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A study of the sensitivity of Lactobacillus species to chlorhexidine

and the effect of local application of chlorhexidine on approximal microflora

by

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A STUDY OF THE SENSITIVITY OF LACTOBACILLUS SPECIES TO CHLORHEXIDINE AND THE EFFECT OF LOCAL APPLICATION OF CHLORHEXIDINE ON APPROXIMAL MICROFLORA

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For Janice

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ABSTRACT

The purpose of this study was to determine the *in vitro* sensitivity of a range of Lactobacillus species to chlorhexidine, Minocycline, and Spiramycin at a range of pH from 5.0-7.4. Strains of Streptococcus were also tested for their sensitivity to chlorhexidine as a comparison between the genera. The effect of a single local application of a 2.0% chlorhexidine gluconate solution applied with Superfloss® on approximal microflora was then tested in a pilot study on 10 subjects. This was compared to the effect of Superfloss alone.

a) *In vitro* sensitivity of Lactobacillus and Streptococcus to chlorhexidine and antibiotics

There was both inter and intra species variation in the sensitivity of the strains tested to chlorhexidine. The strains tested were sensitive at pH 6.7 to the following levels of chlorhexidine (μg/mL) : L. casei (6 strains) 10-60; L. plantarum (4 strains) 40; L. fermentum (13 strains) 2-20; L. brevis (1 strain) 10; L. acidophilus (3 strains) 10-60. The Streptococcus species were sensitive to 1-4 μg/mL (13 strains); 4-10 μg/mL (3 strains); 10-20 μg/mL (2 strains). One strain was able to survive 20 μg/mL. Chlorhexidine was found to be less effective at lower pH levels. The following examples show sensitivity at pH 6.5 followed by sensitivity at pH 5.0: L. casei (ATCC 15008) 40;60; L. plantarum (CH 374) 40;100; L. fermentum (CH 324) 10;40; L. acidophilus (ATCC 4356) 10;40 S. mutans (BM 52) 2;2. A time kill experiment showed a rapid initial loss of viable cells followed by stable levels during the remainder of the test period. This may be attributed to the reduced effectiveness of chlorhexidine in the presence of protein.

All of the strains of Lactobacillus tested with Spiramycin were resistant at pH 5.0. Minocycline was less affected by changes in pH but at pH 7.4 Lactobacillus strains were more resistant as compared to Spiramycin. Both of these antibiotics are bacteriostatic

and therefore may have a more limited effect than a bacteriocidal agent such as chlorhexidine.

The Lactobacillus strains tested required higher concentrations of chlorhexidine than did the strains of Steptococcus for a killing effect *in vitro* and environmental pH will be an important factor in the control of an acidogenic and aciduric oral flora.

b)Pilot study on the the effect of chlorhexidine and Superfloss® on approximal microflora

Ten subjects were included in this study. Two unrestored approximal sites were selected for each subject to be treated with a single application of either Superfloss alone (control sites) or Superfloss soaked in a 2.0% chlorhexidine solution (test sites) for a period of 5 minutes.

Plaque samples were taken from each site prior to treatment (0 hours), 5 minutes after treatment with Superfloss or chlorhexidine/Superfloss (0.08 hours), at 8 hours, 72 hours and at 168 hours.

Superfloss alone was found to be as effective as Superfloss soaked in a solution of 2.0% chlorhexidine in reducing approximal microflora (6.06% and 5.76% of pretreatment counts respectively). The 72 hour sample indicated that the microflora at the sites treated with Superfloss alone returned to pretreatment levels earlier than the sites treated with chlorhexidine/Superfloss (5/10 control sites versus 3/10 test sites). At 168 hours more of the test sites had returned to pretreatment levels (5/10 control sites versus 7/10 test sites). Actinomyces were virtually eliminated from the sites treated with chlorhexidine/Superfloss and did not return to pretreatment levels even after 168 hours. Streptococcus recolonized and were a dominant member of the microflora at these sites. Veillonella and Gram-negative anaerobes were eliminated from the test sites and were not recovered until the 72 hour and 168 hour sample

respectively. Actinomyces, Veillonella and Gram-negative anaerobes persisted in approximately the same proportions at the control sites at each sampling time.

The growth of "S. mitior" appeared to be enhanced at the test sites due to the suppression of Actinomyces by chlorhexidine. "S. mitior" increased at the test sites up to 72 hours and decreased at 168 hours. At the control sites the proportion of "S. mitior" decreased progressively from the 8 hour sample to the 168 hour sample. S. milleri was only detected at the test sites and persisted proportionally throughout the test period virtually unaffected. A group of Streptococcus identified only as "sorbitol fermenters" were eliminated from the test sites until the 72 hour sample. The "sorbitol fermenters" persisted in approximately the same proportion to the other groups of Streptococcus at the control sites but were not detected at the 8 hour sample. S. salivarius persisted proportionally at the control sites and increased at the test sites after chlorhexidine/Superfloss was applied. S. mutans persisted proportionally at the control sites throughout the test period. However, at the test sites, they persisted immediately after chlorhexidine/Superfloss treatment but were not detected at 8 hours and 72 hours. Recovery to pretreatment levels occurred at the 168 hour sample.

CHAPTER 1

1)ETIOLOGY OF CARIES

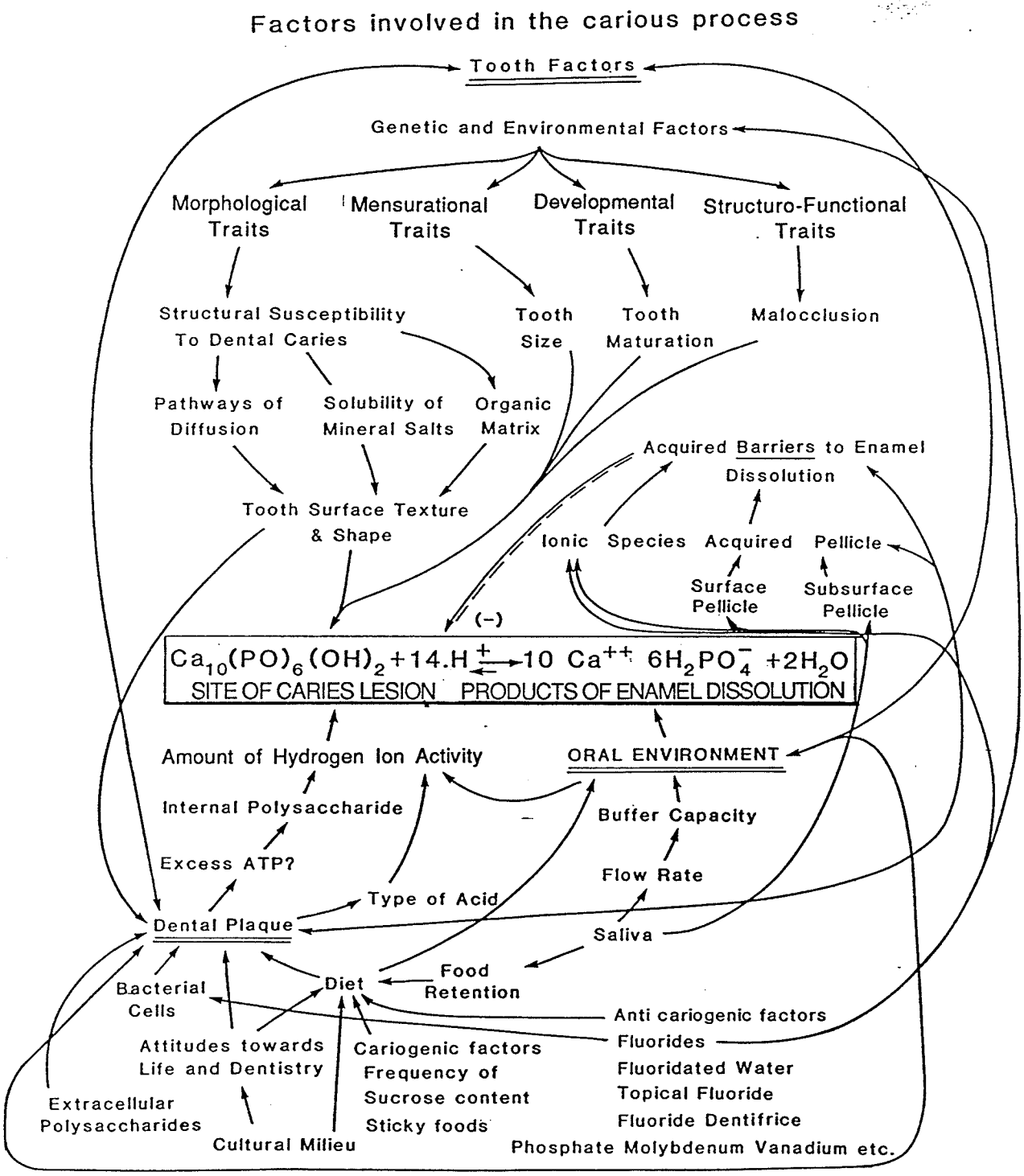
a)HISTORY OF CARIES RESEARCH

The complexity of the interactions between the host, oral microflora, and diet that result in the formation of a caries lesion have given rise to a number of theories on the etiology and pathogenesis of caries (Figure 1.1)[1].

Early theories on the etiology of caries have been summarized by Newbrun[2].The cause of the toothache, according to the ancient Sumerians (circa 5000 B.C.), was thought to be a "worm which drank the blood of the teeth and fed on the roots of the jaw". The ancient Greeks thought that all diseases, including caries, were caused by an imbalance in the proportions of the four humors (sanguine, phlegmatic, melancholic and choleric). The "Vital Theory", proposed in the eighteenth century, suggested that caries originated within the tooth itself. This remained popular until the "Chemical Theory" was suggested by Parmly in 1819. He proposed that caries was caused by an unidentified chemical that formed on the tooth where food putrified. Robertson in 1835 and Regnart in 1838 supported this theory but suggested that the cause of the destruction of enamel and dentin was sulfuric and nitric acids. In 1843 the "Parasitic Theory" was described by Erdl , who was able to isolate filamentous parasites from the tooth surface. He did not, however, explain the relationship of the microorganisms to the carious process.

In 1890, Miller[3] found that caries was caused by acids produced by the fermentation of carbohydrates by oral microorganisms. This has been termed the "Chemico-parasitic Theory" and is the basis for the current theories of caries etiology. Although other 19th century researchers such as Magitot, and Leber and Rottenstein in 1867 as well as Miles and Underwood in 1881[2] also demonstrated that acid could dissolve teeth *in vitro* and implicated oral microorganisms in the etiology of caries, Miller's work firmly established the interaction between bacteria, substrate and host. In

Figure 1.1[a)]



a) adapted from White, G.E. (1975)

his experiments he incubated saliva, bread and an extracted tooth together at 37° C and found that decalcification occurred. When the saliva was heated, thereby killing the microorganisms, no decalcification was observed. He found that at least 30 different microorganisms were able to ferment carbohydrates and concluded that caries was a nonspecific bacterial infection[3]. Treatment was directed towards the reduction or elimination of the oral microorganisms. Mechanical removal of bacterial deposits by toothbrushing was advocated as a means of preventing dental decay.

The search for specific bacteria in the etiology of dental caries followed Miller's work. In 1915 Kligler[4] cultured carious dentin and found a high proportion of Gram-positive rods that appeared to be lactobacilli. He surmised that the microorganism(s) that caused caries must be both aciduric and acidogenic. Using a selective medium with a pH of 5.0 he was able to isolate lactobacilli from carious dentin consistently.

In the 1920's studies were beginning to show a relationship between lactobacilli and caries. Enright *et al.*[5] found that high salivary lactobacilli counts preceded the clinical detection of dental caries. They concluded that lactobacilli were the cause of dental caries.

With improved microbiological methods of quantifying and culturing plaque, it became evident that the lactobacilli comprised a very small proportion of the plaque community. In 1950 Stralfors[6] found that the streptococci outnumbered the lactobacilli by a factor of 10^4/mg of plaque and were found to be more acidogenic with the pH between 5.0 and 6.5. The search for the etiology of caries now became focused on the more numerous streptococcal species.

Clarke[7] isolated an unusual ovoid form of streptococcus from caries lesions in 1924. It was able to produce a sticky extracellular substance in a sucrose medium and could ferment mannitol. Since it appeared to be a mutant streptococcus isolate, it was named Streptococcus mutans. The isolation frequency was found to be higher for S. mutans than lactobacilli. *In vitro* experiments using extracted teeth demonstrated that

lactobacilli could not attach to intact teeth , but S. mutans produced a thick, adherent plaque. Other researchers, however, were not able to differentiate S. mutans from other streptococcal species and supported Miller's nonspecific "Chemoparasitic Theory" of the etiology of caries[8]. This work was generally ignored until the significance of S. mutans was rediscovered in the 1960's.

The production of acid in dental plaque by bacterial metabolism of carbohydrates was studied by Stephan[9]. *In vivo* experiments showed that the resting pH of plaque in caries-active individuals was lower than in individuals who were caries-free. Caries-active individuals who were challenged by a glucose rinse had a greater pH drop which took longer to return to its resting value. The pH remained below 5.0 for more than 30 minutes. This may lead to the dissolution of hydroxyapatite and the development of an early caries lesion.

b) ORAL MICROFLORA IN THE ETIOLOGY OF CARIES

i) ANIMAL MODELS

The complexity of the interactions of the factors involved in the etiology of dental caries has led investigators to search for an appropriate animal model system that might be studied under more controlled conditions.

The critical involvement of microorganisms in dental caries was shown by McClure and Hewitt[10]. Caries was eliminated in caries-susceptible rats on a high sucrose diet by the addition of penicillin to their drinking water while 50% of the control group developed caries. In 1955 Orland *et al.*[11] demonstrated that germ-free rats on a cariogenic high sucrose diet did not develop caries. The implantation of an 'enterococcus' into a group of animals on the same diet, however, resulted in the rapid development of caries.

The use of animal models has provided a great deal of information but caution must be exercised in relating the results of animal studies to humans. The morphology of rat teeth is different from that of human teeth. The enamel of the rat molar is much thinner

(approximately one twentieth that of human enamel), is hypomineralized (especially fissures) at the time of eruption, and does not cover the cusp tips. Post-eruptive mineralization of the enamel occurs for approximately two months, therefore younger rats are considerably more susceptible to caries[11,12,13] than are older animals. Because rats are coprophagic and recycle approximately 35% of their feces each day, the oral flora will contain higher numbers of bacteria usually associated with fecal material such as enterococci and coliform bacteria. The living conditions are such that the wood shavings in the cage may be eaten and serve as a reservoir for substrate, bacteria and bacterial acids. The experimental rat is usually fed a simple diet, high in sucrose *ad libitum* and oral hygiene procedures are normally not introduced[8,14]. Such differences are significant when attempting to relate results of animal experiments to humans, especially since oral hygiene procedures result in a major disruption of plaque[15].

Keyes[16] found that the offspring of caries-susceptible hamsters treated with penicillin or erythromycin remained caries-free. However, when these offspring were caged with offspring of untreated caries-active hamsters, caries developed in both groups. This provided evidence for the transmissibility of caries with the pathogen present in the feces. Fitzgerald and Keyes[17] were able to prove this by labelling the suspected fecal pathogen with a genetic marker. Koch's postulates were met in this study because

1)they isolated a pathogen from a caries lesion.

2)it was introduced to a susceptible host by ingestion of fecal material.

3)it produced a caries lesion in the susceptible host.

4)the genetic marker allowed for reisolation of the organism.

Further studies by Fitzgerald *et al.*[18-20] found that there was a limited number of bacteria that could cause caries in the gnotobiotic rat. A number of acidogenic and aciduric species were introduced but all strains were not able to produce caries *in vivo*. Organisms such as Lactobacillus acidophilus, Lactobacillus fermentum, Streptococcus faecalis var. zymogenes , Streptococcus lactis[19], three strains of Streptococcus sanguis and three strains of Streptococcus salivarius did not cause caries when introduced as a monoinfection in the gnotobiotic rat[20]. One human strain of Actinomyces israelii and Actinomyces naeslundii were also caries-inactive in the experimental rat[20]. A microaerophilic strain of Streptococcus that was not specifically identified to species level was able to produce extensive cavitation. It fermented mannitol and sorbitol and has since been shown to be Streptococcus mutans[18]. A more recent study by Fitzgerald and coworkers[21] tested the cariogenicity of 32 strains of lactobacilli. Seventeen strains were moderately to highly cariogenic and only one strain was found to be non-cariogenic (a strain of Lactobacillus lactis). The cariogenic lactobacilli produced predominantly pit and fissure decay. Involvement of smooth surfaces was secondary due to the extension of fissure lesions. Four strains of Lactobacillus plantarum which were non-cariogenic members of the oral flora of hamster were cariogenic in the gnotobiotic rat[21]. They attributed this to the deep molar fissures in the rat. These studies challenged Miller's nonspecific "Chemoparasitic theory"[3] which stated that the acid production by several types of bacteria was the determinant of cariogenicity.

Krasse[22], using conventional hamsters demonstrated that exogenous bacteria had difficulty in colonizing because the complex, established oral microflora had filled the available niches. However, he was able to establish S. mutans in the presence of a high sucrose diet, because it could then compete successfully.

The establishment of single strains of bacteria in gnotobiotic animals is relatively uncomplicated. Variables are reduced and pathogenic potential can be evaluated. The

results of studies using this type of experimental model, devoid of microbial interaction, must be interpreted carefully if they are to be extrapolated to humans. Experiments by Mikx *et al.*[23] attempted to introduce microbial interactions as a variable. Gnotobiotic rats were fed a cariogenic diet and were inoculated with one of the following:

1)S. mutans

2)S. mutans and Veillonella alcalescens

3)S. sanguis

4)S. sanguis and Veillonella alcalescens

Veillonella species metabolize lactic acid into acetic and propionic acids which are not as effective in decalcifying enamel as lactic acid. Their presence could modify the caries potential of S. mutans. Caries was reduced by 60% when the rats were inoculated with S. mutans and Veillonella alcalescens compared with S. mutans alone. S. sanguis alone produced less caries than S. mutans and the combination of S. sanguis and Veillonella alcalescens reduced that amount by 30%.

The primate is more similar to the human with respect to dental morphology, pattern of caries development and oral flora. It is also possible to maintain them on a human diet[24]. A study by Colman and Hayday[25] using Macaca fascicularis found that the numbers of S. mutans and Lactobacillus species increased prior to the onset of caries as in humans. However, the cost of acquiring and maintaining the animals precludes their widespread use in caries research[8].

Gnotobiotic animal studies have been able to show that

1)caries will not occur without microorganisms.

2)many single microorganisms are capable of inducing caries.

3)not all acidogenic microorganisms are cariogenic but the ability to produce acid is required to produce caries.

4)cariogenic streptococcal strains produce extracellular

polysaccharides but not all strains that produce extracellular polysaccharides are cariogenic[19,20].

Experimental conditions in animal studies can be rigidly controlled and the factors involved in the etiology of caries can be examined in isolation. However, attempts by researchers to find a specific pathogen have neglected the ecological aspects of the disease. Individual strains do not act in isolation in an ecosystem as complex as dental plaque. Dental caries is now considered to be a disease due to an imbalance of the normal oral microflora and not the result of a single pathogen[2,26-28].

ii)HUMAN STUDIES

Studies in humans have mainly been focused on Streptococcus mutans and Lactobacillus species as a result of animal studies that have shown these species to increase in association with the onset and progression of caries[29]. Plaque from caries lesions has been found to have elevated numbers of both Streptococcus mutans and Lactobacillus species when compared with plaque from non-carious tooth surfaces[30-36].

A study by Duchin and van Houte[34] pointed out some of the inherent deficiencies in cross-sectional analyses. Cause and effect relationships are difficult to determine. It may be that Streptococcus mutans is responsible for the initiation of the caries lesion or its presence may be the result of changing environmental conditions that promote its colonization and prominence in the ecosystem. Caries develops slowly in humans and is characterized by periods of demineralization and remineralization[37,38]. Therefore, when plaque samples are analysed in a cross-sectional type of study, the significance of the dynamics of the carious process has not been considered.

A cross-sectional study by Loesche *et al.*[39] in 1975 provided further evidence associating S. mutans and caries. S. mutans was 10% or more of the total cultivable flora in 73% of carious fissures while 70% of caries-free fissures had no detectable levels of S. mutans. This same study, however, also found caries-free fissures with S. mutans