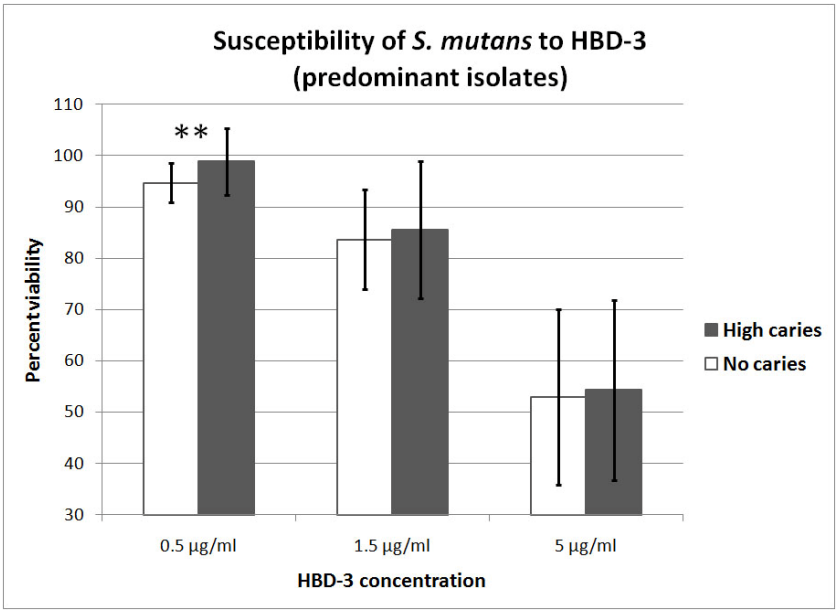


**Figure 3-8 Susceptibility of *S. mutans* to HBD-3 and host caries experience**. (A) Average percent viability based on the predominant amplitypes from each subject; (B) Average percent viability of all amplitypes. Asterisks indicate statistically significant differences ($p<0.05$).

**A.**




**B.**

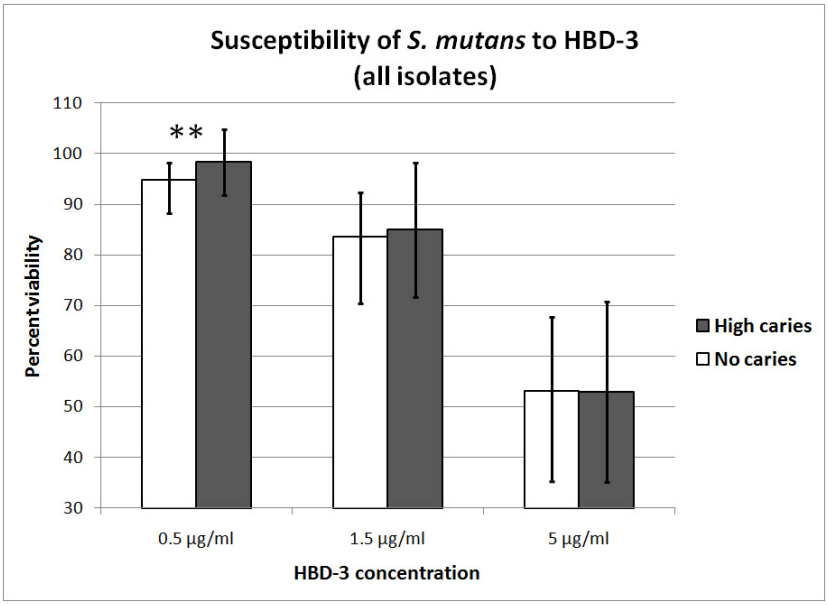
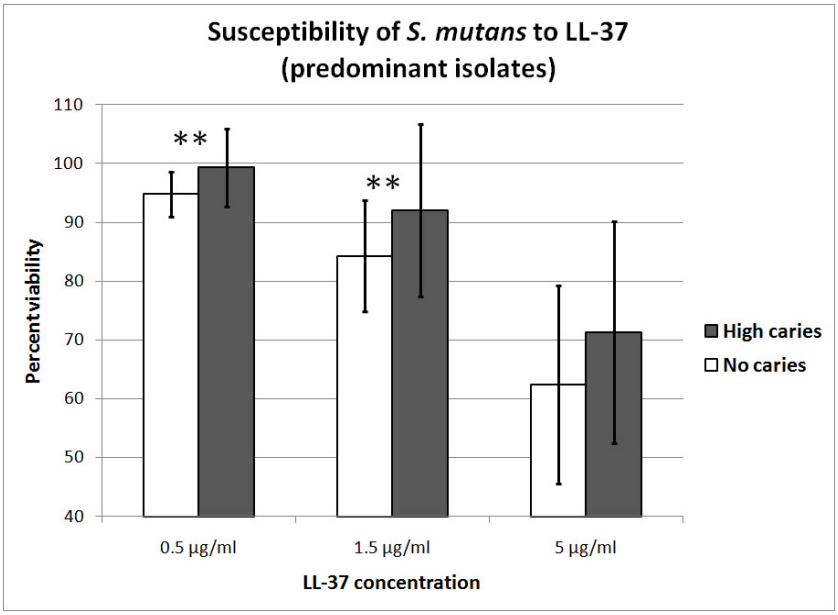


**Figure 3-9 Susceptibility of *S. mutans* to LL-37 and host caries experience.** (A) Average percent viability based on the predominant amplitypes from each subject; (B) Average percent viability of all amplitypes. Asterisks indicate statistically significant differences ($p<0.05$).

**A.**




**B.**

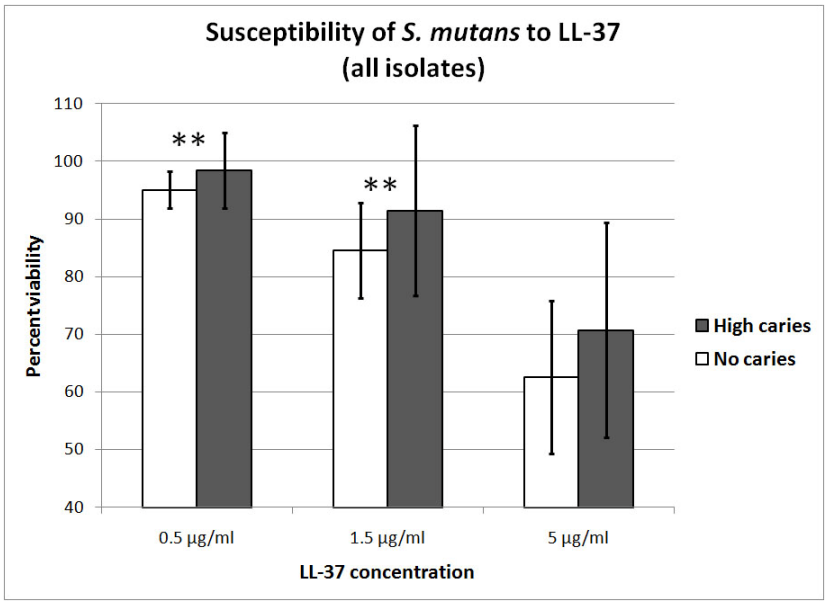


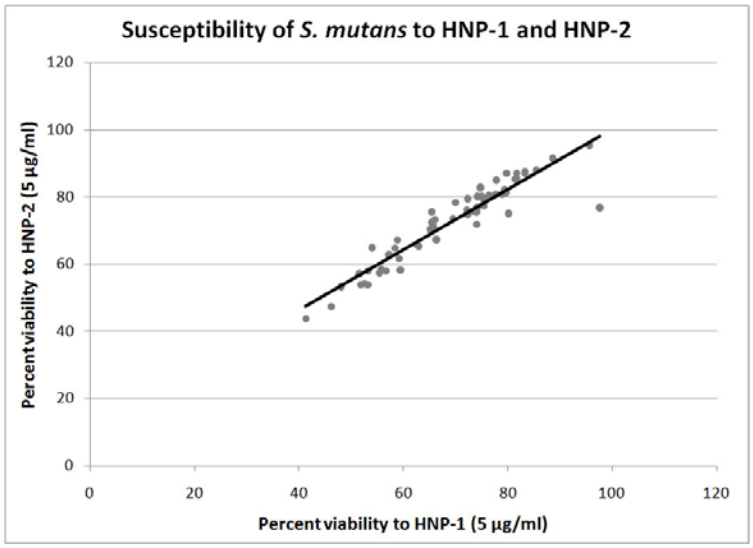
**Table 3-2 Correlations between the susceptibility profiles of *S. mutans* to each antimicrobial peptide at 5 μg/ml.**

| Susceptibility to the peptide pair | Correlation coefficients | P-values |
|---|---|---|
| HNP-1 and HNP-2 | 0.94 | <0.0001* |
| HNP-1 and HNP-3 | 0.69 | <0.0001* |
| HNP-2 and HNP-3 | 0.78 | <0.0001* |
| HNP-1 and HBD-2 | 0.01 | 0.9005 |
| HNP-1 and HBD-3 | -0.09 | 0.4702 |
| HNP-1 and LL-37 | 0.18 | 0.165 |
| HNP-2 and HBD-2 | -0.01 | 0.9653 |
| HNP-2 and HBD-3 | -0.01 | 0.9193 |
| HNP-2 and LL-37 | 0.15 | 0.2687 |
| HNP-3 and HBD-2 | 0.05 | 0.6958 |
| HNP-3 and HBD-3 | -0.01 | 0.9754 |
| HNP-3 and LL-37 | 0.07 | 0.5861 |
| HBD-2 and HBD-3 | 0.56 | <0.0001* |
| HBD-2 and LL-37 | 0.6 | <0.0001* |
| HBD-3 and LL-37 | 0.53 | <0.0001* |

Asterisks indicate statistically significant correlations ($p<0.05$). Pearson correlation coefficients give the strength of correlations (see text).

**Figure 3-10 Correlations between the susceptibility profiles of *S. mutans* to each antimicrobial peptide at 5 µg/ml.** Statistically significant correlations ($p<0.05$) were observed between the following peptide pairs, (A) HNP-1 and HNP-2; (B) HNP-1 and HNP-3; (C) HNP2 and HNP-3; (D) HBD-2 and HBD-3; (E) HBD-2 and LL-37; (F) HBD-3 and LL-37.

**A.**




**B.**

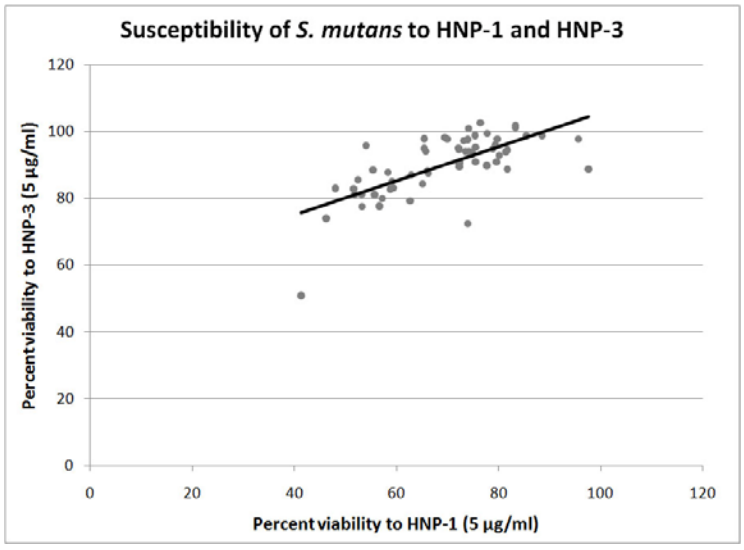
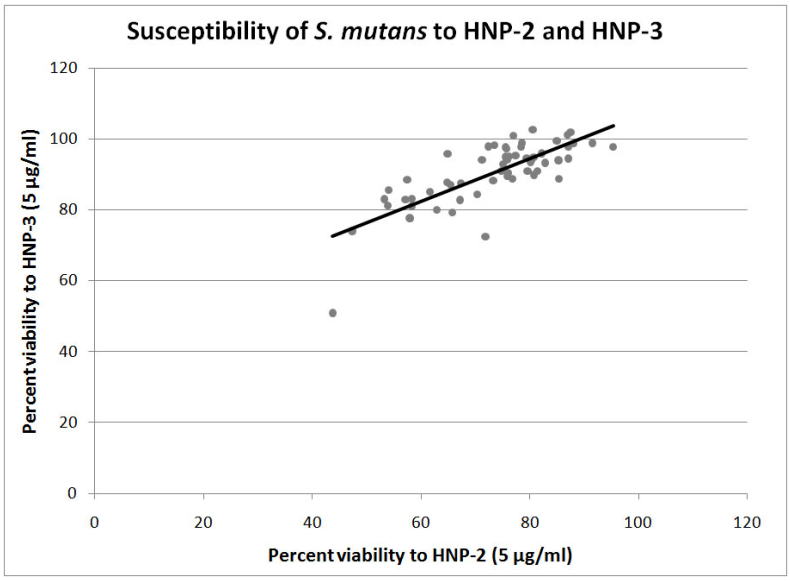


**Figure 3-10** Continued

**C.**




**D.**

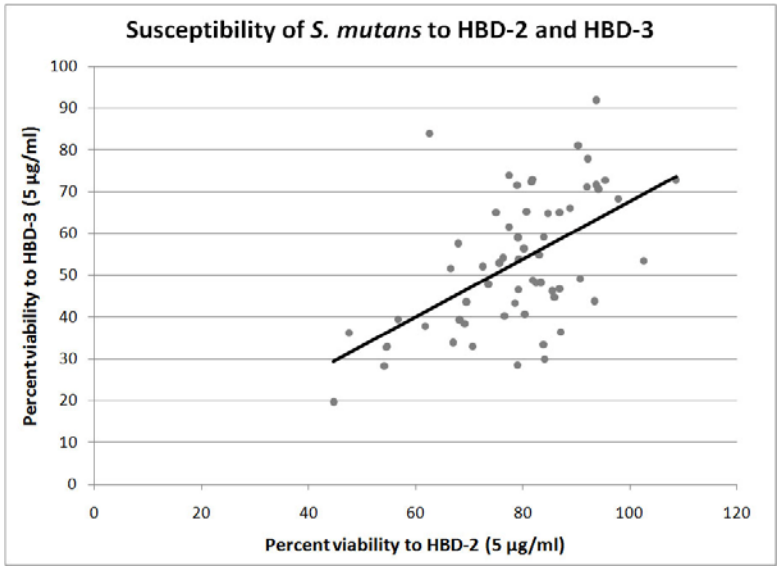
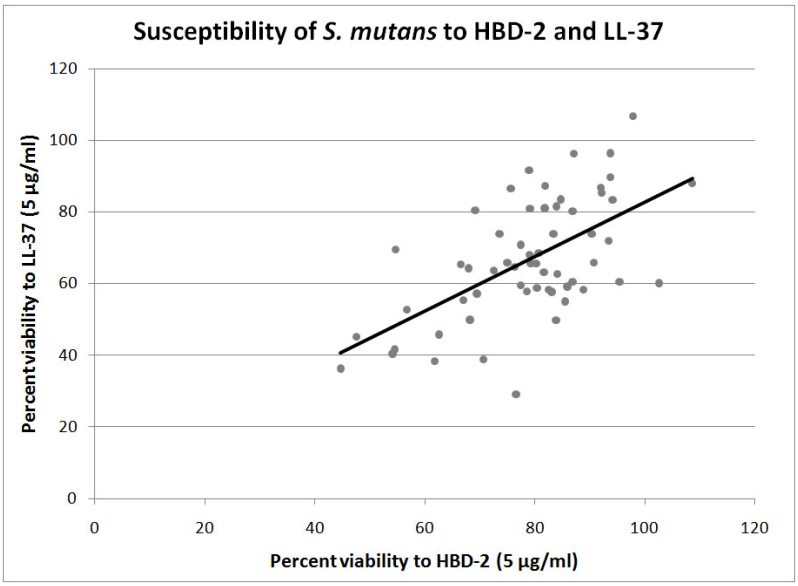


**Figure 3-10** Continued

**E.**




**F.**

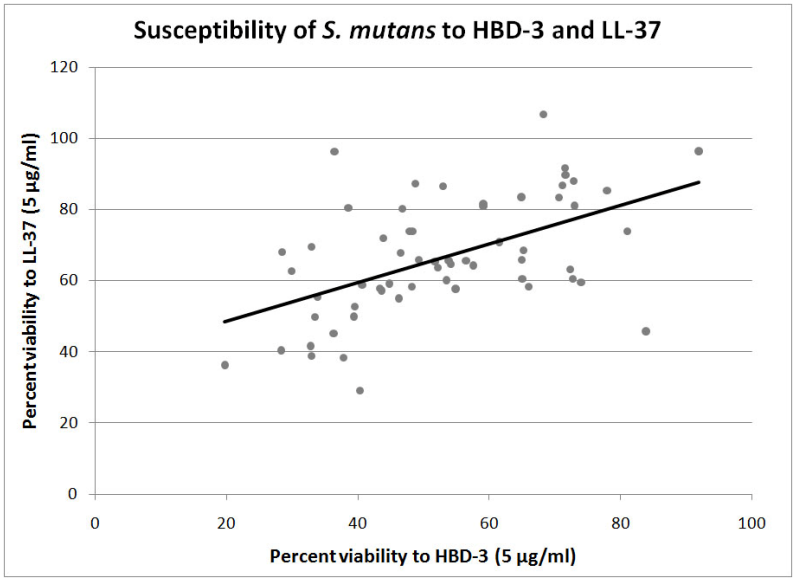
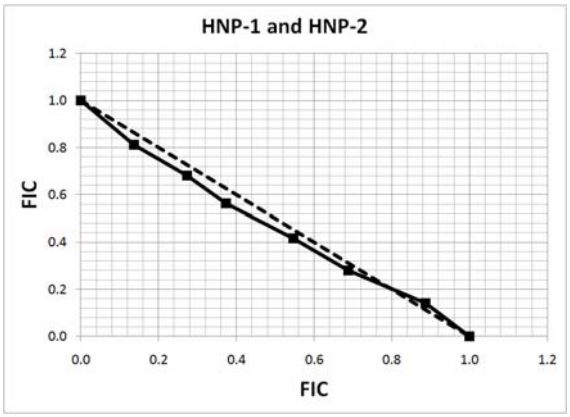


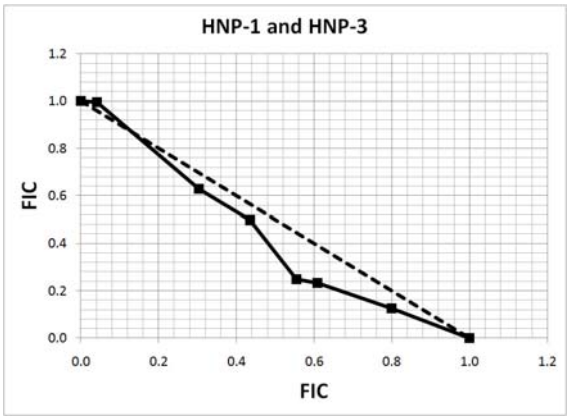
**Figure 3-11 Effect of antimicrobial peptide combinations.** Isobolograms of each peptide combination are shown. (A) HNP-1 and HNP-2; (B) HNP-1 and HNP-3; (C) HNP-2 and HNP-3; (D) HNP-1 and HBD-3; (E) HNP-2 and HBD-3; (F) HNP-3 and HBD-3; (G) HNP-1 and HBD-2; (H) HNP-2 and HBD-2; (I) HNP-3 and HBD-2; (J) HNP-1 and LL-37; (K) HNP-2 and LL-37; (L) HNP-3 and LL-37; (M) HBD-2 and HBD-3; (N) HBD-2 and LL-37 and (O) HBD-3 and LL-37. Experiments were repeated two times with similar results. Results shown are from a representative experiment.

**A.**



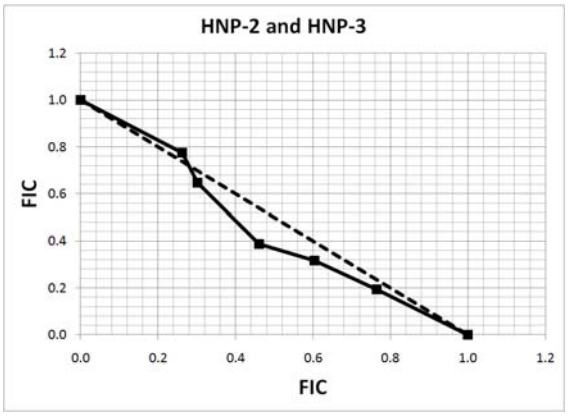

HNP-1 and HNP-2; FIC index = 0.961

**B.**



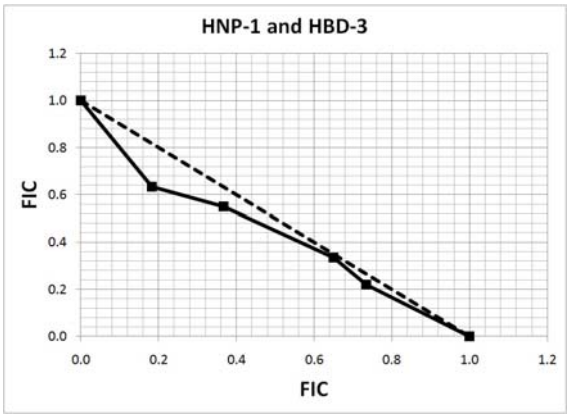

HNP-1 and HNP-3; FIC index = 0.933

**C.**




HNP-2 and HNP-3; FIC index = 0.849

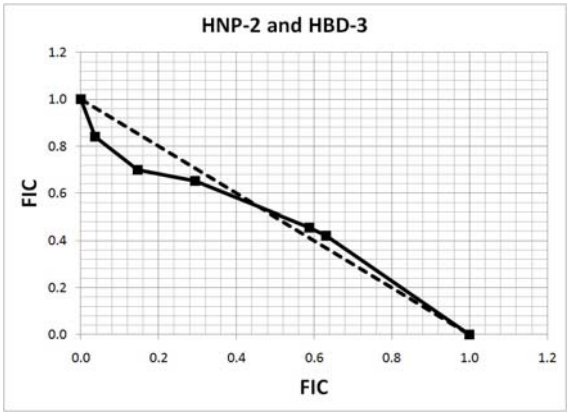
**D.**




HNP-1 and HBD-3; FIC index = 0.918

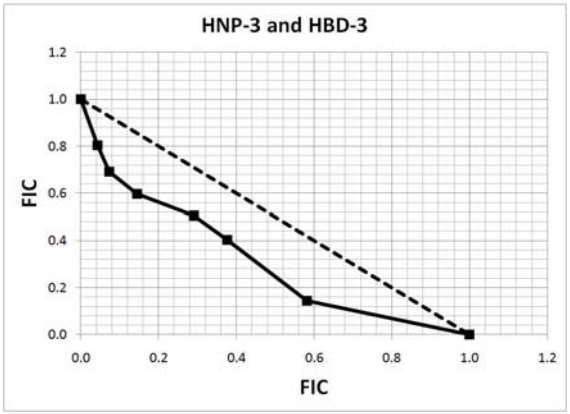
**Figure 3-11** Continued.

**E.**



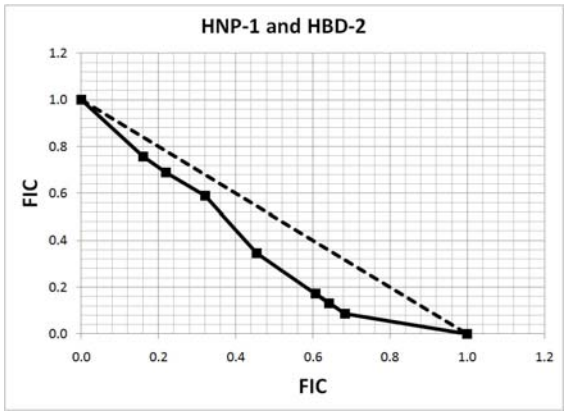

HNP-2 and HBD-3; FIC index = 1.042

**F.**



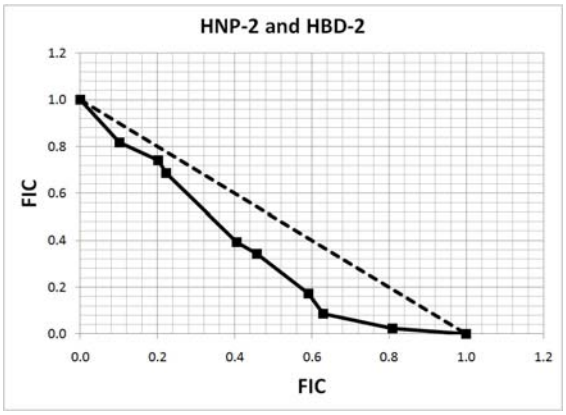

HNP-3 and HBD-3; FIC index = 0.779

**G.**




HNP-1 and HBD-2; FIC index = 0.799

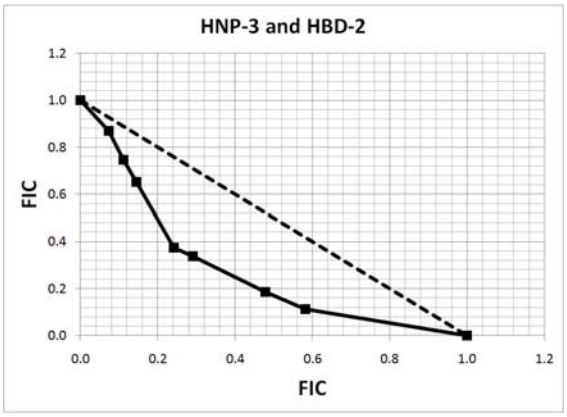
**H.**




HNP-2 and HBD-2; FIC index = 0.715

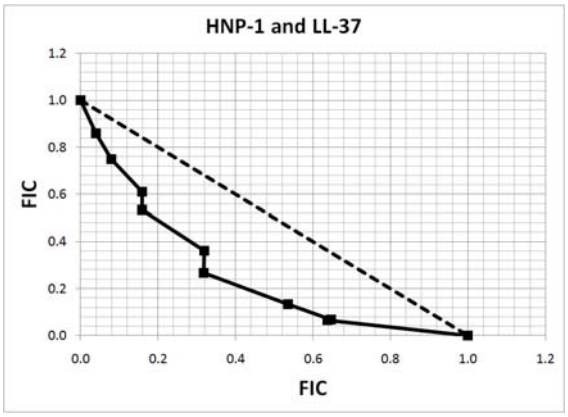
**Figure 3-11** Continued.

**I.**



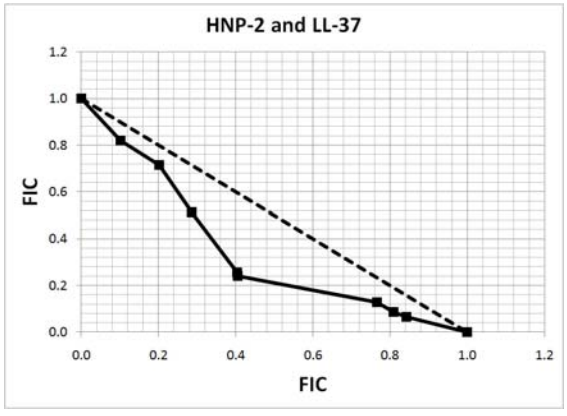

HNP-3 and HBD-2; FIC index = 0.629

**J.**



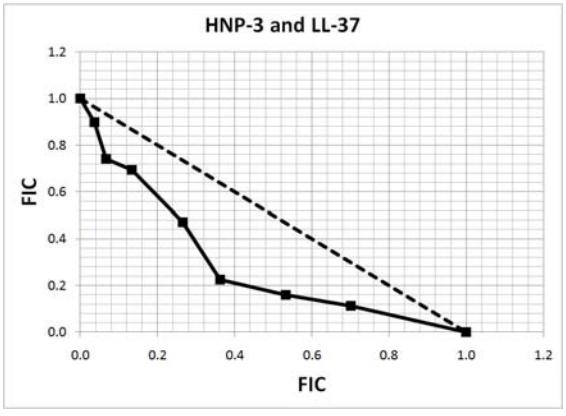

HNP-1 and LL-37; FIC index = 0.586

**K.**




HNP-2 and LL-37; FIC index = 0.645

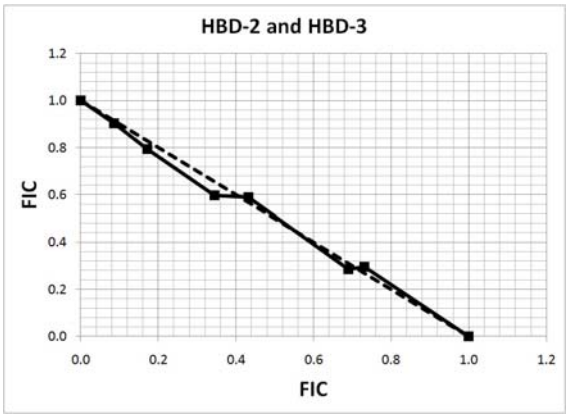
**L.**




HNP-3 and LL-37; FIC index = 0.587

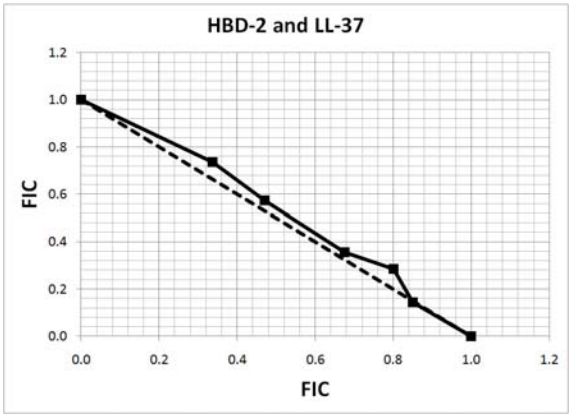
**Figure 3-11** Continued.

**M.**



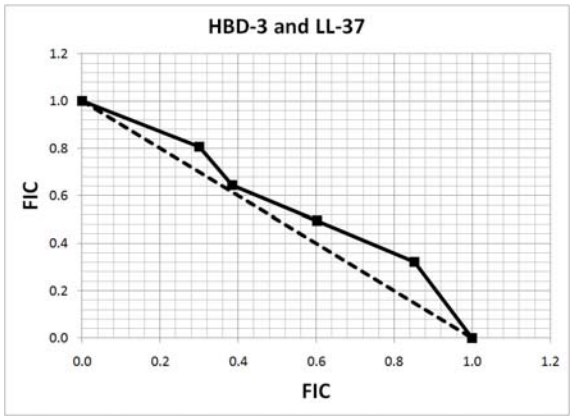

HBD-2 and HBD-3; FIC index = 1.022

**N.**




HNP-2 and LL-37; FIC index = 1.043

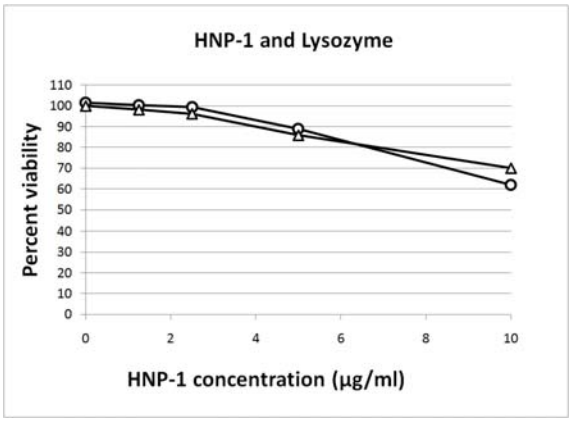
**O.**




HBD-3 and LL-37; FIC index = 1.096

**Figure 3-12 Antimicrobial peptide combinations with either lysozyme or histatin-5.** Susceptibility of *S. mutans* to HNP-1-3, HBD-2-3 or LL-37 was examined in the presence of subinhibitory concentrations (10 μg/ml) of lysozyme or histatin-5. O: antimicrobial activity of the indicated peptide alone; Δ: combined effect of the peptide with either lysozyme or histatin-5, as indicated. Combinations of lysozyme or histatin-5 did not alter the activity of the antimicrobial peptides tested. (A) HNP-1 and lysozyme; (B) HNP-1 and histatin-5; (C) HNP-2 and lysozyme; (D) HNP-2 and histatin-5; (E) HNP-3 and lysozyme; (F) HNP-3 and histatin-5; (G) HBD-2 and lysozyme; (H) HBD-2 and histatin-5; (I) HBD-3 and lysozyme; (J) HBD-3 and histatin-5; (K) LL-37 and lysozyme; and (L) LL-37 and histatin-5. Assays were performed in duplicate, each with similar results. Results are from a representative experiment.

**A.**

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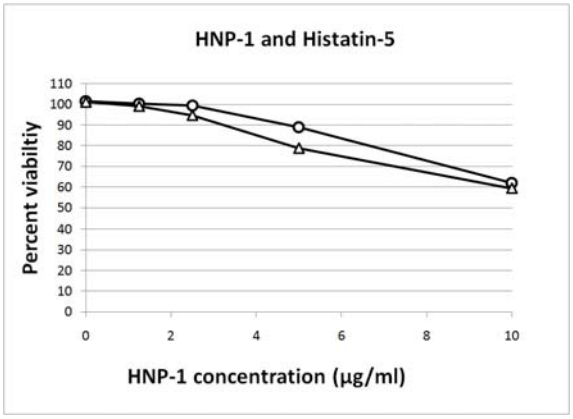


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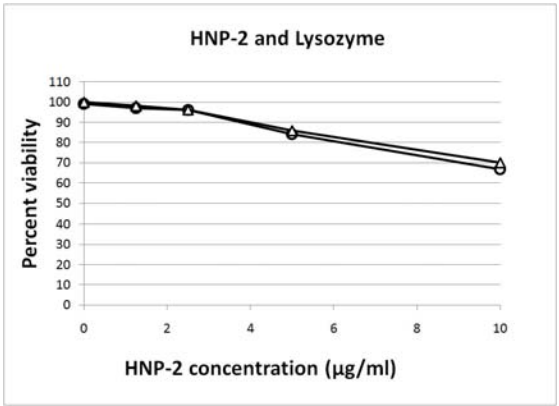



**D.**

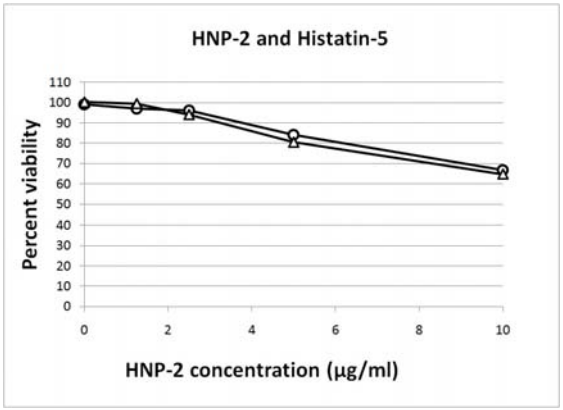
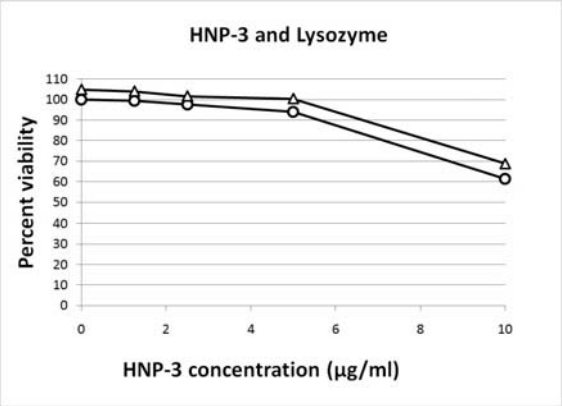
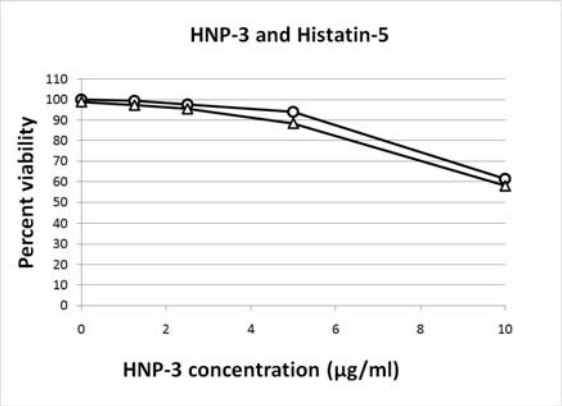


**Figure 3-12** Continued.

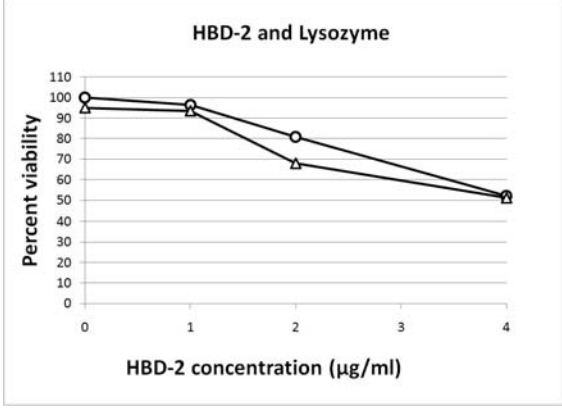
**E.**




**F.**




**G.**




**H.**

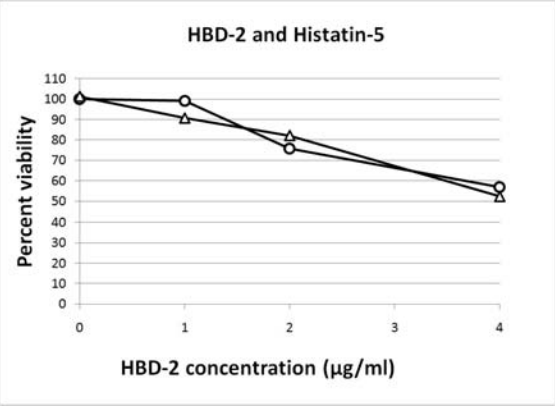
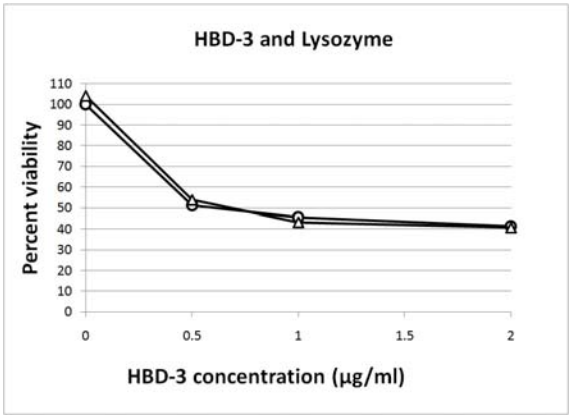
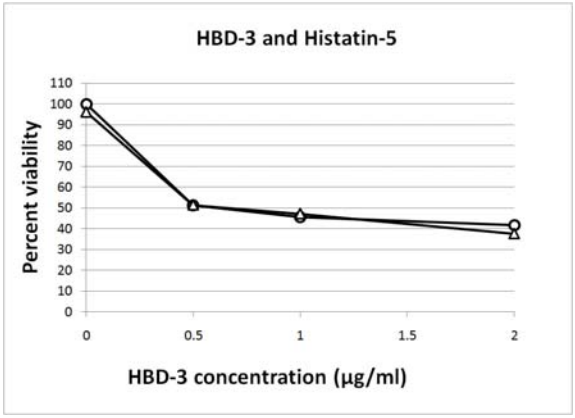


**Figure 3-12** Continued.

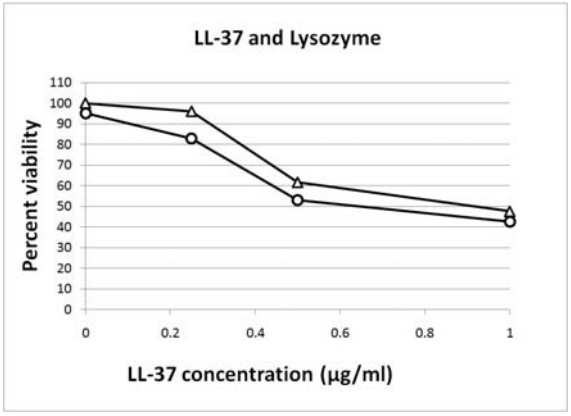
**I.**



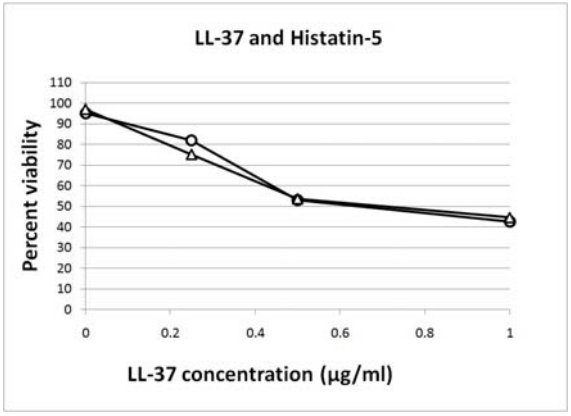

**J.**




**K.**




**L.**



# CHAPTER 4
# LEVELS OF ANTIMICROBIAL PEPTIDES IN SALIVA AND HOST CARIES EXPERIENCE

## Introduction

The oral cavity represents a unique environment in that the wide array of microbial species inhabiting the dental plaque biofilm interacts closely with host saliva. The protective attributes of saliva, such as its physical cleansing and buffering capacities, as well as a number of host innate and adaptive immune components, function collectively to maintain the balance between health and disease[235]. The significance of host saliva to dental caries is well-observed in clinical settings. Individuals with reduced salivary flow such as those with Sjögren syndrome or irradiated patients, suffer tremendously from a rather rapid and widespread development of dental caries[264,265].

Differences in host susceptibility to dental caries have long been recognized, as strongly indicated in several twin studies. It's been observed that monozygotic twins showed statistically significant resemblance in caries incidence, whereas dizygotic twins did not show such resemblance[82,83]. Several potential determinants for this are postulated, including differences in tooth morphology and biomineralization, association with specific immune complex loci, as well as variation in the quantity and quality of salivary protective factors[82].

The potential roles of a number of salivary proteins in the dental caries process have been a subject of interest in dental research, including a variety of salivary cationic antimicrobial peptides, primarily the α-defensins (HNP-1-4), β-defensins (HBD-1-3), LL-37 and histatins. They are expressed from various sources, including salivary gland acini and ducts, oral epithelium and neutrophils. Their levels in saliva are variable among individuals[116,122,237,239,266].

These antimicrobial peptides play a major role in host defense against infection. As clearly shown in *in vivo* studies by Wu *et al.*[267,268], the expression of murine β-defensin genes (mBDs) was differentially regulated in the *Pseudomonas aeruginosa*-infected cornea of resistant BALB/c mice versus susceptible C57BL/6. Following *P. aeruginosa* infection, BALB/c mice showed significantly higher mBD-1-2 expression than B6 mice. In addition, knocking down the expression of mBDs-2-3 worsened the clinical disease and increased the bacterial load in BALB/c cornea. These data indicate that antimicrobial peptides are required for host resistance against certain infections and that differential expression of selected peptides may determine host susceptibility to diseases.

In humans, it has been reported that the genes for α-defensins and β-defensins, which map to chromosome 8q23.1, show extensive copy number variation that positively correlates with levels of their mRNA transcripts[269,270]. This may potentially explain the basis for host genetic susceptibility to infections. A variation in the copy number of β-defensin genes has been associated with differing susceptibility to several diseases, such as Crohn's disease and psoriasis[271,272].

Evidence for a role of salivary antimicrobial peptides in dental caries has been reported. Tao *et al.*[116] found significantly higher HNP-1-3 levels in unstimulated saliva from caries-free children, compared to saliva from caries-active children, whereas no correlations between host caries experience and levels of HBD-3 or LL-37 were observed. These findings suggest a role for HNP-1-3 in dental caries prevention in children. This could be due to differences in host gene expression and also raises the possibility of using salivary levels of HNP-1-3 as a predictor of caries risk.

In order to provide perspective to our investigation of variability in *S. mutans* susceptibility to antimicrobial peptides, we sought to analyze salivary levels of antimicrobial peptides in the same subjects from whom the *S. mutans* isolates were obtained. We therefore measured the salivary levels of HNP1-3, HBD-2-3 and LL-37 and

determined their potential correlations with host caries experience. In this chapter, we show that the levels of antimicrobial peptides were highly variable among individuals. Their concentrations in saliva were not associated with caries experience in this subject group. However, high caries subjects had significantly a higher total protein concentration in saliva than did caries-free subjects. In addition, relationships between salivary levels of particular peptides were evident. Our findings suggest that levels of antimicrobial peptides in stimulated saliva are not specific to dental caries incidence and are therefore not reliable as a means to predict caries risk in children.

## Results

### Antimicrobial peptides in saliva

To measure the salivary levels of HNP-1-3, LL-37, HBD-2 and HBD-3, ELISA assays were performed using saliva from sixty 13 year-old subjects, equally divided between caries-free and caries active groups. The ELISA assay for HNP-1-3 is commercially available and can readily be used to measure their levels in saliva. HNP-1-3 are highly homologous, thus they were measured collectively. To achieve the most accurate measurements of LL-37, HBD-2 and HBD-3, optimal conditions were developed by adding divalent cation (300 mM $MgCl_2$) to the sample diluent to overcome masking by endogenous components in saliva. Recovery experiments were performed in all assays using appropriate recombinant peptides and revealed a mean recovery range of 80-100%, with linear dilution and equivalent quantification across the standard range.

Salivary analysis showed that antimicrobial peptide levels in saliva were highly variable among subjects despite normalization with total salivary protein concentrations. Mean total protein concentration in saliva was 935.189$\pm$273.363 μg/ml (range from 385.573 to 1,661.190 μg/ml). The mean HNP-1-3 concentration was 1,913.559$\pm$1,157.245 ng/ml (range from 548.625 to 5,213.060 ng/ml), or 2,034.333$\pm$1,069.123 ng/ml (range from 631.375 to 5,545.389 ng/mg) when normalized

to total salivary protein. The mean LL-37 concentration was 15.812±10.436 ng/ml (range from 3.931 to 71.015 ng/ml), with a normalized value of 17.482±11.371 ng/mg (range from 4.019 to 77.211 ng/mg). The mean HBD-3 concentration was 2.233±2.183 ng/ml (range from 0.150 to 11.558 ng/ml), and 2.571±3.072 ng/mg (range from 0.109 to 19.359 ng/mg) when normalized to total protein. The mean HBD-2 concentration was 0.734±0.811 ng/ml (range from 0.080 to 3.892 ng/ml), and the normalized value was 0.783±0.814 ng/mg (range from 0.079 to 3.352 ng/mg) (Table 4-1).

## Relationships between salivary AMP levels and host caries experience

To assess the relationships between the levels of antimicrobial peptides in saliva and caries experience in children, two-sample t-tests were used to compare the mean salivary levels of each peptide, with or without normalization to total salivary protein, for caries-free and high caries subjects. We found that there were no significant differences in salivary levels between the two groups for all peptides tested: HNP1-3 ($p=0.3747$), HNP1-3 relative to total protein ($p=0.8417$); LL-37 ($p=0.9353$), LL-37 relative to total protein ($p=0.4366$); HBD-3 ($p=0.8097$), HBD-3 relative to total protein ($p=0.5400$); HBD-2 ($p=0.2332$), and HBD-2 relative to total protein ($p=0.5363$); (Figures 4-1 to 4-4).

The salivary levels of these peptides were highly variable in subjects from both high and no caries groups though the mean and median levels were relatively comparable. The mean HNP-1-3 concentration was 2047.443±1254.763 ng/ml in high caries subjects and 1779.675±1055.034 ng/ml in caries-free subjects. The mean HNP-1-3 relative to total protein was 2006.422±1125.165 ng/mg in high caries subjects and 2062.243±1028.520 ng/mg in caries-free subjects. The mean LL-37 concentration was 15.923±7.497 ng/ml in high caries subjects and 15.701±12.859 ng/ml in caries-free subjects. The mean LL-37 relative to total protein was 16.326±8.287 ng/mg in high caries

subjects and 18.637±13.842 ng/mg in caries-free subjects. The mean HBD-3 concentration was 2.164±1.906 ng/ml in high caries subjects and 2.302±2.461 ng/ml in caries-free subjects. The mean HBD-3 relative to total protein was 2.325±2.393 ng/mg in high caries subjects and 2.817±3.654 ng/mg in caries-free subjects. The mean HBD-2 concentration was 0.860±0.895 ng/ml in high caries subjects and 0.609±0.711 ng/ml in caries-free subjects. The mean HBD-2 relative to total protein was 0.848±0.826 ng/mg in high caries subjects and 0.717±0.810 ng/mg in caries-free subjects. This suggested that the level of each antimicrobial peptide in saliva by itself was not a reliable predictor of caries risk in this population.

Interestingly, our data provided strong evidence that the two groups differed in total protein levels in saliva ($p=0.0178$). The mean total salivary protein level was significantly greater in children with high caries experience than that observed in caries-free children (Figure 4-5). The mean total salivary protein concentrations were 1,018.187±306.328 μg/ml (range from 574.79 to 1,661.190 μg/ml) in high caries subjects and 852.191±209.694 μg/ml (range from 385.573 to 1,240.848 μg/ml) in caries-free subjects. This greater salivary protein level in general may lead to efficient pellicle deposition that promotes development of the dental plaque biofilm, including cariogenic bacterial species, thereby increasing caries risk.

In addition, we also compared the salivary antimicrobial peptide levels between boys and girls. Unexpectedly, we found significant differences in the levels of HNP-1-3 and LL-37 relative to total protein between boys and girls. The data revealed that girls had greater mean levels of both peptides relative to total protein than boys ($p=0.0409$ for HNP1-3; $p=0.0333$ for LL-37). However, when considering the levels of these peptides without normalization, there were no significant differences in levels of either HNP1-3 ($p=0.3861$) and LL-37 ($p=0.2169$) in saliva between girls and boys. Furthermore, within gender comparisons showed no significant differences between high caries and caries-free subjects regarding for either HNP-1-3 or LL-37 concentrations relative to total

protein. No significant differences were found in the levels of HBD-3, HBD-3 relative to total protein, HBD-2, HBD-2 relative to total protein and total protein in saliva between boys and girls ($p>0.05$).

## Relationships between the levels of antimicrobial peptides in saliva

Since a variety of antimicrobial peptides are present in saliva and to some extent may play overlapping and/or collaborative roles in host defense against oral pathogens, it was of interest to evaluate the correlations between antimicrobial peptide levels in saliva within individuals. Therefore, Pearson correlation analyses were performed to assess the correlations among the salivary levels of these peptides and the Pearson correlation coefficient was used to interpret the strength of each correlation. Analyses showed that there were statistically significant correlations between the levels of LL-37 and HNP1-3 ($p<0.0001$) and between the levels of LL-37 relative to total protein and HNP1-3 relative to total protein ($p<0.0001$), with Pearson correlation coefficients of 0.66 and 0.65, respectively, indicative of moderate increasing relationships (Figure 4-6). In addition, statistically significant correlations were found between HBD-2 and HBD-3 levels ($p<0.0001$) and between HBD-2 relative to total protein and HBD-3 relative to total protein ($p<0.0001$), with Pearson correlation coefficients of 0.50 and 0.67, respectively, also indicating moderate increasing relationships (Figure 4-7).

Analyses between HBD-3 and LL-37 revealed a significant correlation between the levels of HBD-3 relative to total protein and LL-37 relative to total protein ($p=0.0466$). The Pearson correlation coefficient of 0.26 indicated a weak increasing relationship between the two peptides. No correlation between non-normalized salivary concentrations of HBD-3 and LL-37 was observed ($p=0.3238$) (Figure 4-8). We also found a significant correlation between HNP1-3 and HBD-2 concentrations in saliva ($p=0.0032$) with Pearson coefficient of 0.37, indicating a weak increasing relationship.

However, no correlation between the HBD-2 relative to total protein and the HNP1-3 relative to total protein was found (p=0.1076); (Figure 4-9).

No significant correlations were observed between the levels of HNP1-3 and HBD-3 (p=0.1920), between the levels of HBD-3 relative to total protein and HNP1-3 relative to total protein (p=0.2618); (Figure 4-10), between the levels of LL-37 and HBD-2 (p=0.3459), and between the levels of LL-37 relative to total protein and HBD-2 relative to total protein (p=0.2059); (Figure 4-11).

### Correlations between the salivary levels of antimicrobial peptides and MS in dental plaque

We further examined the relationship between the levels of antimicrobial peptides in saliva and MS levels within dental plaque. Pearson correlation analyses showed no significant correlations between the ratio of MS relative to total bacteria in dental plaque and the salivary levels of any antimicrobial peptides tested with or without normalization to total salivary protein ($p>0.05$). However, we found a significant correlation between the MS count in dental plaque and HNP-1-3 concentration relative to total protein (p=0.0280). The Pearson correlation coefficient indicated a weak increasing relationship between these two variables. The correlation analysis between MS count and the non-normalized salivary concentration of HNP-1-3 also revealed a similar trend but did not reach statistical significance (p=0.0713). No significant correlations were found between MS counts and salivary concentrations of other antimicrobial peptides (Table-4-2).

In addition, the relationships between the salivary levels of antimicrobial peptides and total cultivable bacteria in dental plaque were also examined. Spearman's rank correlation tests were performed and no statistically significant correlations were observed between the salivary level of each peptide, with or without normalization, and total salivary protein or total plaque bacteria (Table 4-3). However, it should be noted that there was a trend towards an increasing relationship between total plaque bacteria

and the salivary concentration of HBD-3 (p=0.0583) and salivary concentration of HBD-3 relative to total protein (p=0.0946), particularly when considering high caries subjects only.

### Correlations between the salivary levels of antimicrobial peptides and the susceptibility profiles of *S. mutans*

We next asked if there were relationships between the level of each antimicrobial peptide in saliva and the susceptibility or resistance to the corresponding peptide of *S. mutans* isolated from the same individual. It was expected that *S. mutans* strains would be most resistant to the antimicrobial peptides that were represented at the highest levels in saliva. Pearson correlation analyses showed only a single statistically significant correlation between the levels of peptide in saliva and percent viability of *S. mutans*. A significant correlation between the salivary level of HNP-1-3 relative to total protein and the percent viability of *S. mutans* strains to 5 μg/ml HNP-1 (p=0.0438) was observed. The Pearson correlation coefficient of 0.26 indicated a weak increasing relationship between the two variables (Table 4-4).

## Discussion

Several findings were demonstrated in this chapter. We found that salivary concentrations of HNP-1-3, LL-37, HBD-3 and HBD-2 in saliva were highly variable among individuals and their levels were not associated with host caries experience in this population. The peptide concentrations varied considerably, from approximately a 10-fold difference between maximum and minimum values for HNP-1-3 to more than a 70-fold difference for HBD-3. A similar magnitude in variability was still observed after normalization of the salivary concentrations of these peptides with total salivary protein. In fact, the range was even greater for HBD-3 which demonstrated more than a 170-fold difference between maximum and minimum values.

These findings are in agreement with several previous studies that reported variable antimicrobial peptide concentrations within a population[116,117,266,273-278]. HNP-1-3 has been reported in saliva, varying from 0.06 μg/ml to more than 20 μg/ml[116,276,277]. LL-37 has been detected at less than 1 ng/ml to up to 12 μg/ml[116,274]. The β-defensins are found at lower amounts. HBD-2 has been reported to range from undetectable to 0.8 μg/ml, while HBD-3 concentrations have varied from undetectable to 6.21 μg/ml. The marked variations in salivary concentrations of these peptides are evident within the same studies. However, it is noteworthy that the extreme variations between studies may be partly due to differences in methods of measurement. Previous studies that utilized ELISA assays to detect HNP-1-3 and LL-37 reported salivary concentrations that were comparable to ours[116,274]. In contrast, HNP-1 alone has been reported as high as 23.4 μg/ml using liquid chromatography/mass spectrometry (LC/MS) as a method of measurement[276,277]. Likewise, LL-37 concentrations were determined to be in the ng/ml range by ELISA assay[274], but were detected in μg/ml ranges using Western blot or dot blot immunoassays[116,273].

The different analytical methods have their inherent advantages and drawbacks. Western blot and dot blot analyses are only semi-quantitative. LC/MS can detect multiple peptides at a time but may have limited specificity due to co-eluting or isobaric interfering agents. While ELISA assays are the highly sensitive and quantitative, they may be subject to interference that affects binding specificity[275]. As shown in previous studies, the detection level of defensins in saliva is masked by salivary components such as mucin[122,266]. The masking is believed to be due to ionic interactions between the cationic antimicrobial peptides and anionic molecules present in saliva that block the binding of epitopes by antibodies.

In this study, we also observed this masking effect, particularly for the assays of HBD-2-3 and LL-37. Preliminary experiments with spiking varying concentrations of recombinant peptides into saliva samples in these ELISA assays initially yielded poor

peptide recovery rates. However, we found that the recovery was improved to an optimal level by adding divalent cations, in the form of $MgCl_2$, into the assay sample diluent. The final concentration of 300 mM $MgCl_2$ increased the percent recovery to 80-100% with linearity of dilution across the range of standard concentrations. As shown by Ghosh *et al.*[266], there are differences in the degree of the masking effect, with the effect being greater for HBD-3 than that of HBD-2, potentially due to the more positive charge of HBD-3. In addition to these β-defensins, we also noted the masking effect of saliva in the assay for LL-37, whereas such effects were not observed with HNP-1-3. This may be due to the fact that HNP-1-3 are present at higher concentrations in saliva than HBD-2-3 or LL-37, allowing for the samples to be diluted to a level sufficient to overcome masking effect.

It is of note that the salivary levels of HNP-1-3 detected in this study were well within the range of their antimicrobial activity, supporting a physiologically relevant role in oral immune defense. LL-37 and the β-defensins, however, were found within the ng/ml range in saliva in this subject group. These salivary concentrations are generally lower than their effective range *in vitro*, though the actual physiological concentrations of these peptides at different sites in oral cavity are not known. The β-defensins are expressed from oral keratinocytes as well as salivary duct cells, and their expression is particularly strong at regions close to mucosal surfaces such as at gingival margins around the tooth, allowing for close contact with supragingival plaque[130]. Similarly, LL-37 is secreted from multiple sources, including salivary ducts, oral epithelium and neutrophils. In normal individuals, 30,000 neutrophils per minute are estimated to migrate into oral cavity through the junctional epithelium around the teeth[279]. As a result, the levels of HNP-1-3 and LL-37 are concentrated in gingival crevicular fluid, in close proximity to tooth surfaces. This could potentially increase the concentrations of these peptides substantially within the environment surrounding tooth structure and thus closely influence microbial ecology within dental plaque.

In the context of the whole oral environment, a number of other salivary factors are also present together antimicrobial peptides that can affect their activity. For example, salivary mucin is known to form complexes with other proteins, such as IgA, lysozyme, histatin, PrPs, as well as defensins. These complexes may protect small peptides such as defensins or LL-37 from the action of proteases. In addition, mucin also aggregates bacteria and adheres to tooth surfaces as part of the salivary pellicle. This may help localize antimicrobial peptides close to the bacterial community in dental plaque for more effective activity[122,280].

Also, as shown in chapter 3, these antimicrobial peptides can work additively or synergistically to enhance their antimicrobial activity. Furthermore, the α-defensins, β-defensins as well as LL-37, have other immunomodulatory roles, chemoattractant activity, and can stimulate the adaptive immune response including enhancing the production of IgA or IgG[281]. Therefore, the effect of saliva must be considered in its entirety to completely explain its contribution to dental health.

Aside from the markedly variable salivary levels of these antimicrobial peptides among populations, their concentrations may also be altered in response to various factors such as local inflammatory conditions, a person's health, and physical activity. Mizukawa *et al.*[277] found that the level of HNP-1 in saliva is significantly higher in patients with lichen planus, leukoplakia and iron deficiency anemia-associated glossitis than in healthy subjects. This is believed to be due to inflammatory components associated with these diseases, leading to increased expression of HNP. In contrast, patients with glossodynia or oral discomfort who mainly complained of a strange feeling or bitter taste with no actual inflammatory lesions did not show differences in HNP-1 levels compared to healthy subjects. Davison *et al.*[274] also observed significantly increased levels HNP-1-3 and LL-37 in saliva following prolonged strenuous exercise. This is proposed to be a result of an exercise-induced inflammatory response that leads to neutrophil infiltration into oral mucosa. These findings may partly explain the variation

of these peptides among individuals, since complete information on conditions that affect potential fluctuations of these peptides, as well as their circadian rhythms, are not available and were not strictly controlled in most studies. Results from these studies also demonstrate that determining the relationship between salivary immune factors and specific disease conditions may not be a straightforward process. While the role of these peptides in host defense has been clearly established in various *in vitro* or *in vivo* studies using animal models, studying the relationship between peptide expression and host susceptibility to infection may be obscured by the fact that peptide levels are increased following the influx of inflammatory cells into tissue or in response to the presence of causative microorganisms as well as inflammatory stimuli during the disease process.

In this study, we did not find statistically significant differences between salivary levels of HNP-1-3, LL-37, HBD-3 and HBD-2 between caries-free and high caries subjects. The mean concentrations of these peptides between both groups were relatively comparable with extreme variations between the maximum and minimum values. These results differed from a previous study by Tao *et al.*[116], which showed significantly higher HNP-1-3 levels in saliva of caries-free subjects compared to that of high caries subjects. However, they also did not observe significant differences in LL-37 and HBD-3 levels between both groups. This difference between studies may be the result of several factors. In this study we used stimulated saliva as a source for antimicrobial peptide detection, whereas unstimulated whole saliva samples were used in the Tao *et al.*[116] study. Differences between the concentrations of specific antimicrobial peptides in stimulated versus unstimulated saliva have not been reported to date. However, it has been noted that salivary protein composition can differ, depending on whether saliva was collected under the stimulated or unstimulated conditions[282,283]. In general, submandibular glands represent the major source of unstimulated saliva, whereas all major salivary glands make contributions to stimulated saliva. As different glands contribute different spectrums of protein composition to whole saliva, this may also result

in variations in protein contents between stimulated and unstimulated saliva. α-defensins, β-defensins, as well as LL-37, have all been detected in ductal cells of salivary glands. This, however, may account for only a small fraction of these peptides in saliva since neutrophils are believed to be the primary source of HNP-1-3 and LL-37 and oral epithelium may contribute the major pool of HBD-1-3.

Regarding subject demographics, children recruited into this study were within the same age range as the study by Tao *et al.*[116]. Therefore, age-related variation may be of little significance. One notable difference is the subject ethnicity. While the overwhelming proportion of our subjects were Caucasians, subjects in their study were mostly Hispanic. Several studies have observed not only differences in caries prevalence among people with diverse racial backgrounds, but also variation in salivary protein composition[284-287]. However, no racial differences in antimicrobial peptide expression or genetic polymorphisms have yet been confirmed. Future studies may potentially help clarify the effect, if any, of race on antimicrobial peptide expression.

Our results do not support the measurement of salivary antimicrobial peptide levels as a reliable tool to predict caries risk, at least not for stimulated saliva. As noted earlier, increased levels of α-defensins in saliva have been observed in patients with various oral inflammatory conditions[277,288,289]. The infiltration of neutrophils within these lesions is believed to be responsible for the increased defensin levels[118]. These findings support the role of salivary antimicrobial peptides as a first line of host immune response in oral cavity. However, their levels of expression appear to be associated with a wide range of inflammatory conditions in a rather non-specific manner. Any degree of simultaneous gingival or oral mucosal inflammation can contribute to the levels of these peptides in saliva irrespective of host caries activity. In addition, it has been reported that increases in the number of neutrophils in the blood can result in increased HNP levels in saliva[290].

While this clearly does not exclude a role for salivary antimicrobial peptides in dental caries protection, it appears that a variety of other factors or conditions can also induce the expression of these peptides. As with other cross-sectional studies, measuring the salivary levels of specific peptides at a particular time point may provide partial information regarding the quantity of these peptides, but may not be sufficient to predict relative risk for the future development of dental caries. A comprehensive collection of saliva samples longitudinally with accompanying evaluation of dental caries increment over time, as well as the assessment of multiple salivary chemical and physical properties are likely needed to better clarify their potential relationships with dental caries.

Several previous studies have attempted to investigate relationships between various salivary components and dental caries. Despite these attempts, it is the consensus that a relationship between caries experience and any specific salivary protein has not been adequately demonstrated[235] (Table 1-1). There are a number of aspects related to the dental caries process to be considered. Dental caries is a multifactorial disease involving the physicochemical characteristics of dental hard tissue, the interplay between different types of microbial species within dental plaque, and the mechanical and host defense protective properties of saliva. Individual oral hygiene care, fluoride exposure as well as dietary factors also play crucial roles in the caries process. Salivary factors, while vital to defense against pathogens and maintenance of the oral ecological balance, represent only one segment among all contributing factors.

In addition, there is a degree of functional redundancy within the salivary defense system, such that multiple salivary molecules have similar or overlapping functions. This makes it difficult to evaluate the contribution of a single specific salivary component to caries risk[90,97]. As noted by van Nieuw Amerongen *et al.*[102], in a complex oral environment, no single component may be necessary for the overall antimicrobial capacity. These salivary factors likely work collectively to prevent the overgrowth of

microorganisms and maintain the ecological balance in which harmless resident microflora outnumber potentially pathogenic agents.

One subject to bear in mind for all cross-sectional studies, including this one, is the appropriateness of the DMF index to reflect caries experience. Although the DMF index is a well-known and universally accepted means to measure individual caries activity, it represents a lifetime cumulative index of dental caries that also takes into account treated lesions. It may not reflect ongoing caries activity at the specific time period at which saliva or plaque samples were collected for analysis. However, it is believed that past caries experience is a powerful predictor for future caries and several longitudinal studies do support this notion[291,292].

Unexpectedly, when comparing total salivary protein levels between the two subject groups, we found a significantly greater level of total salivary protein in subjects with high caries experience than in caries-free subjects. Since the protein concentrations in saliva are also dependent on salivary flow rate, it is possible that these high caries subjects may have had lower salivary flow rates than caries-free subjects, which led to more concentrated protein in saliva and increased susceptibility to dental caries.

Another explanation is that high caries subjects may have higher concentrations of certain proteins in saliva that facilitate dental caries formation. Several studies have suggested a role for salivary proteins in the adhesion of bacteria onto oral surfaces by forming adherent biofilms, or pellicles[293,294]. The largest chemical components (49%) of the acquired salivary pellicle on tooth surfaces are proteins derived from saliva[295]. Saliva samples from some individuals were found to promote bacterial adhesion more actively than others. The level of total salivary protein was shown to be strongly associated with the adhesion of oral streptococci to saliva-coated plates[97]. Proteins in dental pellicles modulate this interaction by providing chemical moieties that serve as receptors for bacterial adhesins.

A number of salivary components, when adsorbed to oral surfaces, were described to mediate molecular interactions with oral bacteria, including mucins[296], α-amylase[297,298], fibronectin[299] and PrPs[300,301]. Alpha-amylase, for example, is a multifunctional protein present at high concentration in saliva. Its enzymatic activity acts to digest carbohydrate. In addition, α-amylase binds strongly to oral streptococci and when present in solution, is believed to be involved in bacterial clearance. This protein is, however, one of the major components of the acquired enamel pellicle and may facilitate bacterial adhesion to the tooth surface. Amylase bound to bacteria in dental plaque may also promote dietary starch hydrolysis which increases acid formation in close proximity to tooth surface[297,298].

The clinical relevance of saliva-promoted *S. mutans* adhesion in biofilm formation has been shown by a recent study that reported a positive correlation between the total protein and glycoprotein contents in saliva and saliva-promoted *S. mutans* adhesion to hydroxyapatite. The latter was also positively correlated with dental plaque development after professional cleansing and was significantly greater in caries-active subjects than in caries-free subjects[302]. In addition, Stenudd *et al*.[99] found that *S. mutans* adhesion to saliva-coated hydroxyapatite was among the factors most strongly correlated with high dental caries susceptibility. Therefore, this pellicle-mediated bacterial adhesion could provide the basis for the robust formation of dental plaque populated with sufficient proportions of cariogens to increase risk of dental caries.

Moreover, the role of salivary proteins as a source of nutrients to oral bacteria has also been suggested. In the process of dental plaque formation, plaque bacterial colonizers may require specific salivary proteins that they can degrade to provide nutrients for their metabolism, allowing further growth, multiplication and aggregation to occur[254,293].

Interestingly, we found that the salivary levels of several antimicrobial peptides were correlated within an individual. There were significant correlations between the

salivary levels of HNP-1-3 and LL-37, and between HBD-2 and HBD-3, both with and without normalization to total salivary protein. The Pearson correlation coefficients indicated moderate increasing relationship between both peptide pairs. These correlations are consistent with the natural sources of these peptides, since the majority of HNP-1-3 and LL-37 are produced from neutrophils and both HBD-2 and HBD-3 are secreted from oral epithelial cells and salivary duct cells. In addition, we also observed weak increasing relationships between salivary concentrations of HNP-1-3 and HBD-2 and between salivary concentration relative to total protein of LL-37 and HBD-3. Collectively, it appears that these antimicrobial peptides tend to be produced together into saliva, probably due to the similar types of inflammatory stimuli present as a part of the innate immune response. This may provide an appropriate setting for these peptides to work additively or synergistically to increase their antimicrobial activity against pathogens.

Also examined were the relationships between the levels of antimicrobial peptides in saliva and the MS levels in dental plaque. We found mostly no correlation between the two factors, except between HNP-1-3 relative to total protein and the MS count, which showed a statistically significant but weak increasing relationship ($p<0.0280$). The correlation analysis between the non-normalized salivary HNP-1-3 concentration and the MS count also displayed the same trend but did not reach statistical significance. Likewise, the correlation analyses between total bacteria in dental plaque and salivary levels of HBD-3 or HBD-2 showed trends towards increasing relationships between these variables. These results are consistent with previous findings that the expression of HBD-3 and HBD-2 in oral epithelial cells is inducible upon bacterial contact or in response to pro-inflammatory stimuli, including IL-1β, TNF-α or IFN-γ[128,303-306]. Their expression, however, is variable with different bacterial species[305,307-309]. A recent study demonstrated that a *S. mitis* biofilm significantly upregulated HBD-2 gene expression in gingival epithelial cells, whereas *S. mutans* biofilm was a poor inducer[310]. It is possible that the more resistant early colonizers in dental plaque may induce the expression of

antimicrobial peptides, which in turn limit the colonization and survival of more sensitive, potentially pathogenic microorganisms, such as *S. mutans*[130,311].

Lastly, we examined the relationship between levels of salivary antimicrobial peptides in saliva and the susceptibility/resistance profiles of *S. mutans* isolated from the same individuals. A significant correlation was observed between the salivary level of HNP-1-3 relative to total protein and the percent viability of *S. mutans* strains to 5 μg/ml HNP-1 ($p=0.0438$). The Pearson correlation coefficient indicated a weak increasing relationship between both variables, suggesting that individuals with higher levels of HNP-1-3 in saliva tend to harbor *S. mutans* strains that are more resistant to HNP-1. This finding is in the line with the study by Perron *et al.*[312] which found that in a series of selection experiments, microbial agents are able to evolve *in vitro* in response to a gradual increase of antimicrobial peptide concentrations such that over several hundred of generations there is natural selection for organisms that are more resistant to the tested peptide. These data, coupled with our findings from chapter 3, support the hypothesis that increasing resistance to salivary antimicrobial peptides is one mechanism that promotes for *S. mutans* survival in the oral environment potentially elevating the risk of dental caries.

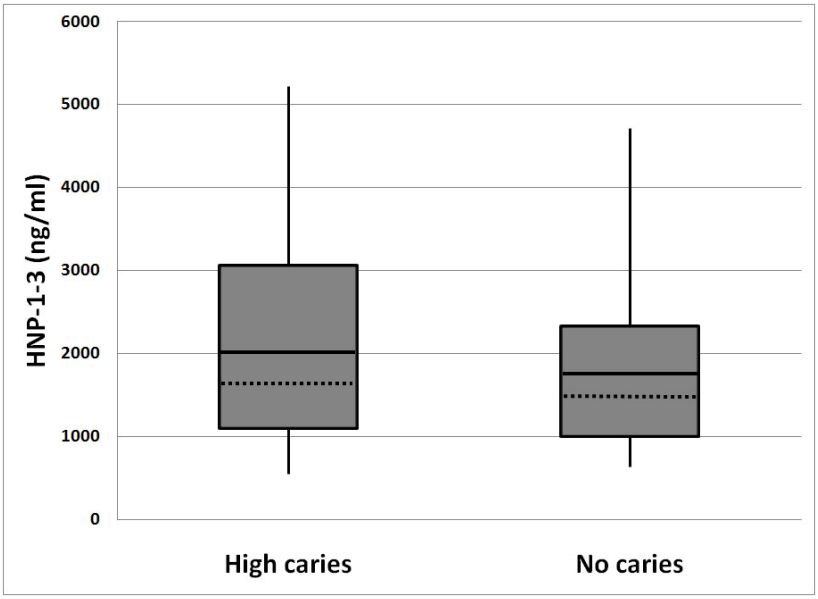
**Table 4-1 Levels of antimicrobial peptides in saliva of subjects.**

| | Total salivary protein (µg/ml) | Salivary concentration (ng/ml) | | | | Salivary concentration relative to total protein (ng/mg) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | HNP-1-3 | LL-37 | HBD-3 | HBD-2 | HNP-1-3 | LL-37 | HBD-3 | HBD-2 |
| **Mean** | 935.189 | 1913.559 | 15.812 | 2.233 | 0.734 | 2034.333 | 17.482 | 2.571 | 0.783 |
| **SD** | 273.363 | 1157.245 | 10.436 | 2.183 | 0.811 | 1069.123 | 11.371 | 3.072 | 0.814 |
| **Median** | 933.757 | 1443.358 | 14.234 | 1.671 | 0.385 | 1704.897 | 15.518 | 1.879 | 0.444 |
| **Maximum** | 1661.190 | 5213.060 | 71.015 | 11.558 | 3.892 | 5545.389 | 77.211 | 19.359 | 3.352 |
| **Minimum** | 385.573 | 548.625 | 3.931 | 0.150 | 0.080 | 631.375 | 4.019 | 0.109 | 0.079 |

Results are shown as the concentration of peptide in saliva (ng/ml) and the salivary concentration of peptide relative to total salivary protein (ng/mg).

**Figure 4-1 Levels of HNP-1-3 in saliva and host caries experience.** No statistically significant differences were found between caries-free and high caries subjects in salivary concentrations of HNP-1-3 (A), and salivary concentrations of HNP-1-3 relative to total protein (B).

**A.**




**B.**

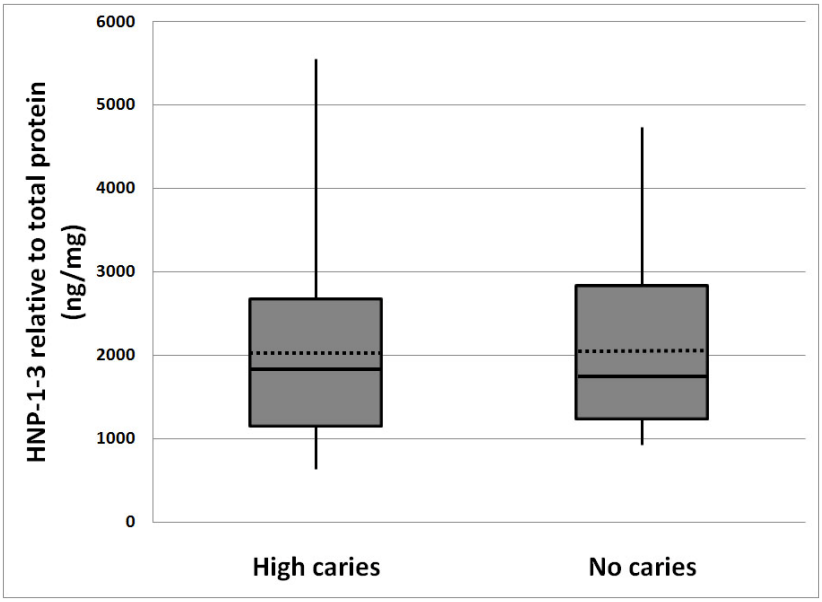
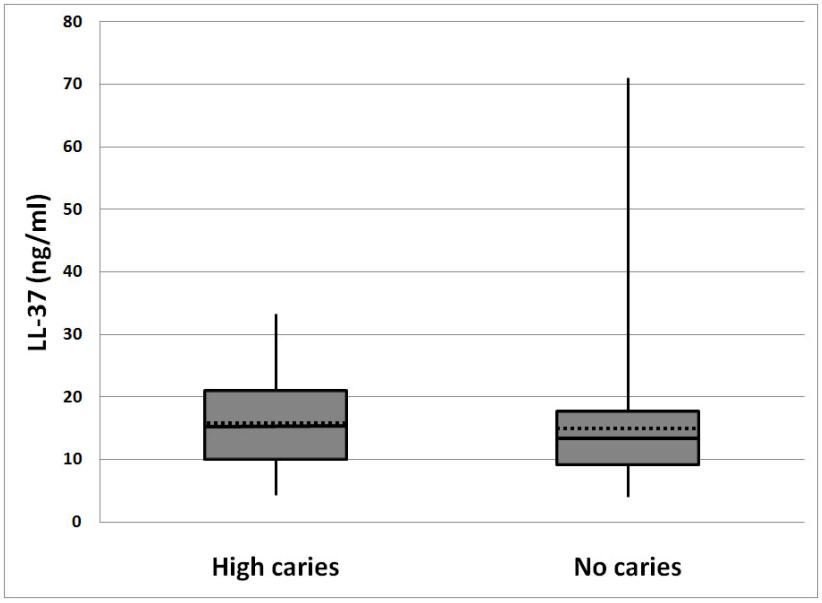


**Figure 4-2 Levels of LL-37 in saliva and host caries experience.** No statistically significant differences were found between caries-free and high caries subjects in salivary concentrations of LL-37 (A), and salivary concentrations of LL-37 relative to total protein (B).

**A.**




**B.**

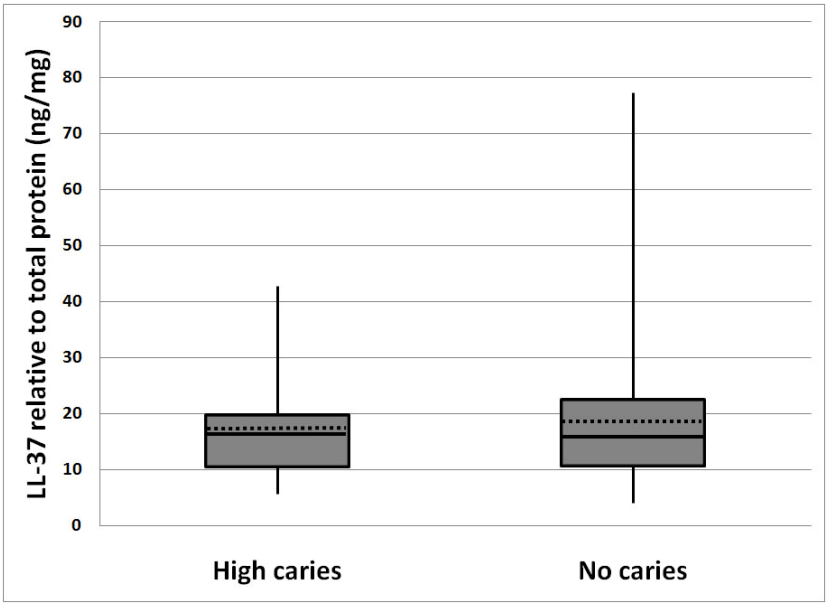
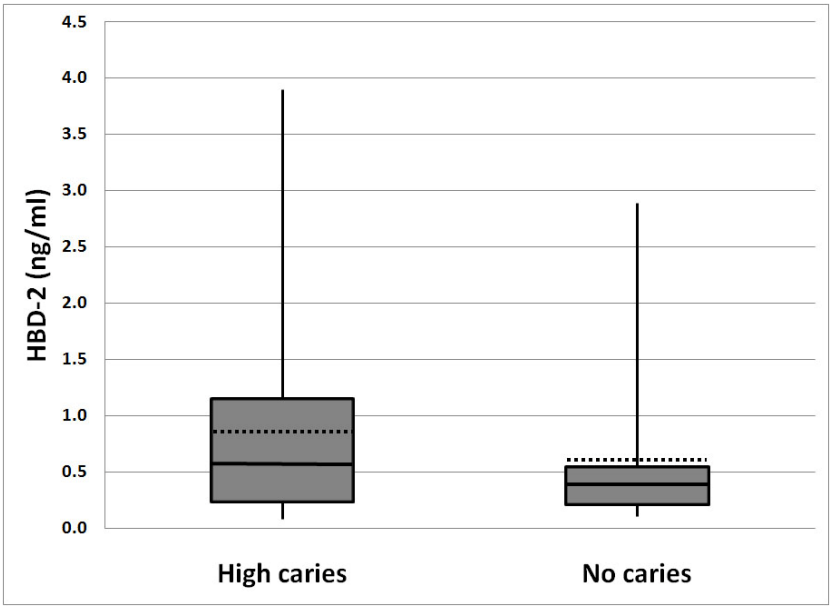


**Figure 4-3 Levels of HBD-2 in saliva and host caries experience.** No statistically significant differences were found between caries-free and high caries subjects in salivary concentrations of HBD-2 (A), and salivary concentrations of HBD-2 relative to total protein (B).

**A.**




**B.**

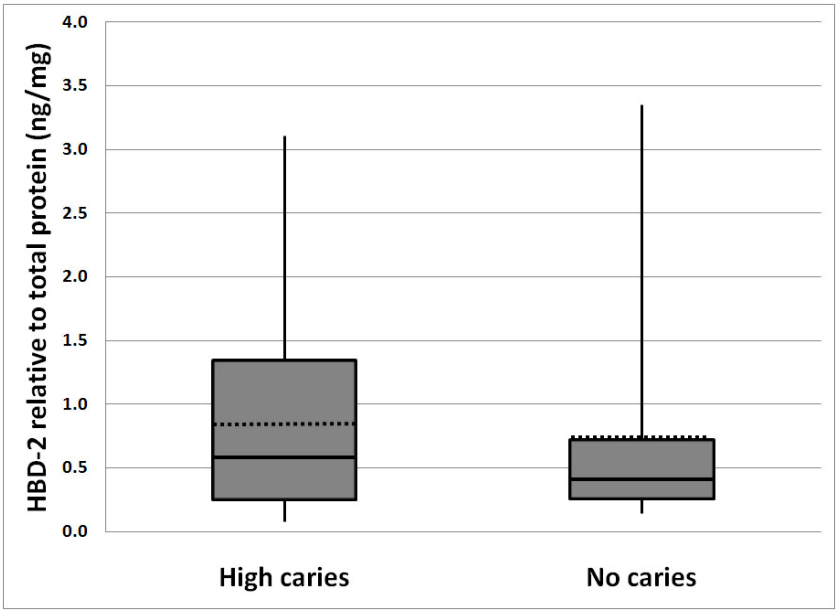
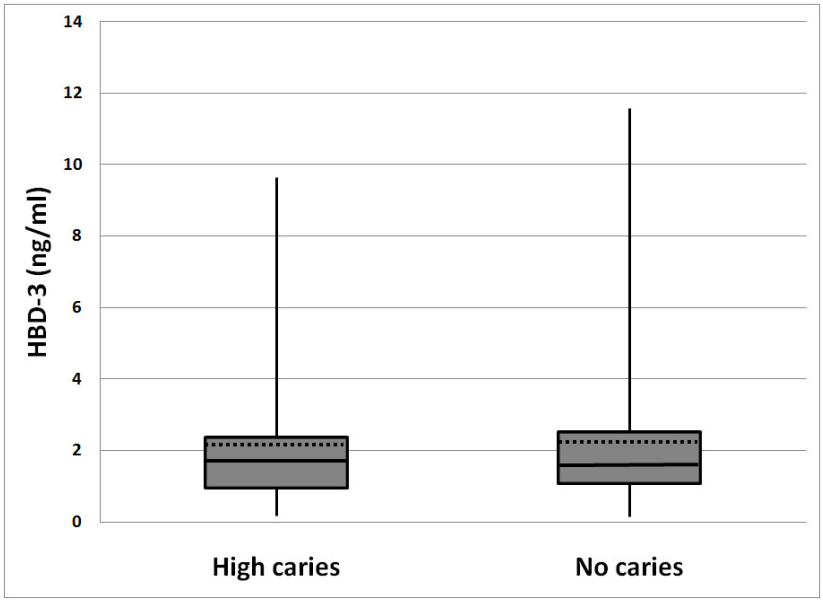


**Figure 4-4 Levels of HBD-3 in saliva and host caries experience.** No statistically significant differences were found between caries-free and high caries subjects in salivary concentrations of HBD-3 (A), and salivary concentrations of HBD-3 relative to total protein (B).

**A.**




**B.**

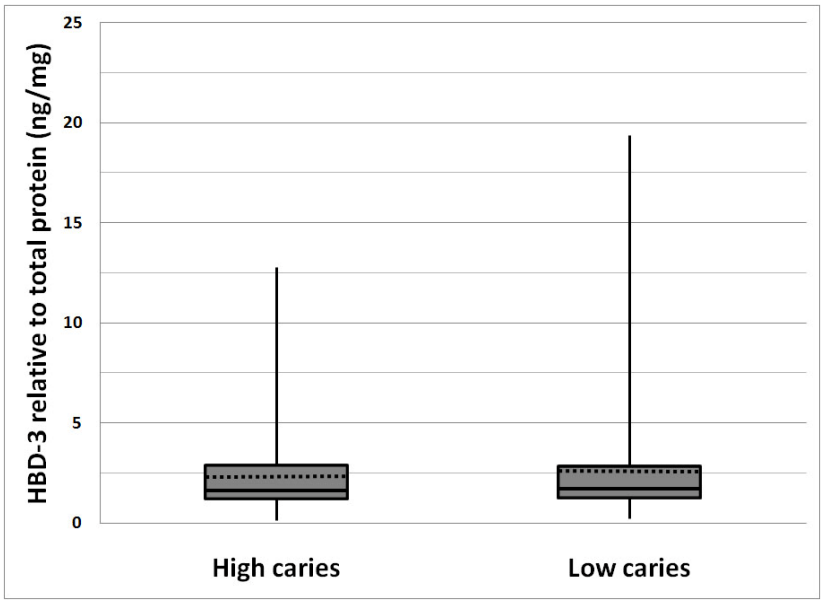


**Figure 4-5 Levels of total protein in saliva and host caries experience.** A statistically significant difference was found in total salivary protein between caries-free and high caries subjects (p=0.0178). High caries subjects had significantly higher total protein in saliva than caries-free subjects.

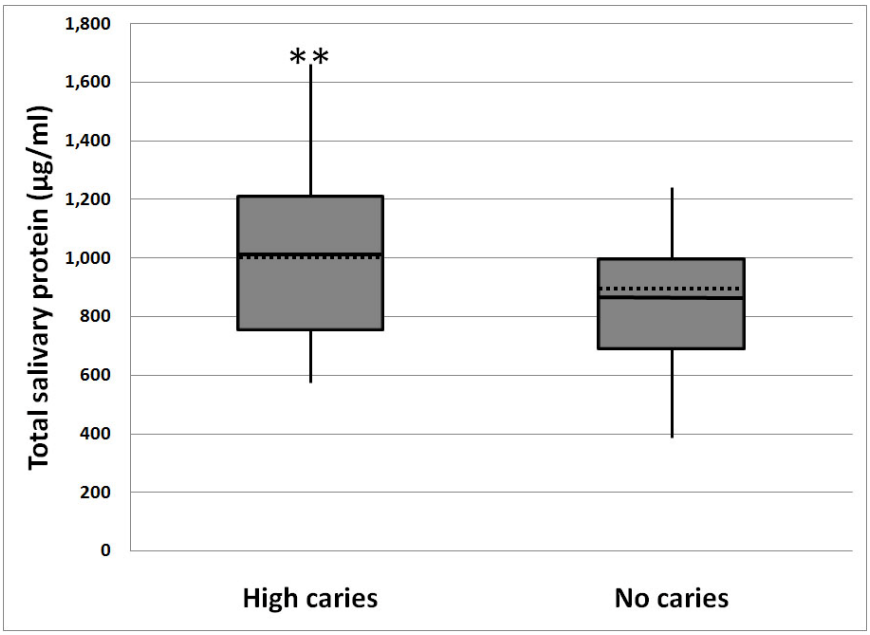
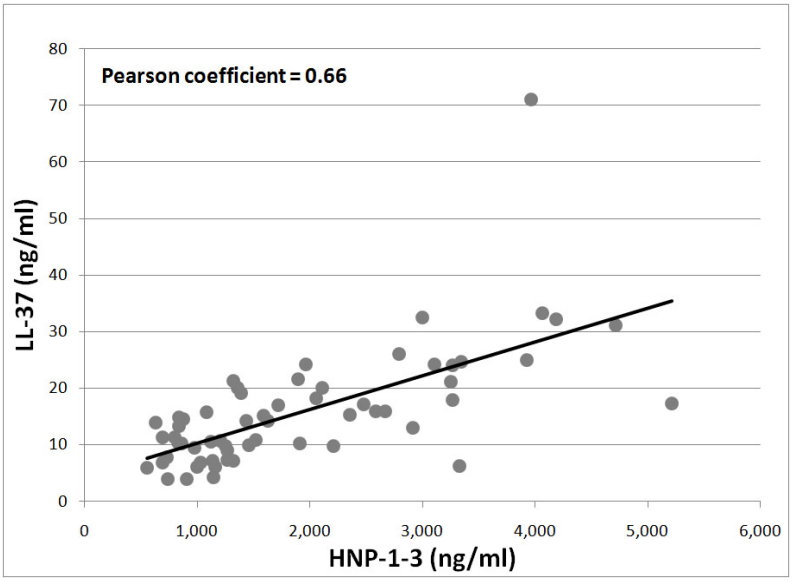


**Figure 4-6 Correlation between the levels of HNP-1 and LL-37 in saliva.** Pearson correlation analyses showed statistically significant correlations between salivary concentrations of HNP-1 and LL-37 ($p<0.0001$) (A); and between salivary concentrations relative to total protein of HNP-1 and LL-37 ($p<0.0001$) (B). Pearson correlation coefficients indicated moderate increasing relationship between these variables.

**A.**




**B.**

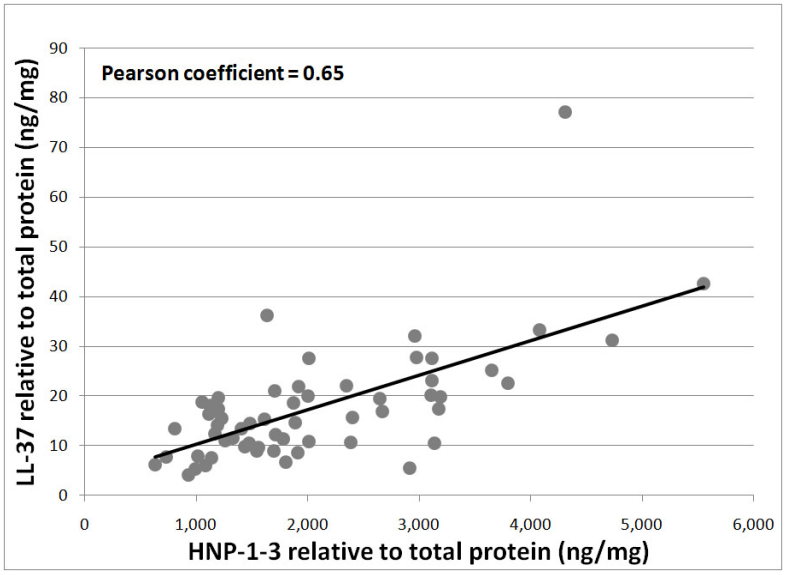
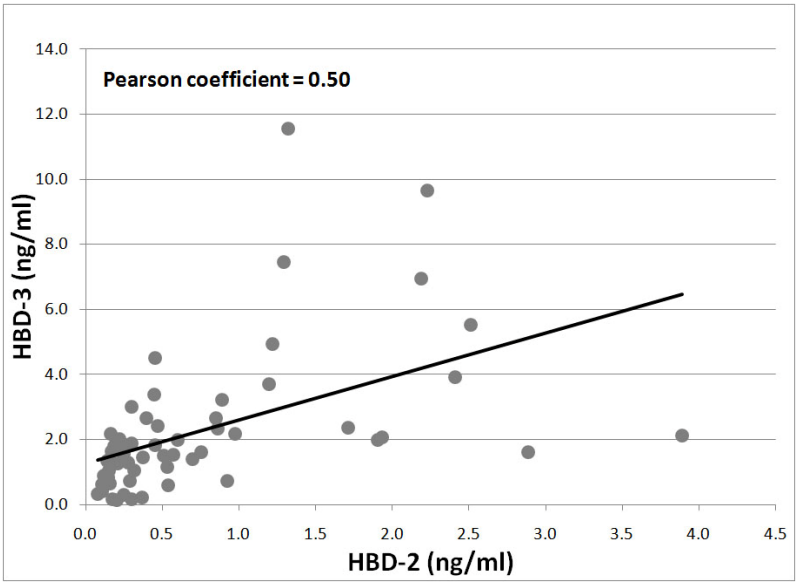


**Figure 4-7 Correlation between the levels of HBD-2 and HBD-3 in saliva.** Pearson correlation analyses showed statistically significant correlations between salivary concentrations of HBD-2 and HBD-3 ($p<0.0001$) (A); and between salivary concentrations relative to total protein of HBD-2 and HBD-3 ($p<0.0001$) (B). Pearson correlation coefficients indicated moderate increasing relationship between these variables.

**A.**




**B.**

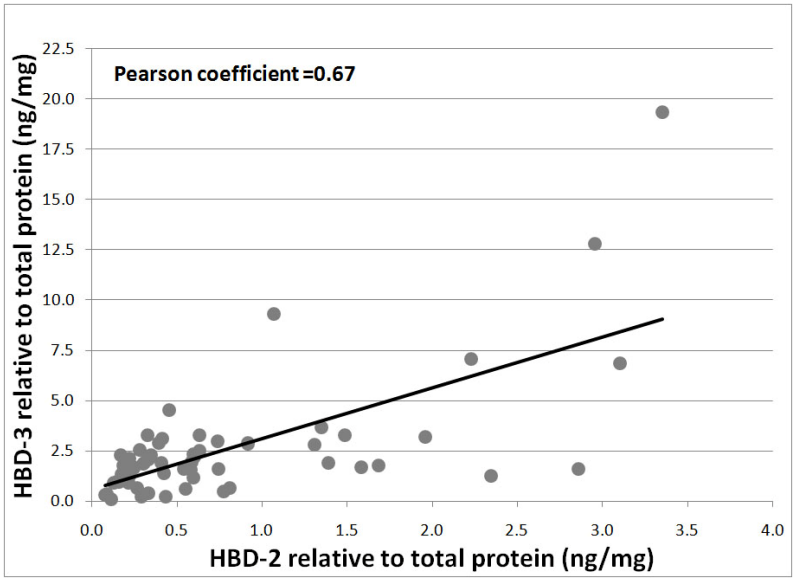
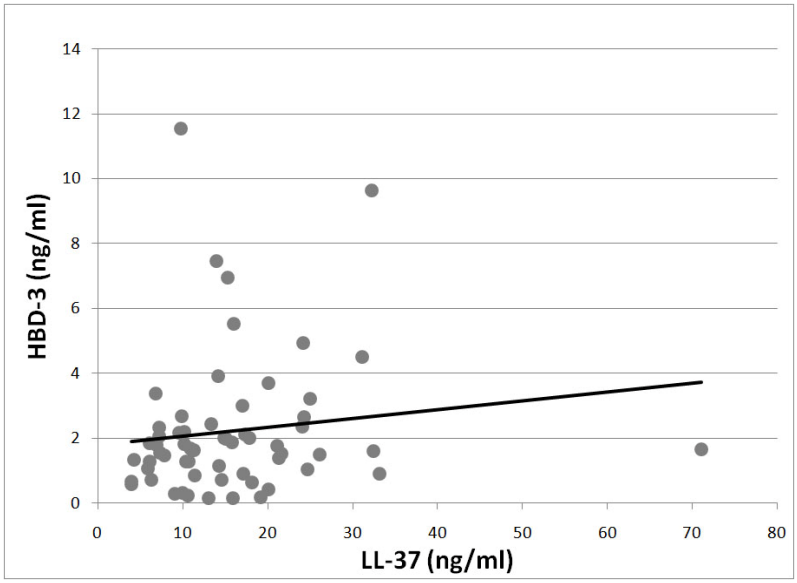


**Figure 4-8 Correlation between the levels of LL-37 and HBD-3 in saliva.** Pearson correlation analysis showed no significant correlations between salivary concentrations of LL-37 and HBD-3 (p=0.3238) (A). However, a significant correlation between salivary concentrations relative to total protein of HBD-2 and HBD-3 was observed (p=0.0466) (B). Pearson correlation coefficients indicated a weak increasing relationship between the two variables.

**A.**




**B.**

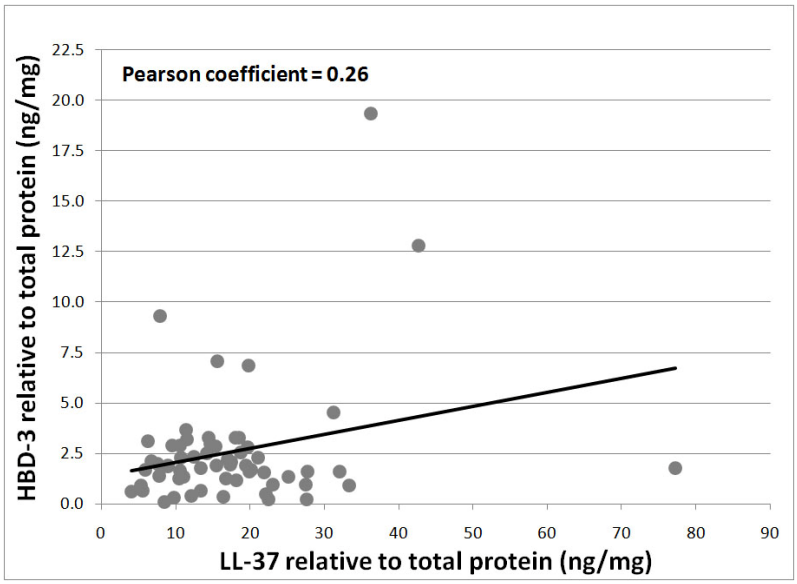
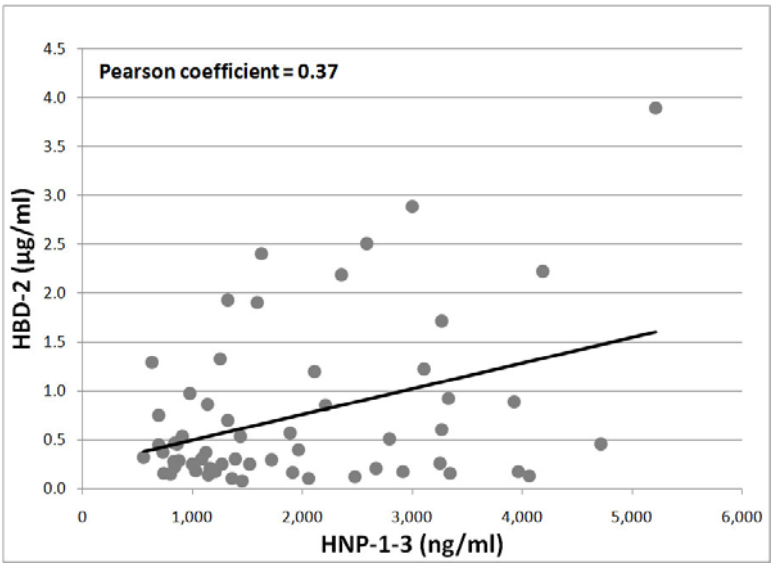


**Figure 4-9 Correlation between the levels of HNP-1-3 and HBD-2 in saliva.** Pearson correlation analysis showed a significant correlation between salivary concentrations of HNP-1-3 and HBD-2 (p=0.0032) (A). The Pearson correlation coefficient indicated a weak increasing relationship between the two variables. However, a significant correlation between salivary concentrations relative to total protein of HNP-1-3 and HBD-3 was not observed (p=0.1076) (B).

**A.**




**B.**

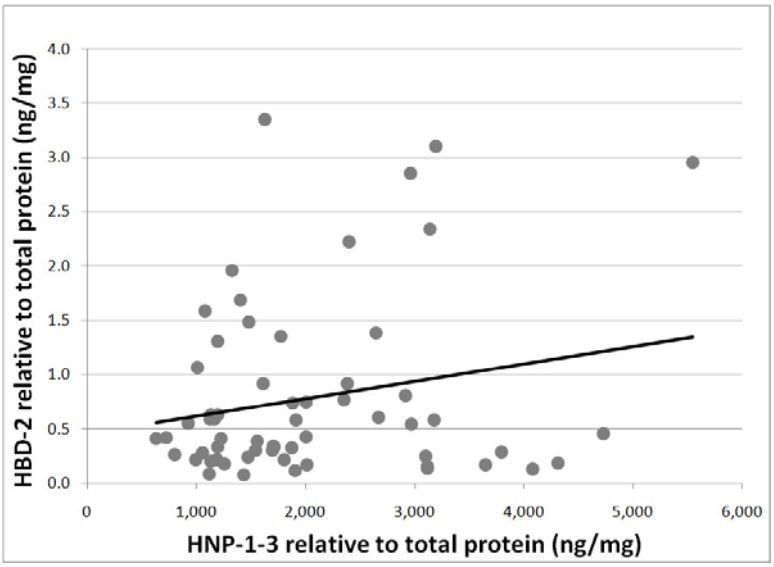
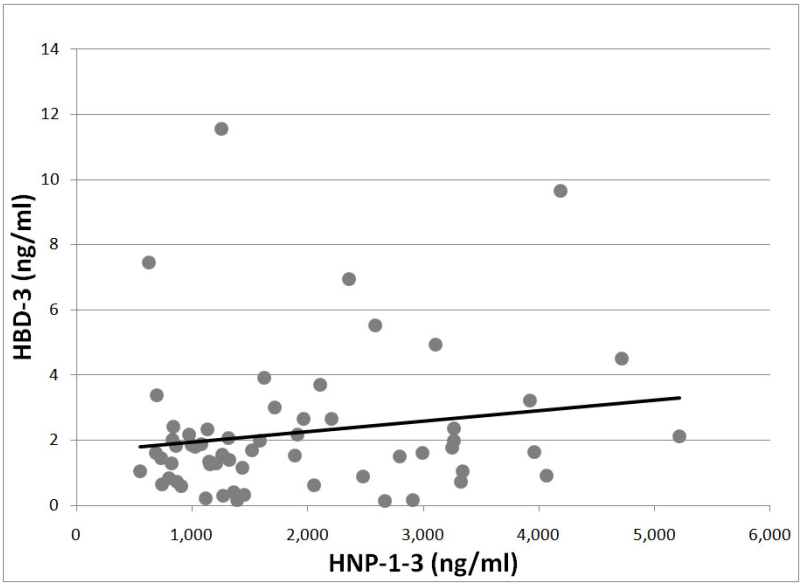


**Figure 4-10 Correlation between the levels of HNP-1-3 and HBD-3 in saliva.** No statistically significant correlations were found between salivary concentrations of HNP-1-3 and HBD-3 (p=0.1920) (A), or between salivary concentrations relative to total protein of HNP-1-3 and HBD-3 (p=0.2618) (B).

**A.**




**B.**

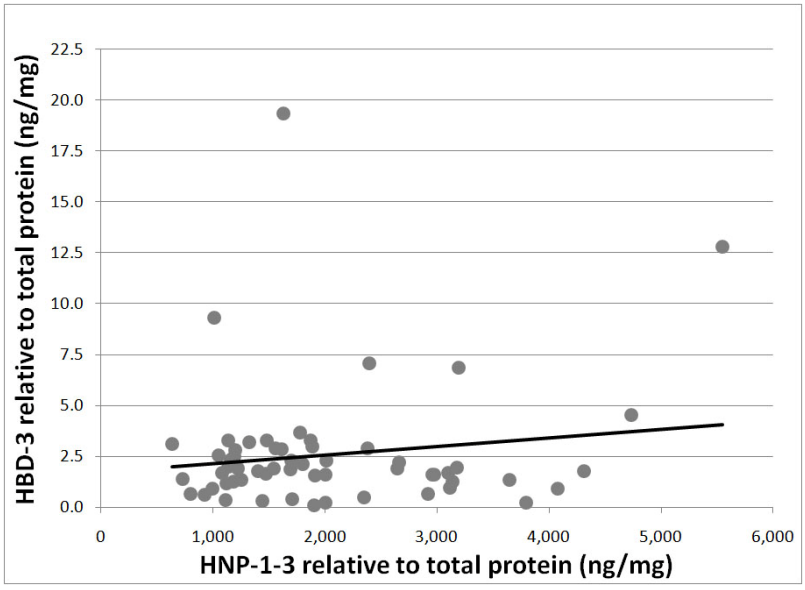
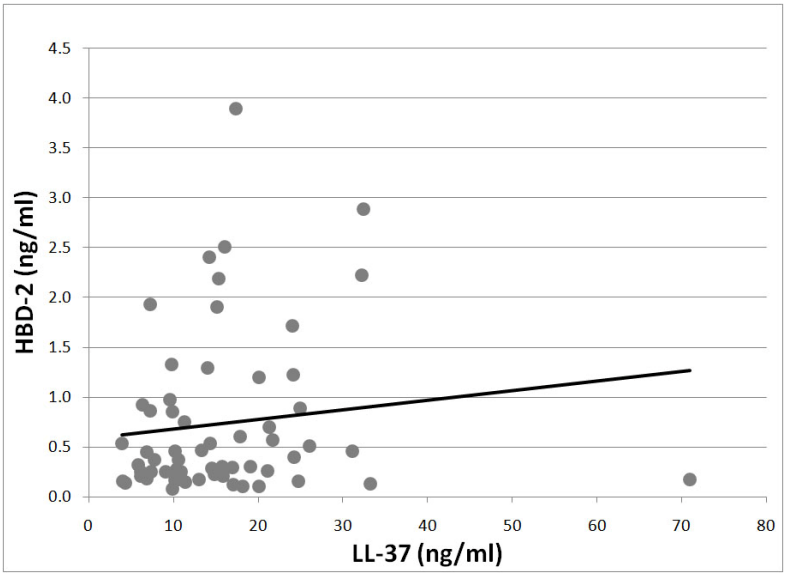


**Figure 4-11 Correlation between the levels of HBD-2 and LL-37 in saliva.** No statistically significant correlations were found between salivary concentrations of HBD-2 and LL-37 (p=0.3459) (A), or between salivary concentrations relative to total protein of HBD-2 and LL-37 (p=0.2059) (B).

**A.**




**B.**

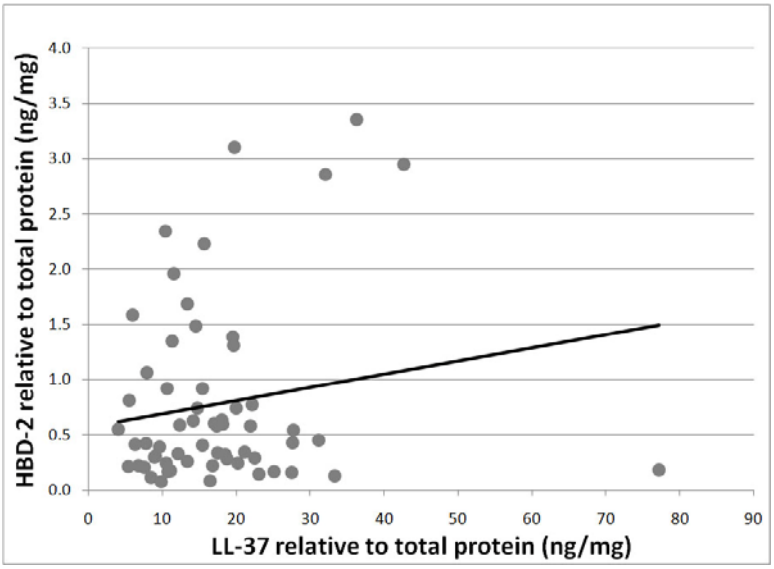


**Figure 4-12 Relationship between levels of HNP-1-3 in saliva and MS count in dental plaque.** A Pearson correlation analysis showed a statistically significant correlation between the salivary concentration of HNP-1-3 relative to total protein and MS count (p=0.0280). The Pearson coefficient indicated a weak increasing relationship between the two variables. Analyses of other peptides showed no significant correlations.

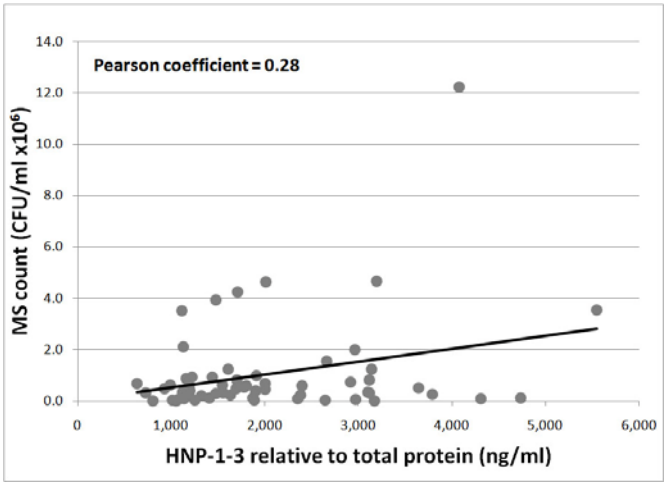



**Figure 4-13 Relationship between levels of HNP-1-3 in saliva and the susceptibility profiles of *S. mutans* to HNP-1.** A Pearson correlation analysis showed a statistically significant correlation between the salivary concentration of HNP-1-3 relative to total protein and the percent viability of *S. mutans* to 5 µg/ml HNP-1 (p=0.0438). Pearson coefficient indicated a weak increasing relationship between the two variables, suggesting that *S. mutans* strains obtained from individuals with higher HNP-1-3 levels in saliva tend to be more resistant to HNP-1. Analyses of other peptides showed no significant correlations.

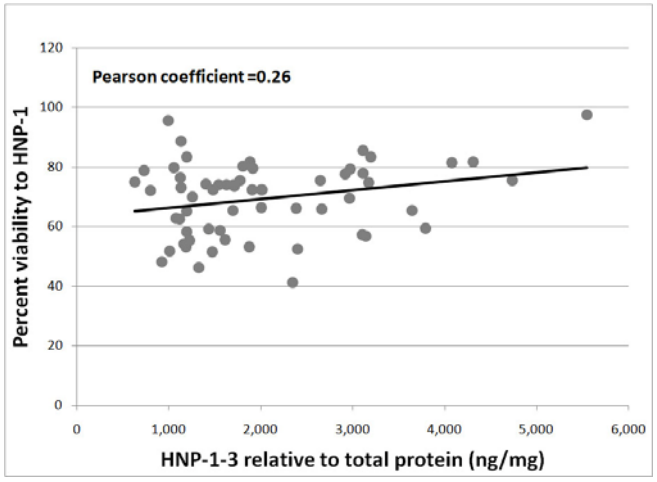


**Table 4-2 Summary of the statistical analyses of the relationships between levels of antimicrobial peptides in saliva and MS levels in dental plaque.**

| Correlations between two variables | P-values |
|---|---|
| HNP-1-3 concentration and MS count | 0.0713 |
| HNP-1-3 concentration relative to total protein and MS count | 0.0280* |
| HNP-1-3 concentration and ratio of MS to total bacteria | 0.7247 |
| HNP-1-3 concentration relative to total protein and ratio of MS to total bacteria | 0.4341 |
| LL-37 concentration and MS count | 0.1816 |
| LL-37 concentration relative to total protein and MS count | 0.2173 |
| LL-37 concentration and ratio of MS to total bacteria | 0.7373 |
| LL-37 concentration relative to total protein and ratio of MS to total bacteria | 0.9200 |
| HBD-3 concentration and MS count | 0.9271 |
| HBD-3 concentration relative to total protein and MS count | 0.8338 |
| HBD-3 concentration and ratio of MS to total bacteria | 0.2230 |
| HBD-3 concentration relative to total protein and ratio of MS to total bacteria | 0.2611 |
| HBD-2 concentration and MS count | 0.7249 |
| HBD-2 concentration relative to total protein and MS count | 0.4970 |
| HBD-2 concentration and ratio of MS to total bacteria | 0.8472 |
| HBD-2 concentration relative to total protein and ratio of MS to total bacteria | 0.7866 |

Pearson correlation analyses were used to assess the correlation between the salivary concentrations of each peptide, with or without normalization, with total salivary protein and the MS count or the ratio of MS relative to total cultivable flora. Asterisks (*) indicate statistically significant findings ($p<0.05$).

**Table 4-3 Summary of the statistical analyses of the relationships between levels of antimicrobial peptides in saliva and total plaque bacteria.**

| Correlations between two variables | Subjects | P-values |
|---|---|---|
| HNP-1-3 concentration in saliva and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.9521<br>0.7445<br>0.5333 |
| HNP-1-3 concentration relative to total salivary protein and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.9114<br>0.8509<br>0.7445 |
| LL-37 concentration in saliva and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.8194<br>0.8932<br>0.7164 |
| LL-37 concentration relative to total salivary protein and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.9409<br>0.8509<br>0.7739 |
| HBD-3 concentration in saliva and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.1000<br>0.0583<br>0.7847 |
| HBD-3 concentration relative to total salivary protein and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.1087<br>0.0946<br>0.5816 |
| HBD-2 concentration in saliva and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.1271<br>0.4234<br>0.1063 |
| HBD-2 concentration relative to total salivary protein and total plaque bacteria | All subjects<br>High caries subjects<br>Caries-free subjects | 0.1110<br>0.3467<br>0.1135 |

Spearman's rank correlation analyses were used to assess the correlation between the salivary concentrations of each peptide, with or without normalization, with total salivary protein and total cultivable plaque flora.

**Table 4-4 Summary of the statistical analyses of the relationships between levels of antimicrobial peptides in saliva and susceptibility profiles of *S. mutans*.**

| Correlations between two variables | P-values |
|---|---|
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml HNP-1 | 0.1524 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml HNP-1 | 0.2012 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml HNP-1 | 0.1916 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 5 μg/ml HNP-1 | 0.0438* |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml HNP-1 | 0.3472 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml HNP-1 | 0.2856 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml HNP-2 | 0.3109 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml HNP-2 | 0.9805 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml HNP-2 | 0.8080 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 5 μg/ml HNP-2 | 0.2155 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml HNP-2 | 0.8905 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml HNP-2 | 0.8309 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml HNP-3 | 0.5827 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml HNP-3 | 0.7343 |
| HNP-1-3 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml HNP-3 | 0.5197 |

**Table 4-4** Continued.

| Correlations between two variables | P-values |
|---|---|
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 5 μg/ml HNP-3 | 0.7251 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml HNP-3 | 0.6520 |
| HNP-1-3 concentration relative to total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml HNP-3 | 0.8158 |
| LL-37 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml LL-37 | 0.1911 |
| LL-37 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml LL-37 | 0.5589 |
| LL-37 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml LL-37 | 0.3706 |
| LL-37 concentration relative total salivary protein and average percent viability of *S. mutans* to 5 μg/ml LL-37 | 0.6100 |
| LL-37 concentration relative total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml LL-37 | 0.7740 |
| LL-37 concentration relative total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml LL-37 | 0.5649 |
| HBD-3 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml HBD-3 | 0.7194 |
| HBD-3 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml HBD-3 | 0.1903 |
| HBD-3 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml HBD-3 | 0.0727 |
| HBD-3 concentration relative total salivary protein and average percent viability of *S. mutans* to 5 μg/ml HBD-3 | 0.3196 |
| HBD-3 concentration relative total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml HBD-3 | 0.0637 |
| HBD-3 concentration relative total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml HBD-3 | 0.0690 |

**Table 4-4** Continued.

| Correlations between two variables | P-values |
|---|---|
| HBD-2 concentration in saliva and average percent viability of *S. mutans* to 5 μg/ml HBD-2 | 0.2999 |
| HBD-2 concentration in saliva and average percent viability of *S. mutans* to 1.5 μg/ml HBD-2 | 0.2713 |
| HBD-2 concentration in saliva and average percent viability of *S. mutans* to 0.5 μg/ml HBD-2 | 0.7021 |
| HBD-2 concentration relative total salivary protein and average percent viability of *S. mutans* to 5 μg/ml HBD-2 | 0.1237 |
| HBD-2 concentration relative total salivary protein and average percent viability of *S. mutans* to 1.5 μg/ml HBD-2 | 0.1235 |
| HBD-2 concentration relative total salivary protein and average percent viability of *S. mutans* to 0.5 μg/ml HBD-2 | 0.4095 |

Pearson correlation analyses were used to assess the correlation between the salivary concentrations of each peptide, with or without normalization, with total salivary protein and the averaged percent viability of *S. mutans* obtained from the same subjects, to each peptide at 5 μg/ml, 1.5 μg/ml and 0.5 μg/ml. Asterisks (*) indicate statistically significant findings ($p<0.05$).

# CHAPTER 5
# CONCLUSIONS AND FUTURE DIRECTIONS

## Conclusions

The oral cavity represents a distinct habitat for a large number of microbial species that reside in a dynamic balance with neighboring microflora as well as host factors. Dental caries develops from an ecological shift of the bacterial population comprising dental plaque towards dominance by specific cariogenic bacteria, primarily *S. mutans*. The main driving force behind this transition is frequent exposure to fermentable carbohydrates, especially sucrose. The subsequent low pH in dental plaque from acids produced by oral bacteria further enriches the colonization of *S. mutans* as well as other aciduric bacteria which are better adapted to survive and prosper under severely acidic conditions that cause demineralization from tooth surfaces.

In maintaining the microbial homeostasis, saliva has a tremendous influence on the microbial ecology of the oral cavity. A number of organic components in saliva can affect the establishment and selection of the oral microflora by limiting the growth or facilitating the clearance of certain organisms, and/or promoting the adhesion of others by forming a selective salivary pellicle on the tooth surface[76]. In this study, we attempted to investigate the influence of a specific subset of salivary proteins, the antimicrobial peptides, on the ecology of *S. mutans* and the relationship with host caries experience.

Firstly, the levels of MS in dental plaque of 13-year old subjects were quantified. The MS counts as well as the ratios of MS to total plaque bacteria were significantly greater in high caries subjects than in caries-free subjects, supporting the principal role of these bacteria in dental caries. Genotyping of *S. mutans* plaque isolates by AP-PCR suggested an increased clonal diversity in high caries individuals. Based on a clustering analysis of AP-PCR fingerprint data, *S. mutans* genotypes from the same subjects tended to show high similarity. This similarity is more likely indicative of mutation or genetic

rearrangement in early established clones than independent acquisition of similar strains from the external environment. The increased clonal diversity of *S. mutans* in high caries subjects may be a consequence of the more favorable host environment for their growth, such as the copious amount of dietary sugars, and/or a less stringent environment with respect to host defense, such as decreased salivary protective factors. As a result, the increased number of *S. mutans* genotypes may enhance their survival advantage over other species, or potentially, the simultaneous interactions among various genotypes within dental plaque may further increase their cariogenic potential *in vivo*.

Various antimicrobial peptides have been detected in saliva, including HNP-1-3, HBD-1-3, LL-37 and histatins. While the assays with HBD-1 and histatin-5 revealed little to no susceptibility, *S. mutans* strains showed varying degrees of susceptibility to HNP-1-3, HBD-2-3 and LL-37. In addition, these peptides, when present together in the local environment, can act additively or synergistically to enhance their antimicrobial activity. Importantly, we found that *S. mutans* strains from high caries subjects were more resistant to salivary antimicrobial peptides than those from caries-free subjects.

As mentioned earlier, the survival and functional activities of microbial inhabitants within dental plaque are ecologically driven. The biofilm species exist in dynamic equilibrium with host defense. From our data, we propose that the relative inherent ability of particular *S. mutans* strains to resist host antimicrobial peptides may provide a distinct advantage over other susceptible strains or species to survive, colonize on tooth surfaces and ultimately contribute to dental caries development. Likewise, host antimicrobial peptides present in saliva may function to maintain a healthy state in the oral cavity by limiting the growth of strains or species that are relatively more susceptible. The antimicrobial activity and the expression of these peptides in saliva and dental plaque may be a factor in the selection of more resistant strains that better populate the plaque thereby increasing the likelihood of initiating or propagating the dental caries process, particularly when the surrounding environment favors their growth.

The correlation analyses between the susceptibility/resistance profiles of *S. mutans* strains and the levels of antimicrobial peptides in saliva also support this notion. We found a significant increasing relationship between the salivary level of HNP-1-3 and resistance to HNP-1 of *S. mutans* strains obtained from the same subjects. Therefore, the expression levels of antimicrobial peptides may provide one of the ecological pressures that dictates the survival and growth of selected microbial species that are able to adapt to, and maintain a balance with, host defense mechanisms. In this case, for the *S. mutans* strains to survive and prosper in hosts with higher expression levels of HNPs, they likely need to possess enhanced resistance mechanisms that better equip them for existing within a more "hostile" environment.

Next, we examined the salivary levels of these antimicrobial peptides in relation to host caries experience. The peptide levels were highly variable among subjects. Significant differences in the mean levels of HNP-1-3, HBD-2, HBD-3 and LL-37 in saliva between high caries and caries-free subjects were not observed. However, we found a significant increasing relationship between MS counts in dental plaque and levels of HNP-1-3 in saliva. Analyzing the relationships between total plaque bacteria and salivary concentrations of HBD-2-3 also showed a similar trend but did not reach statistical significance. The most straightforward explanation of these data is that antimicrobial peptides are produced in saliva in response to an increased plaque bacteria population, including MS. Their expression does not appear to be correlated with host caries experience in these subjects.

Functioning as part of the innate immune system, these antimicrobial peptides exert a broad-spectrum antimicrobial activity against a wide range of microorganisms, including bacteria, fungi and enveloped viruses. Their expression is constitutive or inducible depending on the types of peptides and the local stimuli present. They also exhibit a level of functional redundancy in that a number of peptides may have multiple, and sometimes overlapping functions. As a result, even though, their contributions have

been clearly shown in *in vitro* experiments, it has been difficult to dissect the relationship between the salivary levels of any specific peptide or proteins and host dental caries experience.

In addition to the above findings, we observed significant correlations between salivary levels of these specific types of antimicrobial peptides, specifically HNP-1-3 and LL-37, HBD-2 and HBD-3, HBD-3 and LL-37, and HNP-1-3 and HBD-2. This could be due partly to the overlapping sources of these peptides, such as the neutrophils for HNP-1-3 and LL-37, as well as the oral epithelium and salivary ductal cells for HBD-2, HBD-3 and LL-37. It also suggests that these peptides may be expressed in response to the types of inflammatory stimuli commonly present in the host oral environment at a particular time. The fact that they tended to express concurrently suggests that these peptides may normally function together additively or synergistically *in vivo* to enhance their antimicrobial activity.

In fact, to achieve the optimal protection against dental caries, a variety of host defense molecules, not only salivary antimicrobial peptides, may need to function simultaneously and/or cooperatively to prevent overgrowth of microorganisms as a whole and to maintain a stable ecological system that preferentially favors colonization with harmless microflora over pathogenic organisms.

In summary, our findings support the roles of host salivary antimicrobial peptides in shaping *S. mutans* ecology by restricting the overall growth of this cariogenic bacterium. The relative ability of *S. mutans* to resist the antimicrobial activity of these peptides may constitute a virulence factor for this organism. However, the expression of antimicrobial peptides in saliva is associated with the presence of local inflammatory stimuli, such as the dental plaque microflora, and is not specific to host dental caries experience (Figure 5-1).

## Future directions

Although the role of *S. mutans* in dental caries development is well-established, the effect of host defense factors in the ecology of this bacterium is much less defined. In this thesis, evidence supporting a role for salivary antimicrobial peptides in *S. mutans* ecology in relation to host dental caries experience is presented. Yet it is clear that much remains to be learned regarding the interactions between salivary antimicrobial peptides and dental plaque bacteria as well as their potential roles in host susceptibility to dental caries.

To further delineate the influence of host salivary antimicrobial peptides in dental plaque ecology, it would be valuable to examine the effects of these peptides on the survival and biofilm formation of *S. mutans* as well as other oral species in mixed culture biofilm models that better represent the complexity of dental caries-related plaque. It is well-known that bacteria can behave fundamentally different when they are in aggregates or in biofilms. Neighboring species may interact cooperatively and promote genetic exchange. On the other hand, with limited space and nutrients, a certain degree of bacterial interference or competition among species exists that could affect the overall composition of the bacterial community[313-315].

Several dental plaque models can be performed[316]. The numbers of bacterial species included can be varied but their presence and proportions should be representative of caries-related plaque biofilms. Potential microbial species other than the MS include the early colonizers of dental plaque, such as *S. sanguinis*, *S. gordonii*, *S. mitis*, and *S. oralis*, as well as acid-tolerant and potentially cariogenic bacteria, such as Lactobacilli and *Actinomyces* species. To date, as many as 40 microbial species characteristic of dental caries-related plaque can be grown simultaneously and quantitatively analyzed[317,318]. Such experiments would help characterize the differential impact of salivary antimicrobial peptides individually or in combination, on the survival of each species within biofilms in the presence or absence of fermentable sugars.

Our data, as well as that from others, indicated that the susceptibility of *S. mutans* to antimicrobial peptides was strain-specific. It would be of interest to investigate which components are responsible for these differences. The mechanisms of *S. mutans* resistance to antimicrobial peptides have not been studied. The ability of cationic antimicrobial peptides to bind and integrate into the bacterial cell membrane is crucial for their action and depends largely on the net negative charge of bacterial surface[106]. One potential candidate for this resistance is the function of the *dlt* operon which is involved in the D-alanylation of lipoteichoic acids (LTAs), one of the major membrane targets of these peptides in Gram-positive bacteria[104]. Introduction of D-alanine onto LTAs partially neutralizes the net negative charge on the bacterial cell membrane, and has been shown to increase the resistance to antibiotics and/or cationic antimicrobial peptides in a number of Gram-positive bacteria[169-174]. The knowledge of *S. mutans* resistance to host salivary antimicrobial peptides may be beneficial for the future development of more effective antimicrobial peptides in the clinical setting.

Our studies have also shown that the levels of antimicrobial peptides in saliva are highly variable. The basis for these marked variations is not yet known. To better understand the biology of these peptides in saliva, several aspects of the expression of these peptides in saliva should be characterized, including potential variations based on age, sex, race, or time of a day. It has been shown that the levels of HNP-1-3 and LL-37 are increased following exercise[274], but their associations with other physical and/or mental conditions are not known. In addition, variations of peptide levels in unstimulated versus stimulated saliva should also be evaluated.

As mentioned earlier, levels of salivary peptides and proteins depend a great deal on an individual's salivary flow rate. In addition, simultaneous gingival or other mucosal inflammation may increase the levels of these peptides regardless of dental caries status[319-321]. As a result, the detection of peptide levels may not be the ideal means for studying the potential role of these peptides in host susceptibility to dental caries.

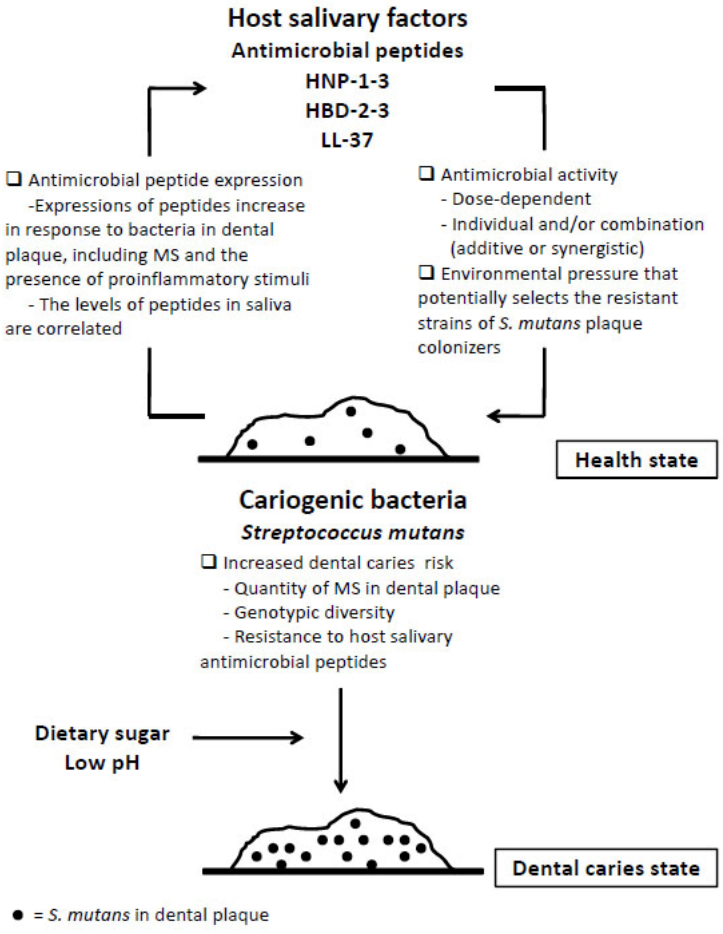
Previous studies have identified copy number polymorphisms for both α-defensins genes (*DEFA1* and *DEFA3*) and β-defensin genes (*DEFB4*, *DEFB103* and *DEFB104*)[269,270,322]. The copy numbers of *DEFA1/3* and *DEFB4/103* varied independently of each other within a subject. Importantly, the gene copy numbers of both α-defensins and β-defensins were significantly correlated with their mRNA transcript levels[269,322]. Studies on copy number polymorphisms of these genes in representative populations with variable caries experience could potentially help determine their role in host genetic susceptibility to dental caries.

Finally, longitudinal-designed studies should be conducted to comprehensively analyze the relationships between salivary antimicrobial peptides, dental plaque bacteria and dental caries development. The study population should include unrelated children of identical age. Ideally, subjects should be at a young age prior to the initiation of the dental caries process. Baseline health history and oral examination, including mucosal and dental-related conditions, should be thoroughly recorded. Saliva and plaque samples should be collected at baseline and periodically throughout the cohort period of several years. Additionally, genetic studies of defensin genes may also be performed at baseline as mentioned previously. The changes in bacterial populations in dental plaque and the salivary levels of antimicrobial peptides in relation to the caries increments could then be analyzed to determine their associations with dental caries risk. Furthermore, functional studies of subject saliva in terms of their collective antibacterial activity against *S. mutans* or mixed-species cultures could be helpful in better demonstrating the biological significance of the various antimicrobial peptides when all are together in the presence of other salivary components.

In summary, this thesis has begun to characterize the influence of salivary antimicrobial peptides, in particular HNP-1-3, HBD-2-3 and LL-37, to *S. mutans* ecology in dental plaque and their relationships to host caries experience. These findings have provided the significant groundwork for future studies to further elucidate the roles of

these peptides in shaping the complex microbial ecology of dental plaque. In the end, our knowledge in this regard will facilitate the development of improved and efficacious preventive and treatment protocols for dental caries.

**Figure 5-1 Diagram summarizing the proposed role of salivary antimicrobial peptides in shaping *S. mutans* ecology and their contribution to dental caries development.** Dental plaque biofilm is a complex microbial community consisting of a wide range of species. Microbial species in dental plaque maintain the ecological balance with neighboring species as well as with host factors in saliva. Under healthy conditions, potentially pathogenic microorganisms, in this case *S. mutans*, are present at low quantity, whereas the non-pathogenic species predominate in dental plaque and restrict the growth of cariogens. The plaque microflora induces the expression of antimicrobial peptides in saliva, which exert their antimicrobial activity both individually and in combinations against *S. mutans*. As a result, the resistance of *S. mutans* strains to host antimicrobial peptides in saliva is crucial for their survival and continued persistence in the oral cavity. When and if a host chooses to consume copious amounts of fermentable sugars for extended periods of time, an advantage is extended to resident *S. mutans* as part of a shift in dental plaque ecology. A number of bacterial species can metabolize the sugars to produce organic acids. As the plaque pH continues to decrease, the acid-tolerant bacteria, particularly *S. mutans*, are increasingly enriched, more genotypically diverse, and eventually become the predominant species in dental plaque. This leads to further production of acid in close proximity to the tooth surface, causing tooth demineralization and dental caries.



# APPENDIX A

## SUBJECT DEMOGRAPHICS, HEALTH HISTORY AND CARIES STATUS

Demographic data, caries scores as well as health history, including past serious illnesses, surgery and antibiotic usage, of all subjects are shown. Table A) High caries subjects; Table B) Caries-free subjects, F=female; M=male.

**Table A-1 Demographics, health history and caries status of high caries subjects.**

| Subject IDs | Sex | Race | $D_2FS$ scores | Antibiotics usage | History of previous surgery or serious illness |
|---|---|---|---|---|---|
| 31 | F | White | 9 | Never | |
| 153 | F | White | 14 | Past usage | |
| 212 | F | White | 7 | Past usage | Frequent infections & dehydration as infant treated with IVIG and antibiotics |
| 386 | F | White | 10 | Never | |
| 498 | M | White | 3 | Past usage | |
| 507 | F | White | 14 | Past usage | |
| 721 | F | White | 5 | Past usage | Eye surgery to correct intermittent crossing |
| 740 | F | White | 5 | Never | |
| 818 | F | White | 10 | Past usage | |
| 866 | M | White | 7 | Past usage | |
| 934 | F | White | 4 | Past usage | |
| 992 | M | White | 4 | Past usage | |
| 1128 | M | White | 7 | Past usage | |
| 1132 | F | White | 5 | Past usage | |
| 1182 | F | White | 9 | Past usage | Ear tubes |
| 1255 | F | White | 4 | Past usage | Pneumonia |
| 1274 | F | White | 4 | Past usage | |
| 1310 | M | White | 3 | Past usage | Ear tubes, hernia, nose cauterized |
| 1327 | F | White | 3 | Past usage | |
| 1409 | M | White | 3 | Past usage | Surgery to repair torn lip |
| 1423 | M | White | 5 | Past usage | |
| 1430 | F | White | 10 | Past usage | |
| 1649 | F | White | 4 | Past usage | |
| 1678 | M | White | 8 | Past usage | Tonsillectomy, Adenoidectomy |
| 1709 | F | White | 4 | Never | |
| 1721 | F | White | 6 | Past usage | |
| 1739 | F | White | 3 | Past usage | |
| 1774 | F | White | 3 | Never | |
| 1838 | M | Hispanic | 3 | Past usage | |
| 1860 | M | Black | 5 | Past usage | |

**Table A-2 Demographics, health history and caries status of caries-free subjects.**

| Subject IDs | Sex | Race | $D_2FS$ scores | Antibiotics usage | History of serious illness or surgery |
|---|---|---|---|---|---|
| 4 | F | White | 0 | Past usage | |
| 12 | F | White | 0 | Current usage | Ear tubes |
| 53 | M | White | 0 | Past usage | |
| 260 | M | White | 0 | Past usage | |
| 271 | F | White | 0 | Past usage | |
| 301 | M | White | 0 | Past usage | |
| 544 | M | White | 0 | Past usage | Bilateral ureteral placement |
| 556 | F | White | 0 | Past usage | Tonsillectomy |
| 645 | F | White | 0 | Never | |
| 654 | M | White | 0 | Past usage | Tonsillectomy, adenoidectomy |
| 710 | F | White | 0 | Past usage | |
| 765 | F | White | 0 | Past usage | Tonsillectomy |
| 770 | F | White | 0 | Past usage | |
| 784 | F | White | 0 | Never | |
| 794 | M | White | 0 | Never | |
| 817 | F | White | 0 | Never | |
| 971 | F | White | 0 | Past usage | |
| 1058 | M | White | 0 | Past usage | Ear tubes, tonsillectomy |
| 1166 | M | White | 0 | Past usage | |
| 1236 | F | White | 0 | Past usage | |
| 1282 | M | White | 0 | Never | |
| 1347 | F | White | 0 | Past usage | |
| 1404 | M | White | 0 | Past usage | |
| 1465 | F | White | 0 | Past usage | |
| 1602 | F | White | 0 | Past usage | |
| 1609 | M | White | 0 | Never | |
| 1653 | F | White | 0 | Past usage | |
| 1744 | F | White | 0 | Past usage | |
| 1787 | F | White | 0 | Never | |
| 1810 | M | White | 0 | Past usage | |

# APPENDIX B

## DENTAL CARIES-RELATED DIETARY HISTORY OF SUBJECTS

Pertinent information regarding dental caries risk factors are shown, including dietary information as well as personal oral hygiene performance. Percent carb indicates the percentage of carbohydrate in the diet of the subject per day. Percent sugar indicates the percentage of sugar-based foods in the diet of the subject per day. Snacks per day represents the number of snacks the subject consumed per day. Dietary energy showed the daily energy intake (Kcal/day). Dietary carb indicates the quantity of carbohydrate intake per day (g/day). Fluoride intake represents the cumulative fluoride intake from water consumed as beverages and added during preparation of foods, other beverages, dietary fluoride supplements and fluoride dentifrices (mg/day). NA: data not available. Details on the collection and abstraction of the dietary data from these subjects were explained elsewhere[323-326].

**Table B-1 Dental caries-related dietary history of high caries subjects.**

| Subject IDs | Percent Carb | Percent sugar | Snacks per day | Dietary energy (Kcal/day) | Dietary Carb (g/day) | Fluoride intake (mg/day) | Daily tooth brushing frequency |
|---|---|---|---|---|---|---|---|
| 31 | 67.08 | 48.72 | 1 | 1062.83 | 178.24 | 0.709 | 2 |
| 153 | 46.86 | 9.51 | 2 | 1283.20 | 150.33 | 0.400 | 2 |
| 212 | 58.89 | 18.14 | 2 | 1318.44 | 194.09 | 2.044 | 2 |
| 386 | 52.84 | 27.17 | 1 | 1904.72 | 251.60 | 0.764 | 1 |
| 498 | 47.24 | 25.47 | 2 | 980.04 | 115.75 | 0.594 | 2 |
| 507 | 52.69 | 32.88 | NA | 1067.87 | 140.65 | 0.335 | 2 |
| 721 | 47.31 | 10.51 | 1 | 1668.77 | 197.37 | 0.796 | 0.5 |
| 740 | 50.18 | 25.42 | 1 | 1583.61 | 198.68 | 0.502 | 2 |
| 818 | 50.49 | 24.06 | 1 | 1310.07 | 165.36 | 0.239 | 2 |
| 866 | 38.15 | 16.30 | 2 | 2478.78 | 236.44 | 1.093 | 0.5 |
| 934 | 48.60 | 18.51 | 2 | 877.05 | 106.56 | 0.438 | 2 |
| 992 | 53.38 | 23.08 | 2 | 1724.79 | 230.19 | NA | 2 |
| 1128 | 46.56 | 20.61 | 2 | 1968.10 | 229.11 | 0.711 | 1 |
| 1132 | 31.72 | 18.00 | 3 | 3036.88 | 240.83 | 0.823 | 0.5 |
| 1182 | 48.00 | 15.07 | NA | 2016.16 | 241.92 | NA | NA |
| 1255 | 47.64 | 26.69 | 1 | 2267.55 | 270.06 | 0.523 | 2 |
| 1274 | 55.54 | 31.73 | 2 | 2133.07 | 296.20 | 1.799 | 2 |
| 1310 | 46.16 | 23.61 | NA | 1692.49 | 195.29 | NA | NA |
| 1327 | 57.37 | 26.65 | 2 | 2003.63 | 287.36 | 0.306 | 0.5 |
| 1409 | 51.47 | 31.30 | 1 | 2780.24 | 357.72 | 1.020 | 1 |
| 1423 | 55.37 | 29.73 | 4 | 1576.01 | 218.14 | 0.452 | 2 |
| 1430 | 40.38 | 20.22 | 4 | 2823.30 | 285.04 | NA | 2 |
| 1649 | 50.38 | 29.03 | 2 | 1264.83 | 159.29 | NA | 1 |
| 1678 | 52.64 | 16.67 | 1 | 1286.83 | 169.35 | 0.761 | 2 |
| 1709 | 53.02 | 22.17 | 2 | 1200.40 | 159.13 | 0.286 | 2 |
| 1721 | 43.07 | 21.29 | 4 | 1183.72 | 127.47 | 0.448 | 1 |
| 1739 | 40.06 | 15.50 | 3 | 2616.08 | 262.02 | 1.379 | 1 |
| 1774 | 48.33 | 33.57 | 2 | 6922.02 | 836.42 | 0.843 | 2 |
| 1838 | NA | NA | 1 | NA | NA | NA | NA |
| 1860 | 53.10 | 24.09 | 2 | 1165.22 | 154.69 | 0.521 | 1 |

**Table B-2 Dental caries-related dietary history of caries-free subjects.**

| Subject IDs | Percent Carb | Percent sugar | Snacks per day | Dietary KCAL (Kcal/day) | Dietary Carb (g/day) | Fluoride intake (mg/day) | Daily tooth brushing frequency |
|---|---|---|---|---|---|---|---|
| 4 | 40.61 | 19.36 | 1 | 868.92 | 88.22 | 0.548 | 3 |
| 12 | 48.78 | 20.19 | 1 | 1344.81 | 164.00 | 0.306 | 2 |
| 53 | 53.70 | 19.75 | 2 | 1977.81 | 265.53 | 0.746 | 2 |
| 260 | 51.03 | 24.28 | 2 | 1762.36 | 224.84 | 1.087 | 2 |
| 271 | 47.92 | 14.98 | 1 | 1691.97 | 202.72 | 0.634 | 2 |
| 301 | 47.09 | 21.75 | 1 | 955.31 | 112.46 | 0.421 | 2 |
| 544 | 47.57 | 16.41 | NA | 2095.39 | 249.17 | 0.484 | 1 |
| 556 | 43.56 | 23.84 | 2 | 1923.49 | 209.46 | 1.098 | 0.5 |
| 645 | 50.78 | 38.55 | NA | 910.68 | 115.61 | 0.916 | 1 |
| 654 | 55.17 | 21.83 | 3 | 2126.85 | 293.33 | 0.854 | 1 |
| 710 | 51.92 | 15.60 | 2 | 1789.68 | 232.31 | 0.521 | 2 |
| 765 | 50.94 | 13.95 | 2 | 2317.84 | 295.20 | 0.422 | 2 |
| 770 | 47.61 | 12.51 | 2 | 1714.76 | 204.12 | NA | NA |
| 784 | 49.98 | 34.45 | 2 | 1445.32 | 180.58 | 0.635 | 1 |
| 794 | 41.57 | 35.13 | 3 | 1801.45 | 187.22 | 1.113 | 1 |
| 817 | 52.37 | 7.86 | NA | 2802.48 | 366.92 | 0.079 | 1 |
| 971 | 61.83 | 28.80 | 2 | 1503.83 | 232.44 | 0.695 | 2 |
| 1058 | 47.14 | 12.46 | 2 | 2379.05 | 280.35 | 0.424 | 3.5 |
| 1166 | 43.31 | 23.13 | 2 | 2695.82 | 291.92 | 1.392 | 0.5 |
| 1236 | 49.34 | 11.26 | 2 | 1228.25 | 151.51 | 0.314 | 1 |
| 1282 | 53.30 | 32.50 | 0 | 1586.57 | 211.41 | 0.679 | 0.5 |
| 1347 | 50.75 | 17.85 | 1 | 1990.28 | 252.53 | 0.403 | 1 |
| 1404 | NA | NA | 2 | NA | NA | 0.284 | 2 |
| 1465 | 53.02 | 27.07 | 2 | 2102.71 | 278.71 | 0.487 | 2 |
| 1602 | 51.12 | 19.04 | 3 | 2108.44 | 269.46 | 0.859 | 2 |
| 1609 | 51.91 | 22.77 | 1 | 1661.49 | 215.60 | 0.578 | 2 |
| 1653 | 51.59 | 26.07 | 2 | 1423.73 | 183.61 | 1.179 | 3.5 |
| 1744 | 55.02 | 23.87 | 1 | 935.36 | 128.65 | NA | NA |
| 1787 | NA | NA | 4 | NA | NA | 1.547 | 2 |
| 1810 | 56.68 | 21.86 | 2 | 1433.60 | 203.15 | 1.175 | 1 |

# APPENDIX C

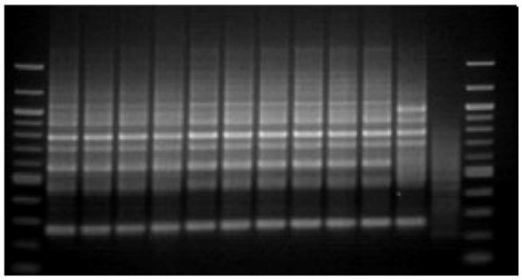
## AP-PCR FINGERPRINTING PROFILES OF *S. MUTANS* ISOLATES.

AP-PCR fingerprinting profiles of all *S. mutans* isolates from plaque samples of 60 subjects are shown. Following biochemical testing to confirm their identity, 10 *S. mutans* isolates per subject were genotyped by AP-PCR using two random primers, OPA2 and the OPA13.

Numbers 1-10 represent the numbers designated for *S. mutans* isolates obtained from a particular subject. A blank lane with no template DNA was included in each experiment (B). DNA template from a laboratory strain of *S. mutans* ATCC25175 was used as a control *S. mutans* DNA (C).

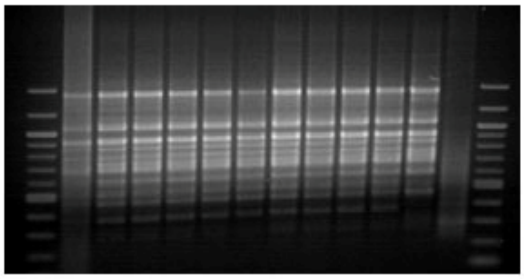
**Figure C-1 AP-PCR Fingerprinting profiles of *S. mutans* isolates**

Sample 4
OPA2



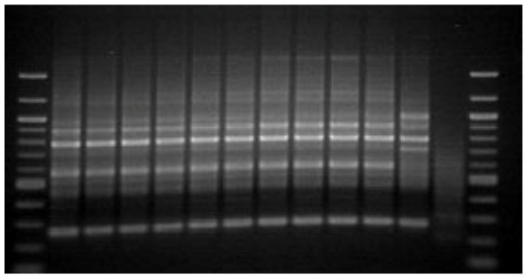
1 2 3 4 5 6 7 8 9 10 C B

OPA13



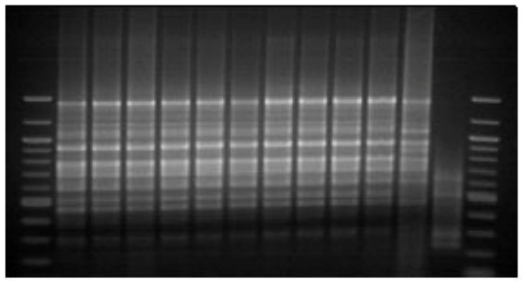
1 2 3 4 5 6 7 8 9 10 C B

Sample 12
OPA2



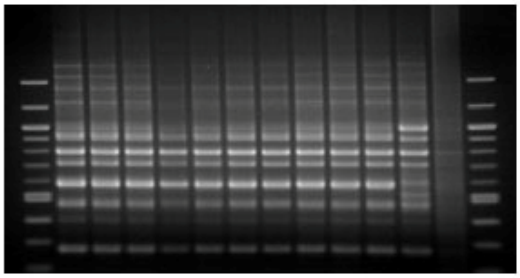
1 2 3 4 5 6 7 8 9 10 C B

OPA13



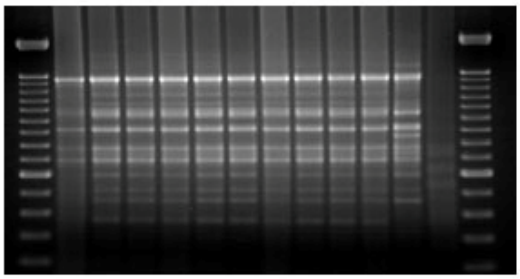
1 2 3 4 5 6 7 8 9 10 C B

Sample 31
OPA2



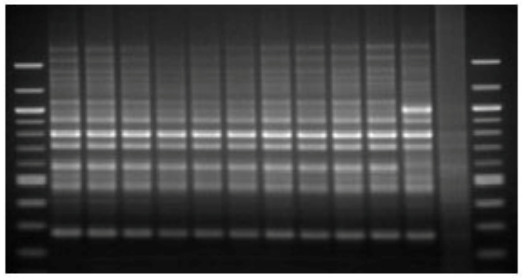
1 2 3 4 5 6 7 8 9 10 C B

OPA13



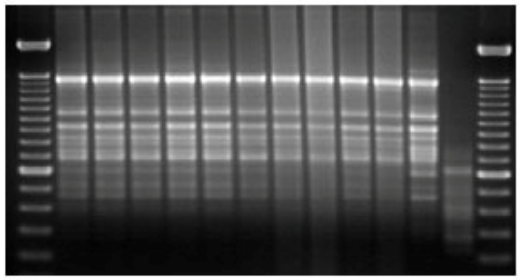
1 2 3 4 5 6 7 8 9 10 C B

Sample 53
OPA2



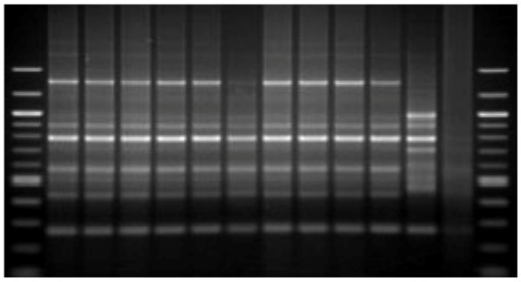
1 2 3 4 5 6 7 8 9 10 C B

OPA13



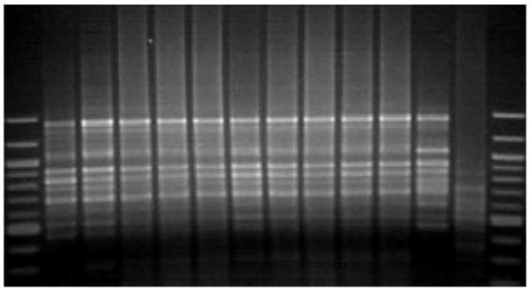
1 2 3 4 5 6 7 8 9 10 C B

Sample 153
OPA2



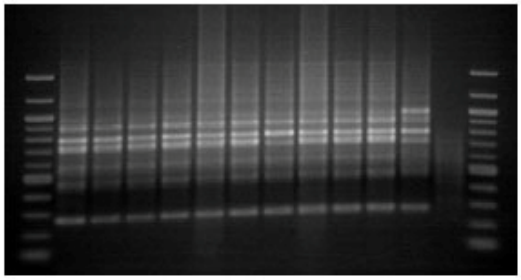
1 2 3 4 5 6 7 8 9 10 C B

OPA13



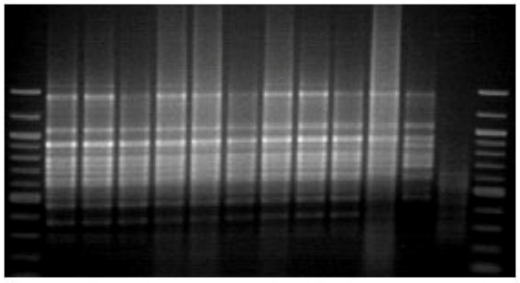
1 2 3 4 5 6 7 8 9 10 C B

Sample 212
OPA2



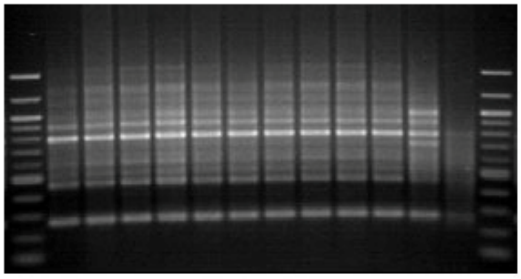
1 2 3 4 5 6 7 8 9 10 C B

OPA13



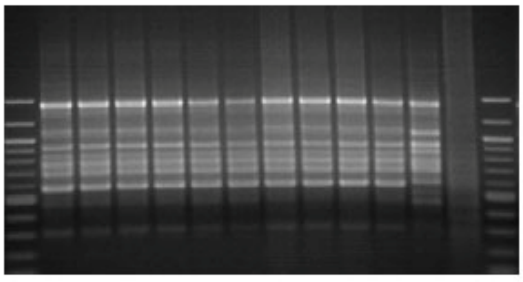
1 2 3 4 5 6 7 8 9 10 C B

Sample 260
OPA2



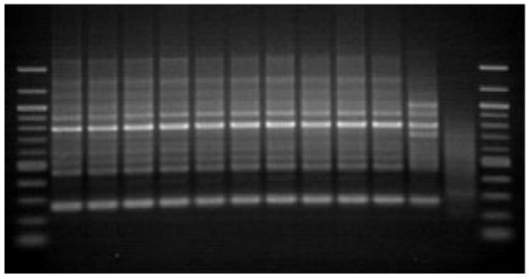
1 2 3 4 5 6 7 8 9 10 C B

OPA13



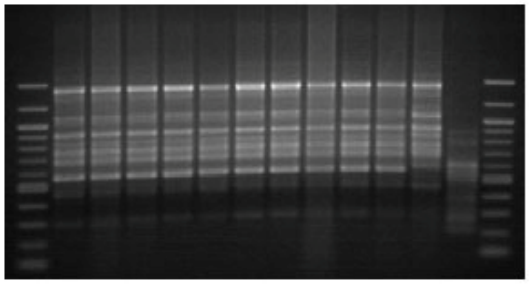
1 2 3 4 5 6 7 8 9 10 C B

Sample 271
OPA2



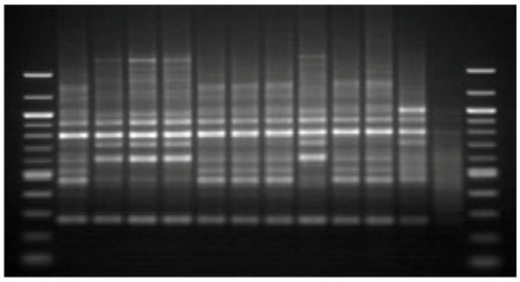
1 2 3 4 5 6 7 8 9 10 C B

OPA13



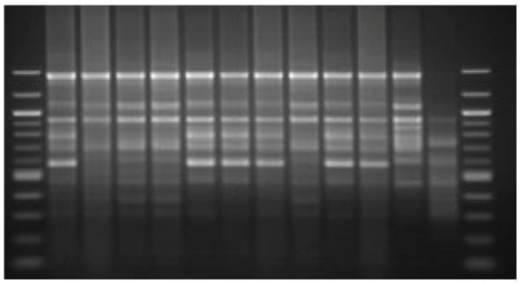
1 2 3 4 5 6 7 8 9 10 C B

Sample 301
OPA2



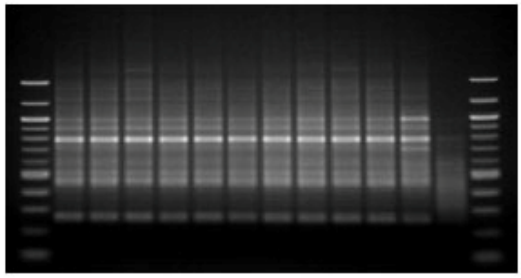
1 2 3 4 5 6 7 8 9 10 C B

OPA13



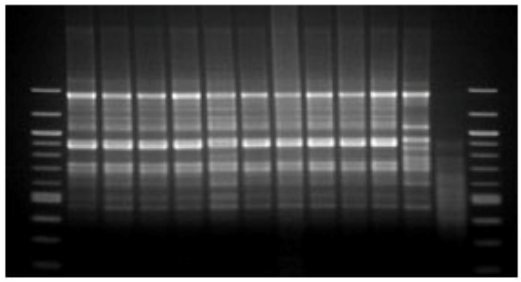
1 2 3 4 5 6 7 8 9 10 C B

Sample 386
OPA2



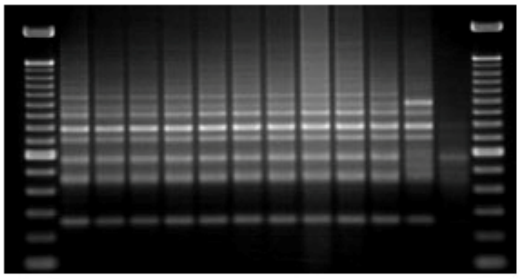
1 2 3 4 5 6 7 8 9 10 C B

OPA13



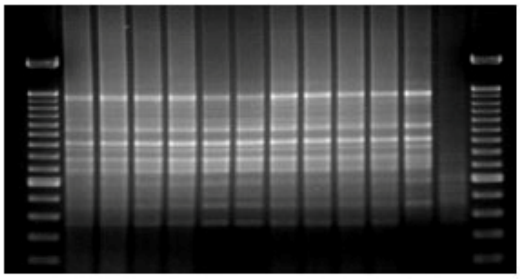
1 2 3 4 5 6 7 8 9 10 C B

Sample 498
OPA2



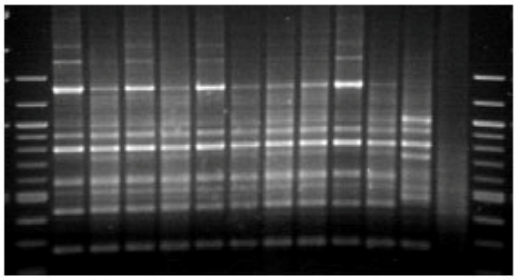
1 2 3 4 5 6 7 8 9 10 C B

OPA13



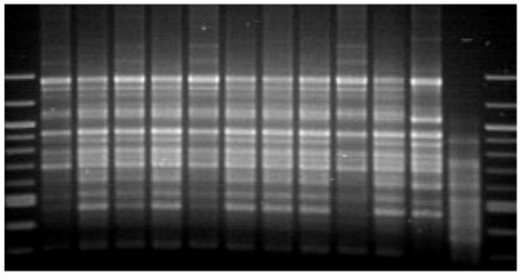
1 2 3 4 5 6 7 8 9 10 C B

Sample 507
OPA2



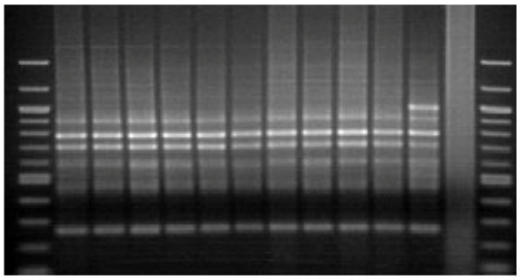
1 2 3 4 5 6 7 8 9 10 C B

OPA13



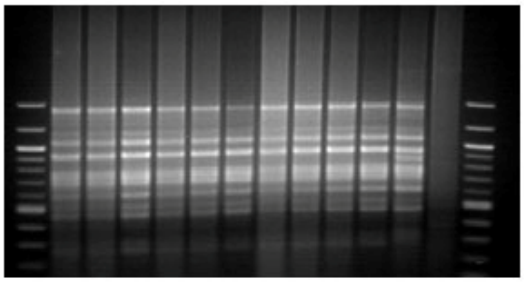
1 2 3 4 5 6 7 8 9 10 C B

Sample 544
OPA2



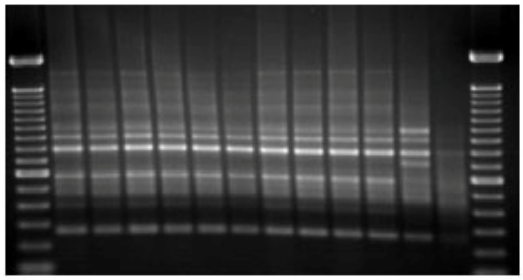
1 2 3 4 5 6 7 8 9 10 C B

OPA13



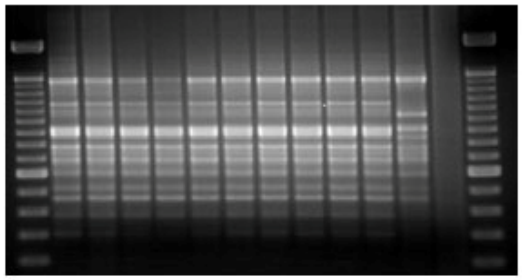
1 2 3 4 5 6 7 8 9 10 C B

Sample 556
OPA2



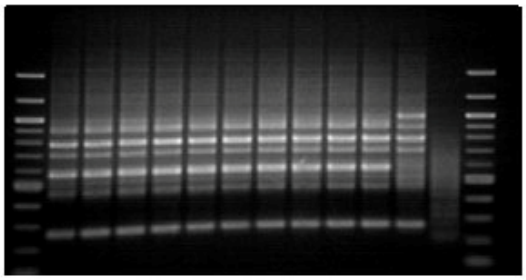
1 2 3 4 5 6 7 8 9 10 C B

OPA13



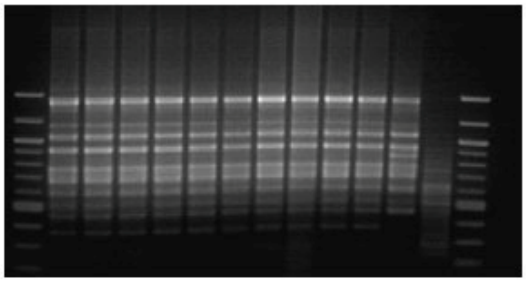
1 2 3 4 5 6 7 8 9 10 C B

Sample 645
OPA2



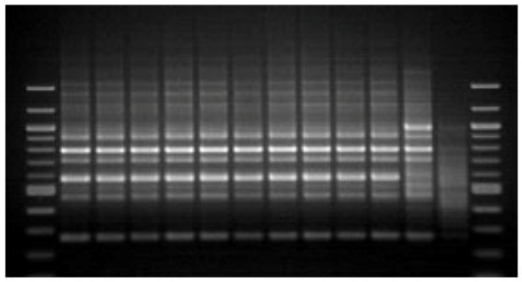
1 2 3 4 5 6 7 8 9 10 C B

OPA13



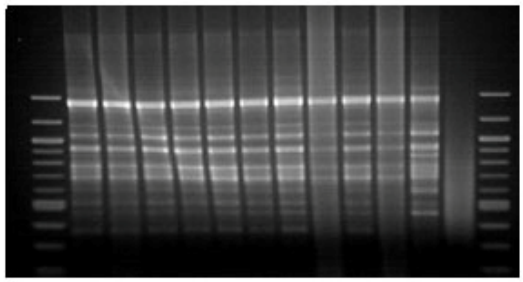
1 2 3 4 5 6 7 8 9 10 C B

Sample 654
OPA2



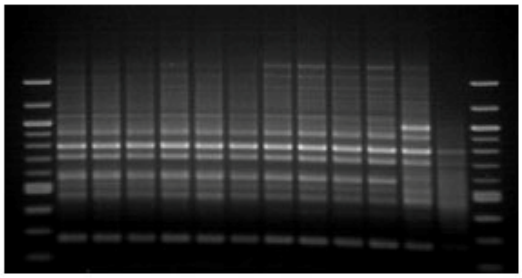
1 2 3 4 5 6 7 8 9 10 C B

OPA13



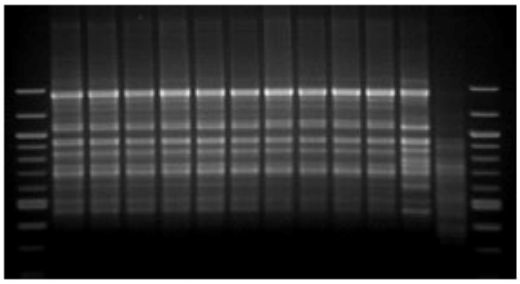
1 2 3 4 5 6 7 8 9 10 C B

Sample 710
OPA2



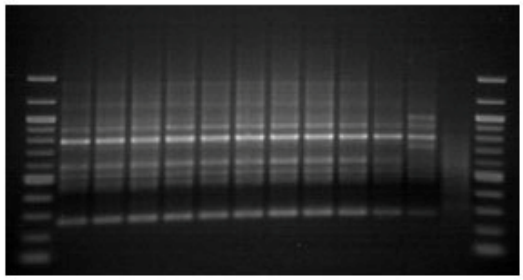
1 2 3 4 5 6 7 8 9 10 C B

OPA13



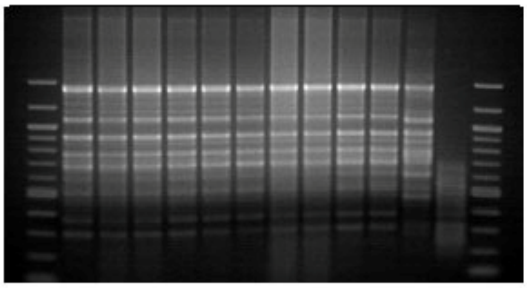
1 2 3 4 5 6 7 8 9 10 C B

Sample 721
OPA2



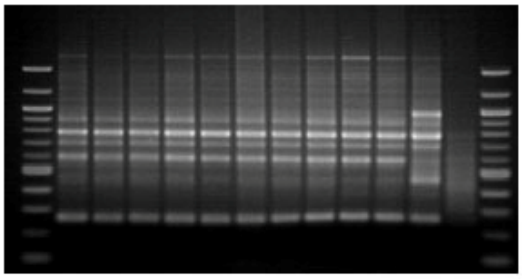
1 2 3 4 5 6 7 8 9 10 C B

OPA13



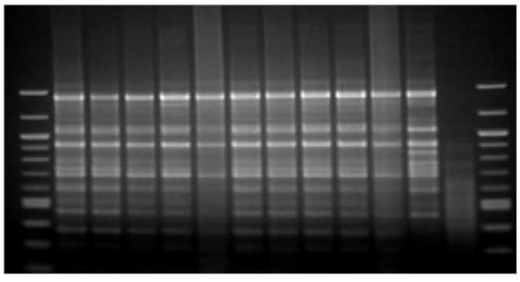
1 2 3 4 5 6 7 8 9 10 C B

Sample 740
OPA2



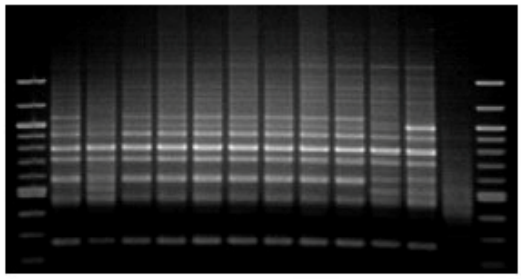
1 2 3 4 5 6 7 8 9 10 C B

OPA13



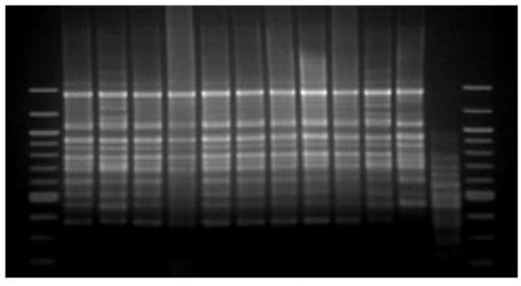
1 2 3 4 5 6 7 8 9 10 C B

Sample 765
OPA2



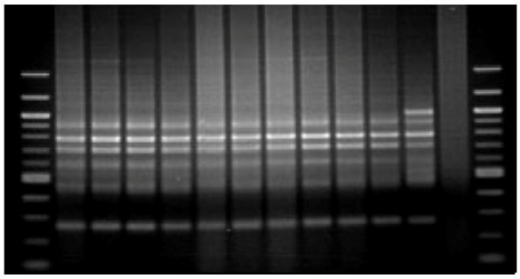
1 2 3 4 5 6 7 8 9 10 C B

OPA13



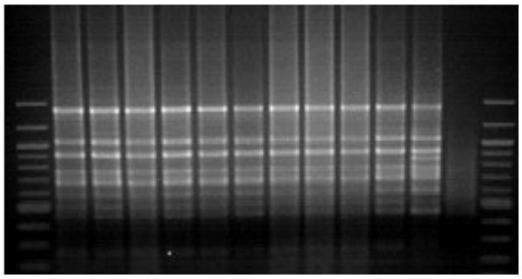
1 2 3 4 5 6 7 8 9 10 C B

Sample 770
OPA2



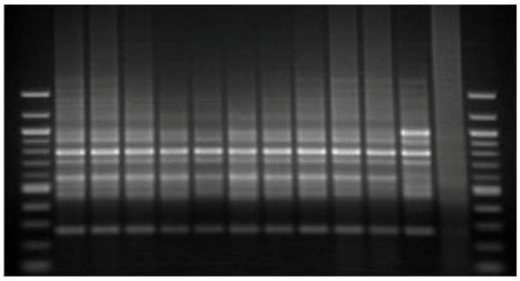
1 2 3 4 5 6 7 8 9 10 C B

OPA13



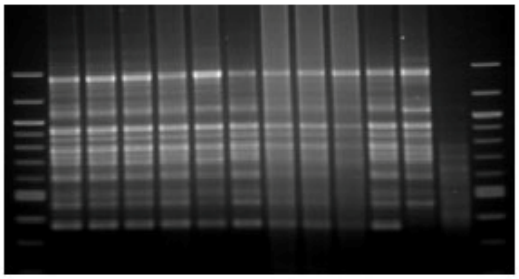
1 2 3 4 5 6 7 8 9 10 C B

Sample 784
OPA2



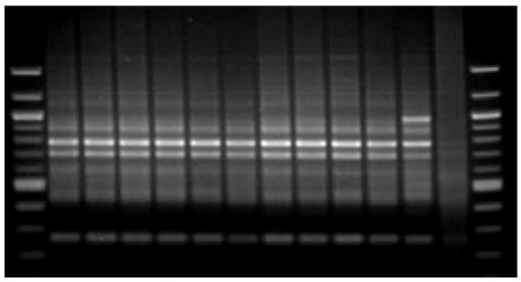
1 2 3 4 5 6 7 8 9 10 C B

OPA13



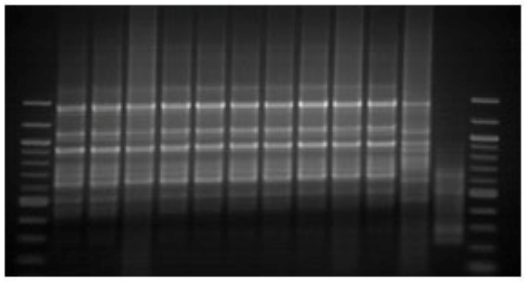
1 2 3 4 5 6 7 8 9 10 C B

Sample 794
OPA2



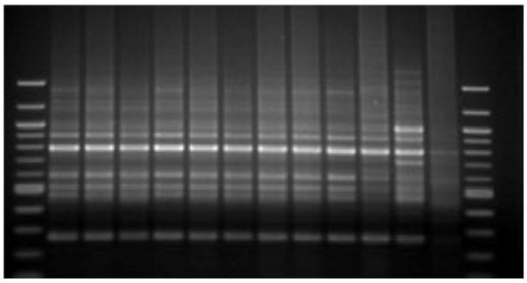
1 2 3 4 5 6 7 8 9 10 C B

OPA13



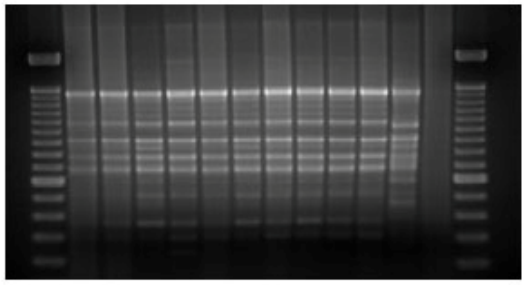
1 2 3 4 5 6 7 8 9 10 C B

Sample 817
OPA2



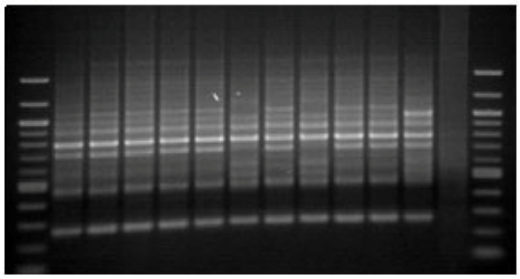
1 2 3 4 5 6 7 8 9 10 C B

OPA13



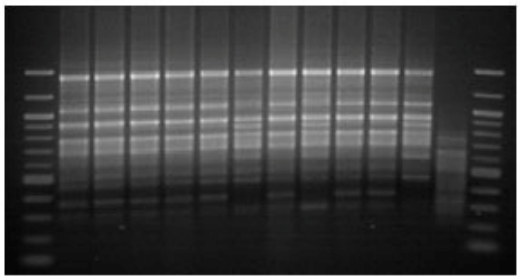
1 2 3 4 5 6 7 8 9 10 C B

Sample 818
OPA2



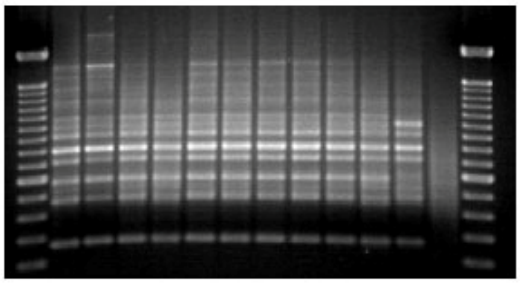
1 2 3 4 5 6 7 8 9 10 C B

OPA13



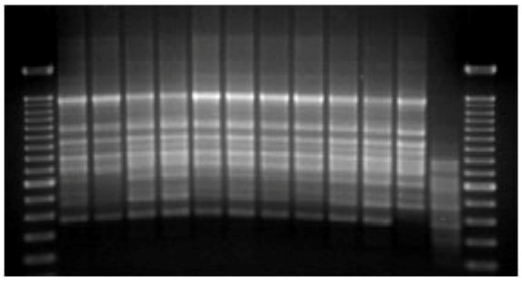
1 2 3 4 5 6 7 8 9 10 C B

Sample 866
OPA2



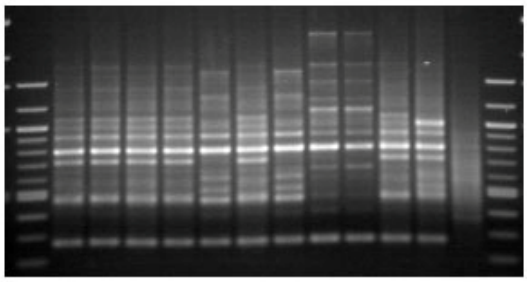
1 2 3 4 5 6 7 8 9 10 C B

OPA13



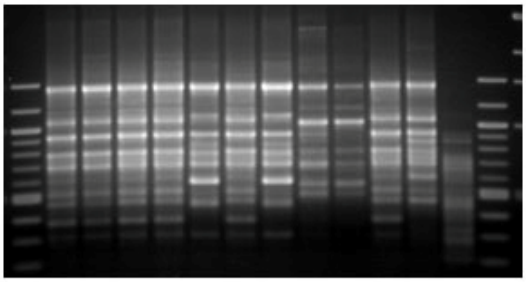
1 2 3 4 5 6 7 8 9 10 C B

Sample 934
OPA2



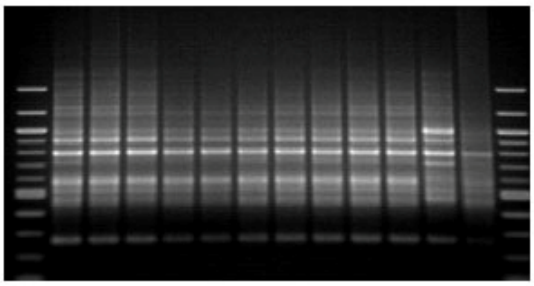
1 2 3 4 5 6 7 8 9 10 C B

OPA13



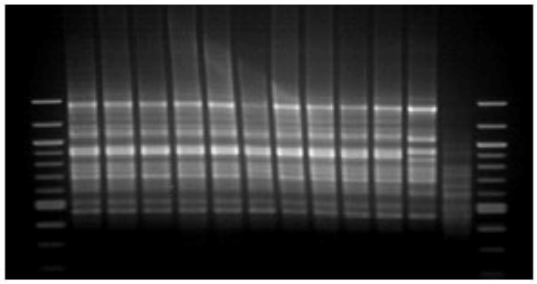
1 2 3 4 5 6 7 8 9 10 C B

Sample 971
OPA2



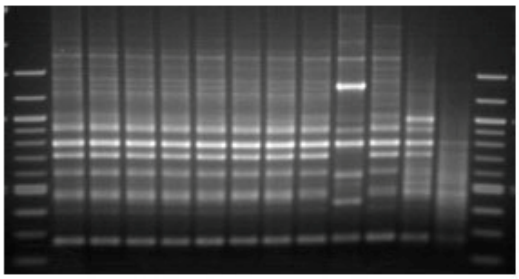
1 2 3 4 5 6 7 8 9 10 C B

OPA13



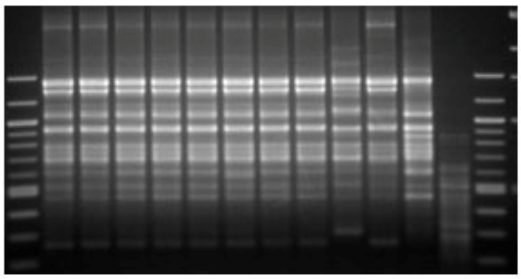
1 2 3 4 5 6 7 8 9 10 C B

Sample 992
OPA2



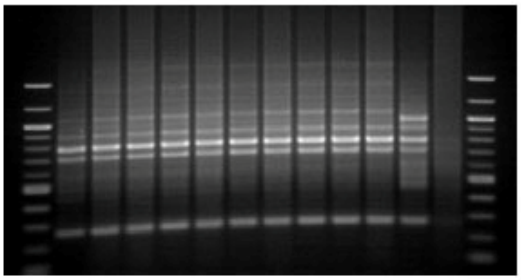
1 2 3 4 5 6 7 8 9 10 C B

OPA13



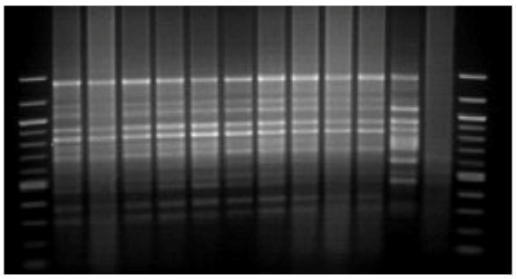
1 2 3 4 5 6 7 8 9 10 C B

Sample 1058
OPA2



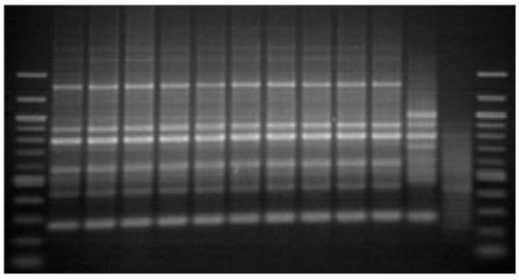
1 2 3 4 5 6 7 8 9 10 C B

OPA13



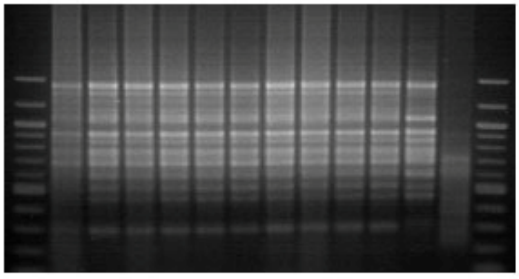
1 2 3 4 5 6 7 8 9 10 C B

Sample 1128
OPA2



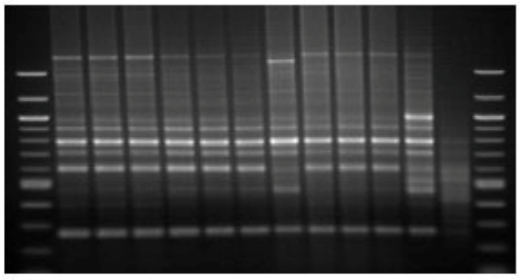
1 2 3 4 5 6 7 8 9 10 C B

OPA13



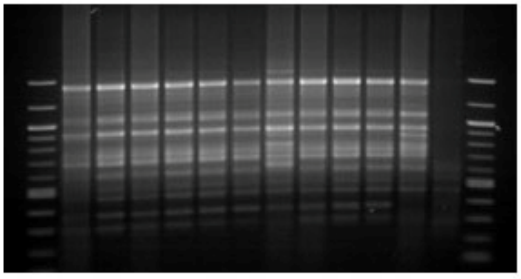
1 2 3 4 5 6 7 8 9 10 C B

Sample 1132
OPA2



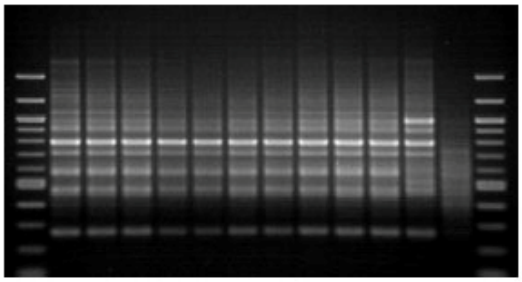
1 2 3 4 5 6 7 8 9 10 C B

OPA13



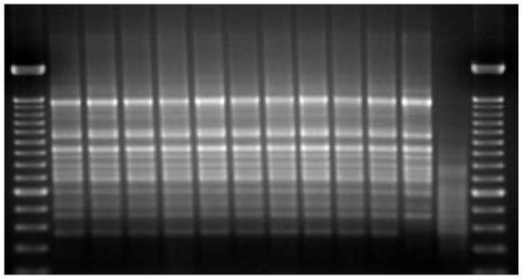
1 2 3 4 5 6 7 8 9 10 C B

Sample 1166
OPA2



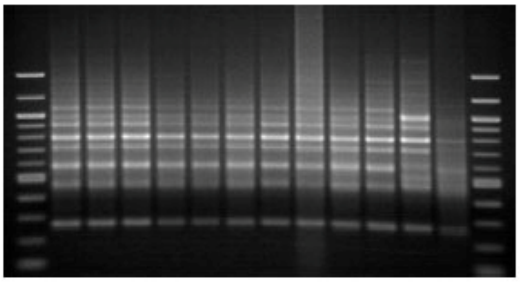
1 2 3 4 5 6 7 8 9 10 C B

OPA13



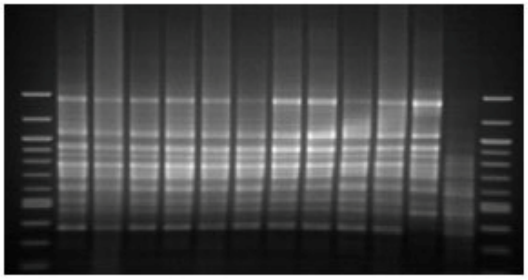
1 2 3 4 5 6 7 8 9 10 C B

Sample 1182
OPA2



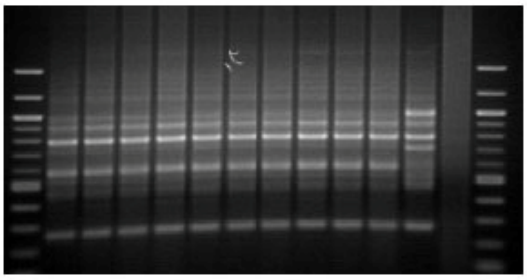
1 2 3 4 5 6 7 8 9 10 C B

OPA13



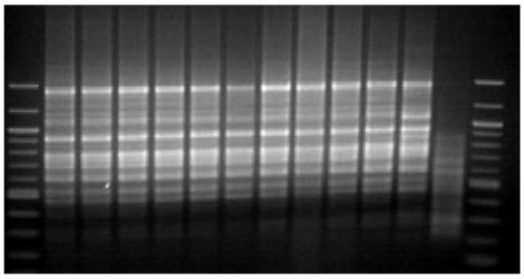
1 2 3 4 5 6 7 8 9 10 C B

Sample 1236
OPA2



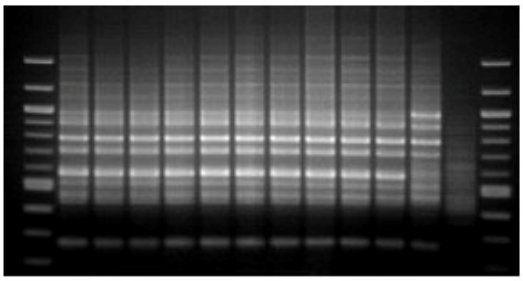
1 2 3 4 5 6 7 8 9 10 C B

OPA13



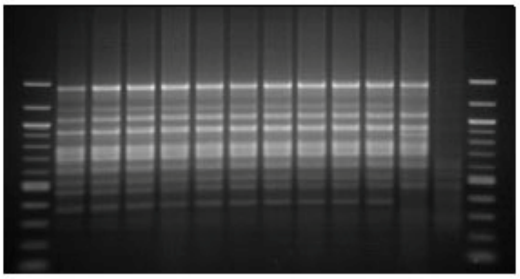
1 2 3 4 5 6 7 8 9 10 C B

Sample 1255
OPA2



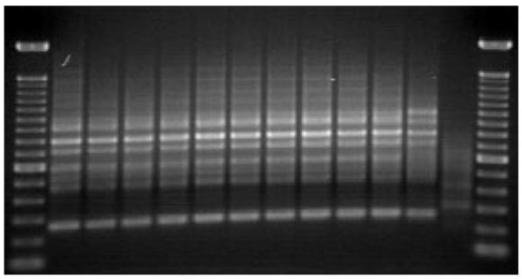
1 2 3 4 5 6 7 8 9 10 C B

OPA13



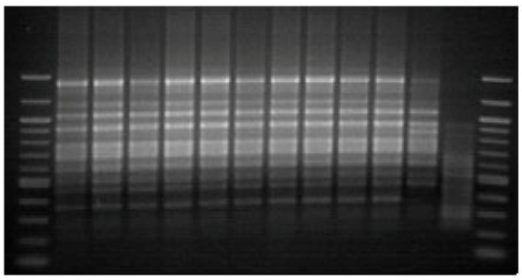
1 2 3 4 5 6 7 8 9 10 C B

Sample 1274
OPA2



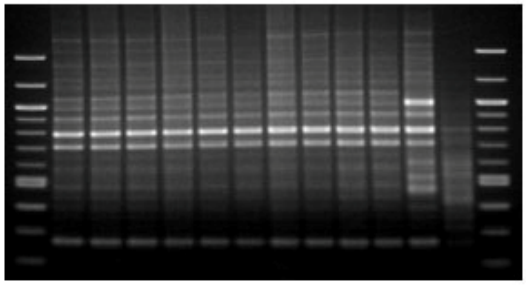
1 2 3 4 5 6 7 8 9 10 C B

OPA13



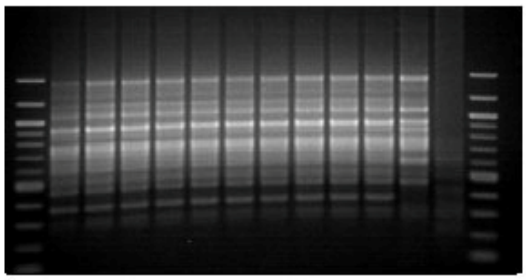
1 2 3 4 5 6 7 8 9 10 C B

Sample 1282
OPA2



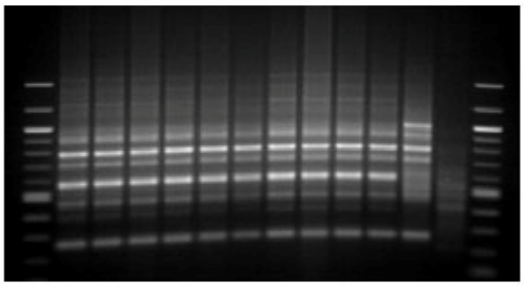
1 2 3 4 5 6 7 8 9 10 C B

OPA13



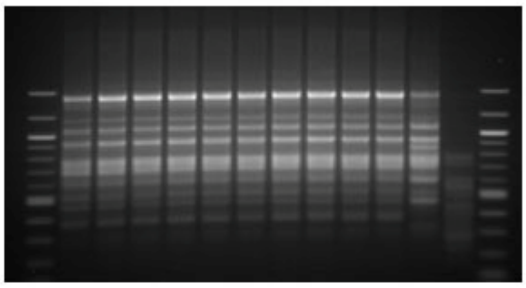
1 2 3 4 5 6 7 8 9 10 C B

Sample 1310
OPA2



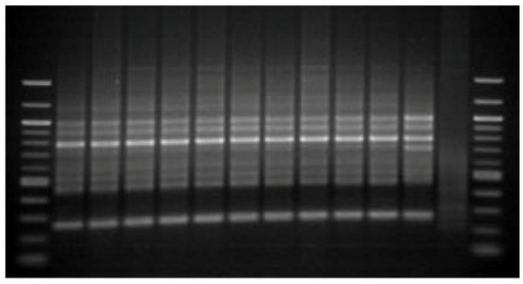
1 2 3 4 5 6 7 8 9 10 C B

OPA13



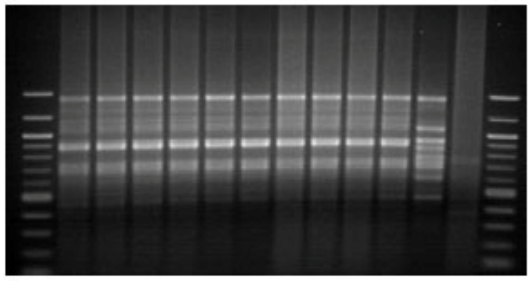
1 2 3 4 5 6 7 8 9 10 C B

Sample 1327
OPA2



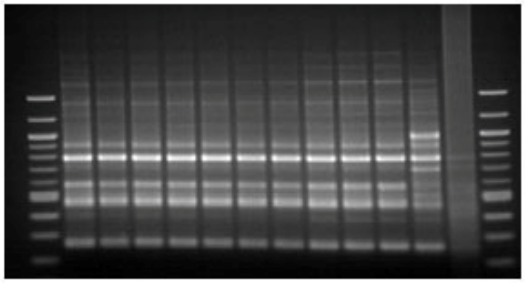
1 2 3 4 5 6 7 8 9 10 C B

OPA13



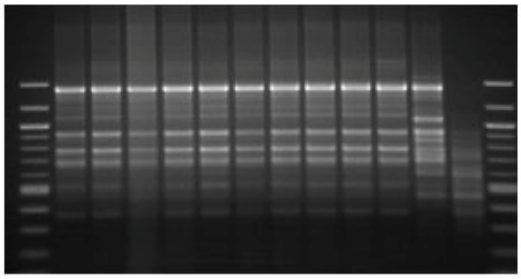
1 2 3 4 5 6 7 8 9 10 C B

Sample 1347
OPA2



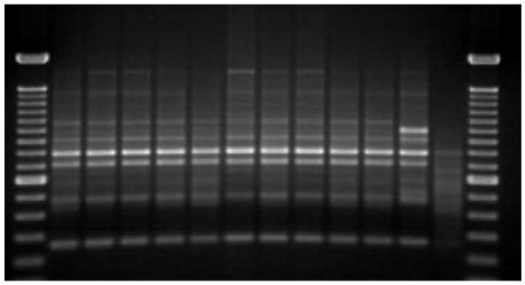
1 2 3 4 5 6 7 8 9 10 C B

OPA13



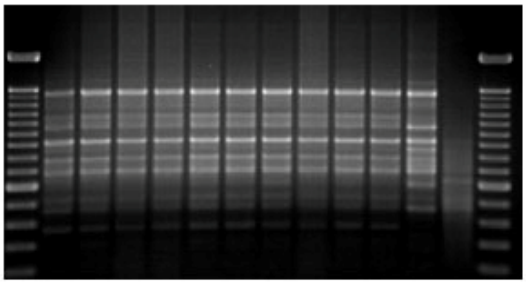
1 2 3 4 5 6 7 8 9 10 C B

Sample 1404
OPA2



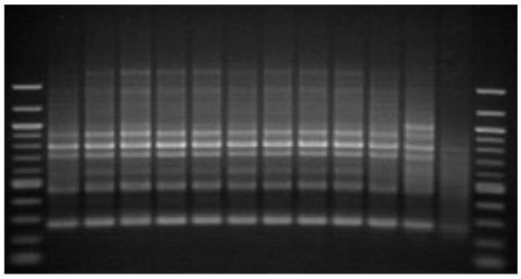
1 2 3 4 5 6 7 8 9 10 C B

OPA13



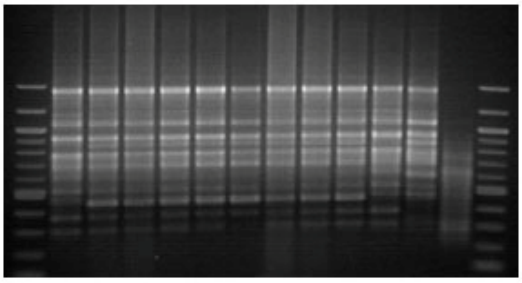
1 2 3 4 5 6 7 8 9 10 C B

Sample 1409
OPA2



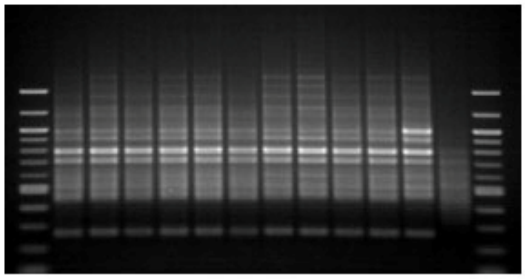
1 2 3 4 5 6 7 8 9 10 C B

OPA13



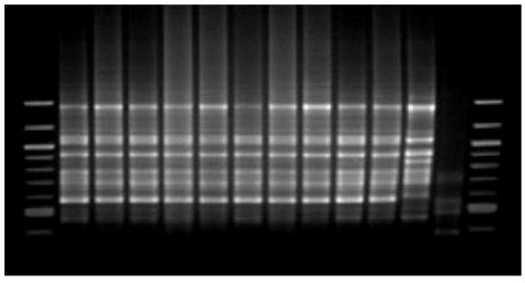
1 2 3 4 5 6 7 8 9 10 C B

Sample 1423
OPA2



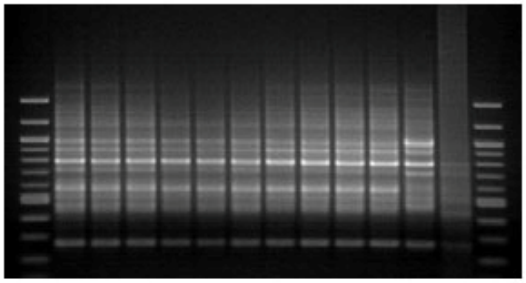
1 2 3 4 5 6 7 8 9 10 C B

OPA13



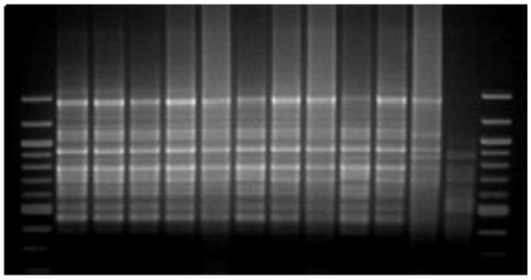
1 2 3 4 5 6 7 8 9 10 C B

Sample 1430
OPA2



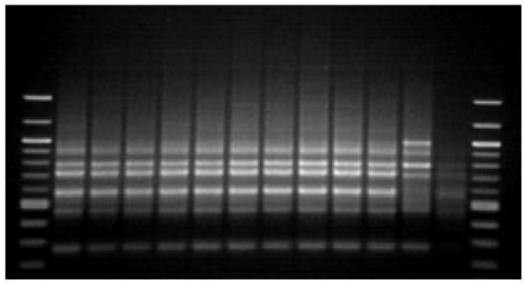
1 2 3 4 5 6 7 8 9 10 C B

OPA13



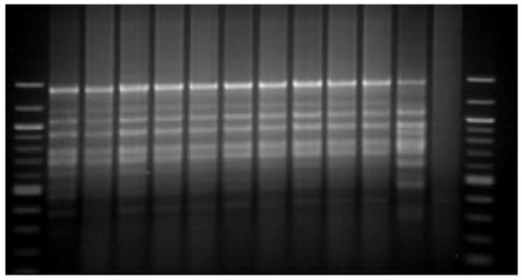
1 2 3 4 5 6 7 8 9 10 C B

Sample 1465
OPA2



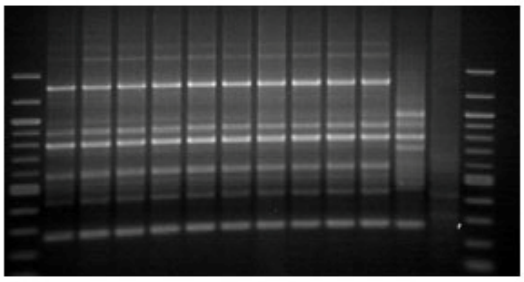
1 2 3 4 5 6 7 8 9 10 C B

OPA13



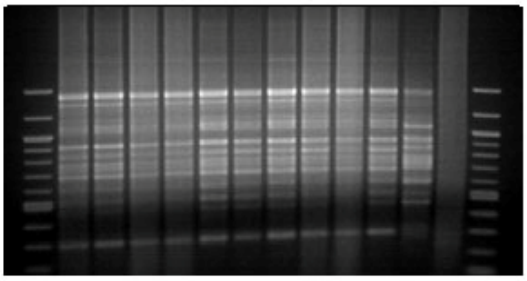
1 2 3 4 5 6 7 8 9 10 C B

Sample 1602
OPA2



1 2 3 4 5 6 7 8 9 10 C B

OPA13



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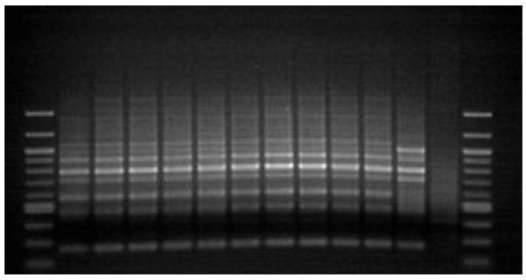

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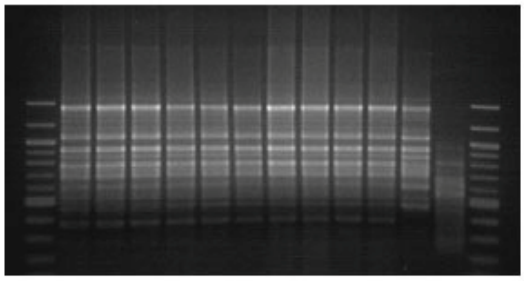

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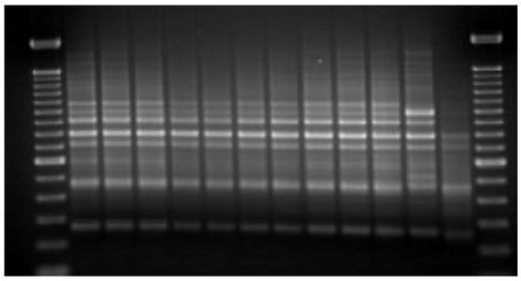

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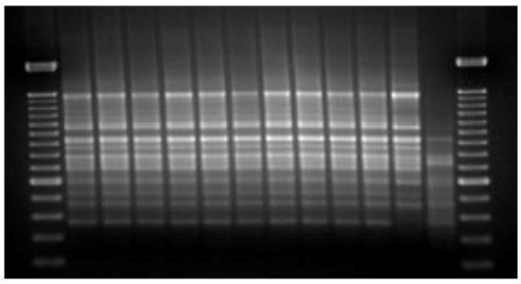

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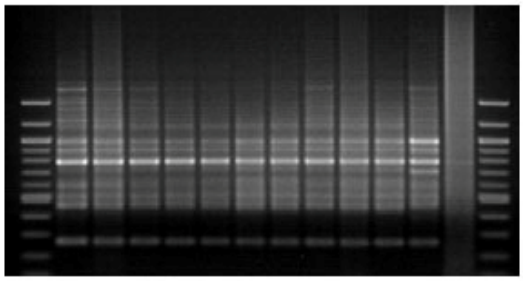

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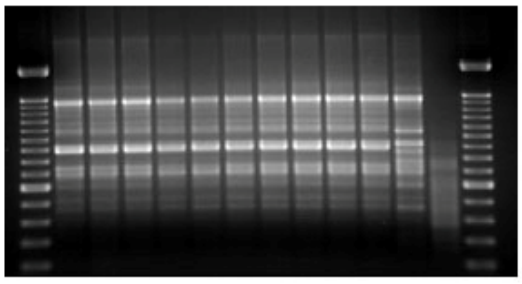

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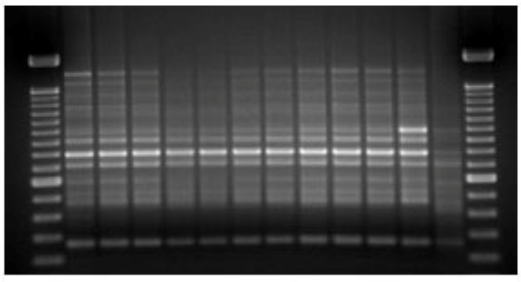

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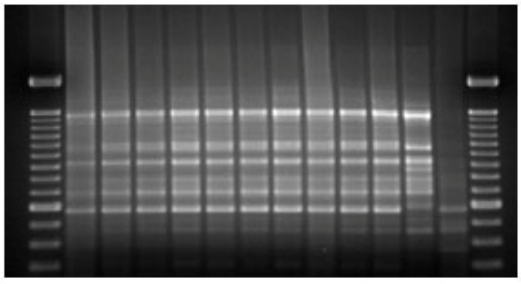

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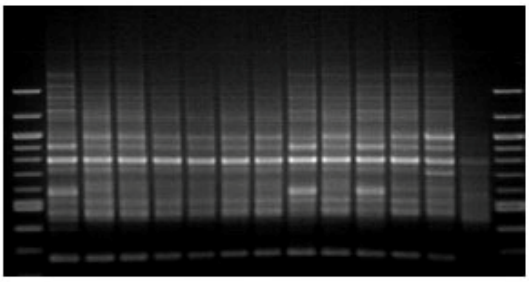

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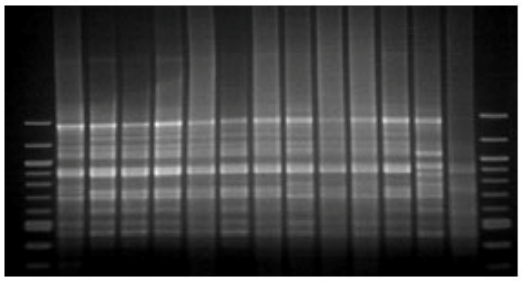

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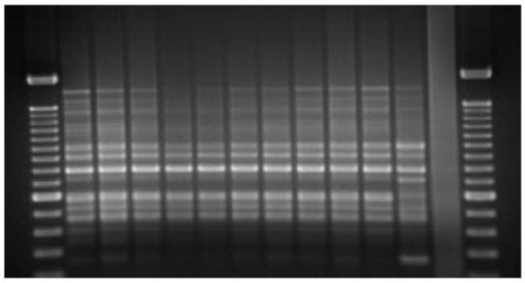

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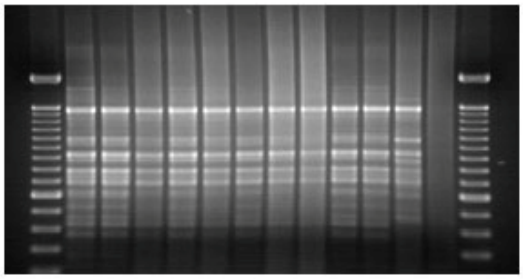

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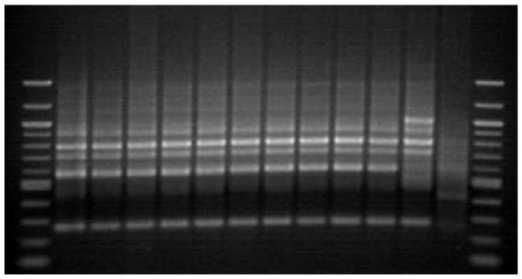

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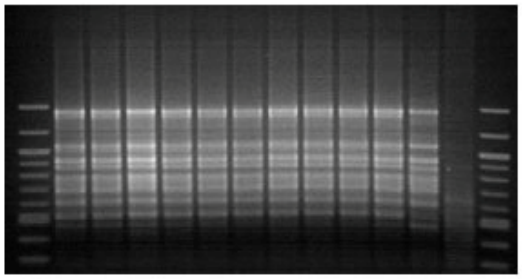

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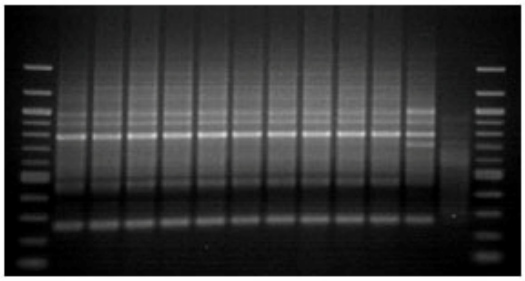

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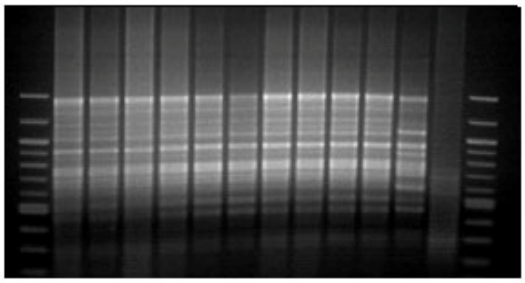

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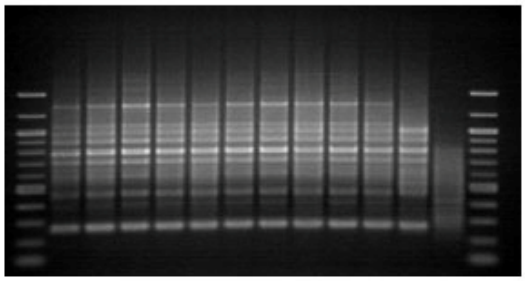

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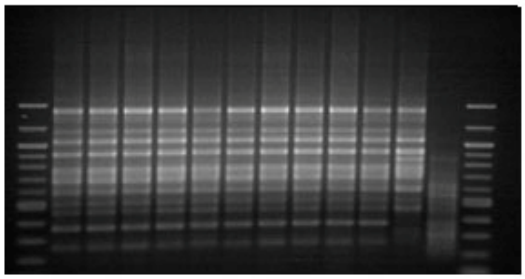

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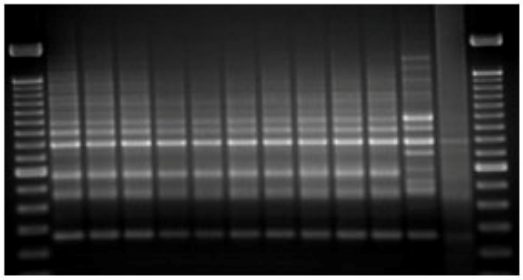

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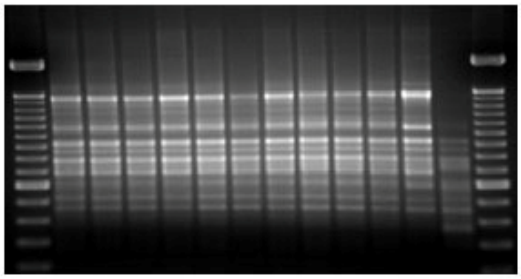

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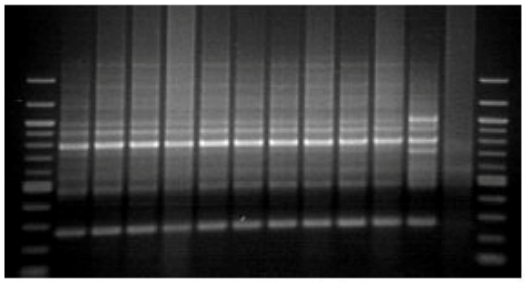

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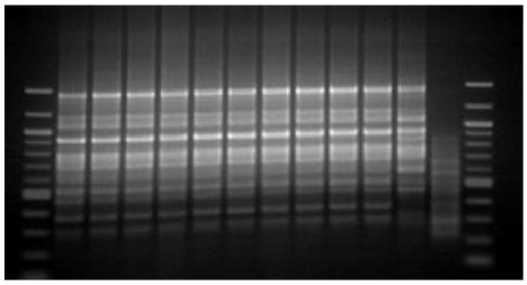

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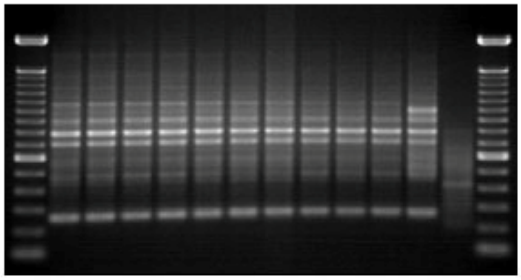

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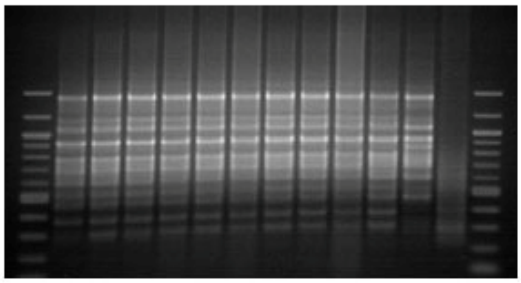

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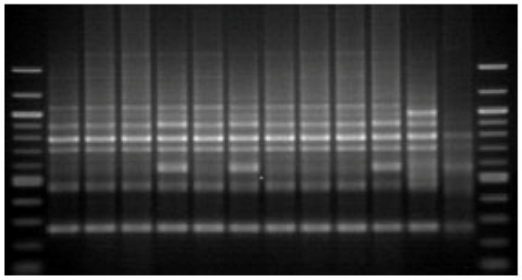

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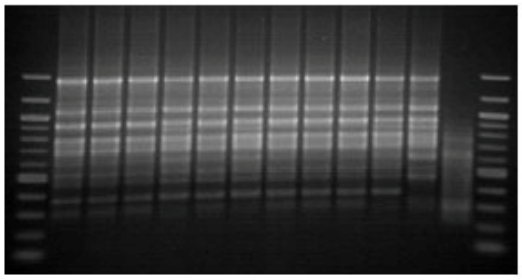

1 2 3 4 5 6 7 8 9 10 C B

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