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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

Blaine Murray Cleghorn

A thesis submitted to

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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

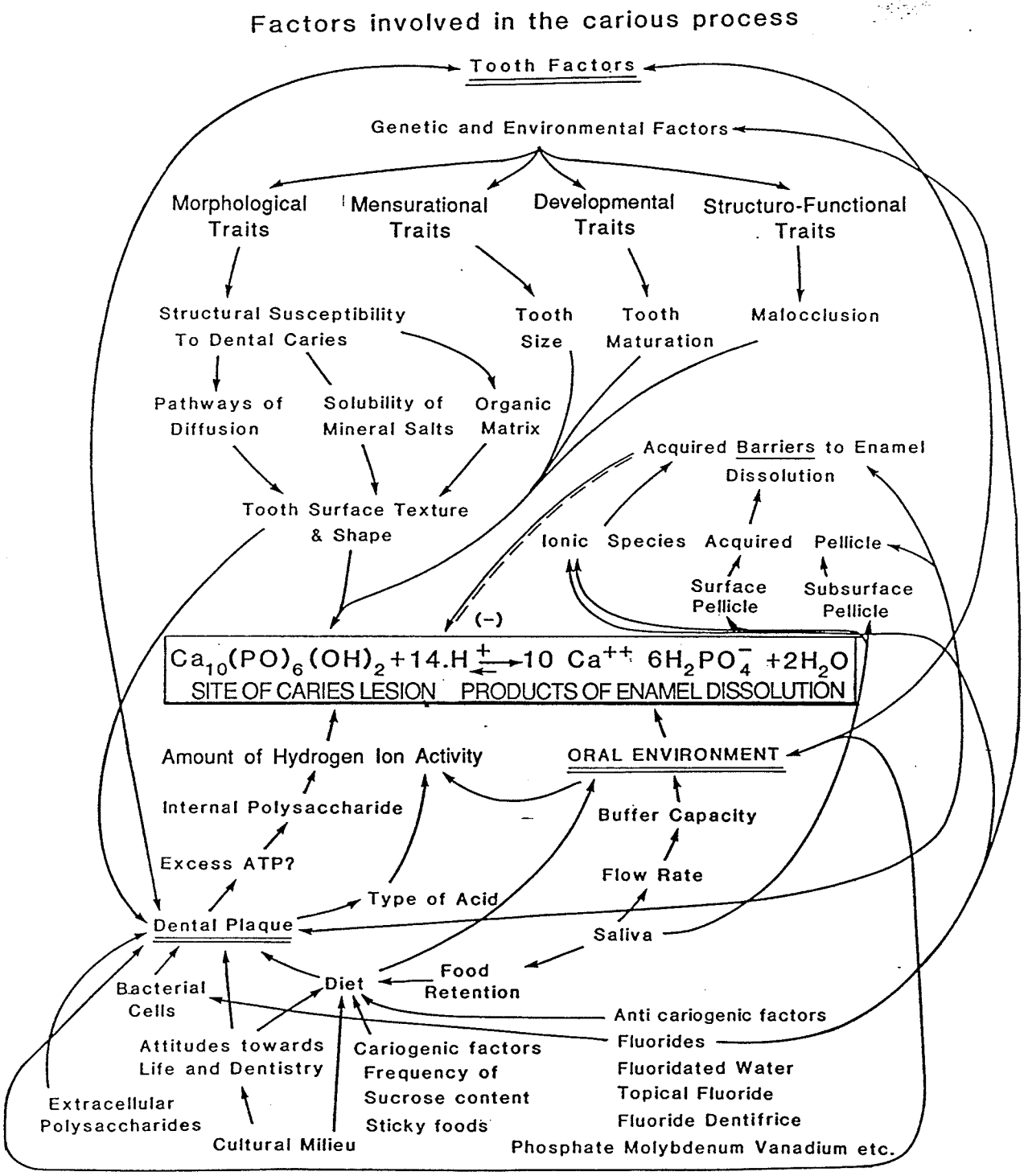
a)HISTORY OF CARIES RESEARCH

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

accounting for 33% of the total cultivable flora and carious fissures without S. mutans. Results of studies such as this point out the necessity of longitudinal studies that are able to follow shifts in an oral ecosystem from a caries-free state to a caries lesion.

An important longitudinal study was undertaken by Hemmens and co-workers[40] in 1946. They suggested that microbial succession could occur in the development of a caries lesion as it does in other ecosystems. A total of 939 plaque samples were taken from 85 children. Sites were examined at 6 week intervals until a lesion developed and were followed for a further 6 months. There were some sites that were followed over a 4 year period. Detailed microbiological analyses were carried out but the results were negatively affected by the limited taxonomy, media, isolation and culturing methods of the day. Although anaerobic techniques were used, all subsequent culturing was done aerobically after the investigators found that the aerobic and anaerobic counts were comparable. The results were analysed by means of percent isolation frequencies and not actual counts of bacteria in pre-carious, transitional and carious states. They found increases in Veillonella, aciduric streptococci, Staphylococcus albus, Gram-negative filaments and diphtheroids in the pre-carious stage. They found that Lactobacillus had the most profound increase as caries developed but the aciduric streptococci had the highest isolation frequency in the carious plaque. The majority of microorganisms isolated had a negative association with the development of caries. This included Actinomyces, Leptotrichia and Streptococcus viridans.

In 1973 Ikeda *et al.*[36] examined the dental plaque from occlusal fissures, interproximal and buccal surfaces of mandibular first permanent molars in 12 children. All sites were initially caries-free and were studied over an 18 month period. They found that the levels of S. mutans and Lactobacillus species increased prior to the initiation of a caries lesion. Caries was found without detectable levels of Lactobacillus species but not in the absence of S. mutans. When Lactobacillus species were detected, they comprised a very small proportion of the total cultivable flora (approximately

0.1%). They concluded that S. mutans was important in the initiation of caries and Lactobacillus species were involved in lesion progression.

In 1979 Loesche and Straffon[31] studied occlusal fissure decay in children over a two year period. They also found that S. mutans increased prior to the diagnosis of caries. However, unlike the study by Ikeda *et al.*, five caries lesions developed in the absence of S. mutans and the levels of Lactobacillus species were over 25% of the total cultivable flora prior to the clinical diagnosis of caries and 4% at the time of diagnosis. They also found that in 92 of the 195 teeth studied, the level of S. mutans was approximately 10% of the total cultivable flora, but the fissures remained caries-free. They concluded that

1)S. mutans and fissure decay are closely associated.

2)fissure decay can occur in the absence of S. mutans.

3)fissures may remain caries-free in the presence of high levels of S. mutans (>10% of the total cultivable flora).

A study by Loesche and co-workers[41] in 1984 examined the microbiology of fissure plaque in 368 children over a period of three years with samples taken every six months. They found that the levels of S. mutans were 24% and 25% of the total cultivable flora when caries was diagnosed in the low-caries group and high-caries group respectively. Some teeth had high levels of S. mutans but did not develop decay. Lactobacillus species were not a consistent finding but increased six months prior to the diagnosis of caries and had levels of 7.1% and 5.2% of the total cultivable flora in the low and high-caries group respectively at the time of diagnosis. Veillonella species increased from 1.4% in caries-free fissures to 5.0% in decayed fissures. Their increase appears to be due to the presence of lactic acid produced by the acidogenic S. mutans and Lactobacillus species. S. sanguis was found to be negatively associated with caries.

Although they concluded that S. mutans is positively associated with fissure decay and Lactobacillus species with lesion progression, the six month interval between samples may have missed other critical shifts in the ecosystem[15]. The samples were actually "pooled samples" because the entire fissure was sampled instead of the specific area that developed caries. Therefore the actual counts do not actually reflect the ecosystem at the site of the fissure caries[42].

Bowden *et al.*[30] and Hardie *et al.*[35] provided an extensive microbiological analysis of developing approximal caries lesions in maxillary first premolars. Bowden and colleagues reported on the interim results after one year while Hardie and colleagues had two year results from a three year study. Samples were collected from the distal surfaces of upper first premolars three times a year in 19 subjects. Initially all sites were caries-free as determined by radiographic examination. Fifteen sites developed caries during the test period. At a generic level all sites were similar with Streptococcus, Actinomyces, Veillonella and Bacteroides having the highest counts. Actinomyces was the most predominant genus. Other organisms commonly found but in smaller proportions include Neisseria, Lactobacillus, Haemophilus, Fusobacterium, Rothia, Bacterionema, Leptotrichia and Eubacterium. They observed that caries may occur at sites where S. mutans is dominant or with moderate levels of S. mutans and Lactobacillus species. S. mutans was found to have increased prior to the detection of caries in two sites. In general, however, both S. mutans and Lactobacillus species increased after the detection of caries. The presence of low levels of Veillonella was thought to indicate the inability of the plaque to degrade lactic acid. No single species could be associated with the onset of a caries lesion. In fact, the microbial flora at a caries-free site may be quite similar to a site that develops a lesion. Therefore other non-bacterial factors must be involved in the initiation of a caries lesion.

Boyar and Bowden[31,33] studied the microflora of incipient approximal caries lesions in 22 children ranging in age from 4-9 years over a one year period.

Lactobacillus species were detected in 28 of 32 lesions that progressed and were never present in non-progressive incipient lesions. The progression of lesions was consistently associated with increased levels of S. mutans, Lactobacillus, Veillonella and A. odontolyticus. Lesion progression was also associated with a decrease in A. naeslundii, A. viscosus and "S. mitior". Loesche and Straffon[31] used a ratio between S. mutans and S. sanguis to describe the reduction of S. sanguis and increase in S. mutans associated with caries. This study suggests that the relationship is actually between S. mutans and "S. mitior" with the possible difference being in the use of different nomenclature. Boyar and Bowden suggested that the presence of Lactobacillus species may be a more accurate indicator than radiographs that a lesion has progressed to the point where restoration is required.

Milnes and Bowden[32] studied the microflora of nursing caries in children between the ages of 10 and 16 months. The test group consisted of 9 children from the Cross Lake Indian Reserve who were at high risk of developing nursing caries. The control group of 9 children were from Winnipeg and in a low risk group. Samples were taken 6 weeks apart and were analysed to the level of 34 species including Streptococcus, Lactobacillus, Actinomyces, Neisseria, Veillonella, Rothia, Bacterionema, Fusobacterium, Leptotrichia, Micrococcus, Haemophilus, Actinobacillus, Bacteroides, Capnocytophaga and Yeasts. Those sites that developed caries lesions had increased levels and isolation frequencies of S. mutans, Lactobacillus species, Veillonella species and Actinomyces viscosus as compared to control sites in the same mouth and sites in the control children that did not develop caries. They also found that susceptible sites in the test group that did not develop caries had a very similar microflora to the sites that did become carious. This suggests that other, as yet unknown, environmental factors are of critical importance in the development of a lesion because a "pathogenic" microflora does not always lead to caries.

A negative correlation was found between S. mutans and "S. mitior" and between A. viscosus and A. naeslundii. No single microorganism was found to be the etiological agent. They concluded that, under appropriate conditions, any number of combinations of acidogenic and aciduric micoorganisms could produce caries[15,32].

c)ECOLOGICAL CONCEPTS

The oral flora is relatively simple in newborn children with S. salivarius becoming established within a few hours after birth[43]. It is the most predominant microorganism for the first year of life. Transient organisms such as fecal lactobacilli have been detected but do not become established. These pioneer microorganisms must have the ability to overcome host defense mechanisms and become established. The oral microflora continues to develop in complexity with age. At one year of age moderate numbers of Neisseria and Veillonella and lower numbers of Actinomyces, Bacteroides, Leptotrichia, Rothia and Fusobacteria have been found[44]. S. sanguis was not detected until teeth began to erupt and S. mutans was first detected after one year[45]. The isolation frequency of Spirochetes and Bacteroides melaninogenicus increase with age and have been found to be ubiquitous in adults[46]. Whether or not a species is able to colonize successfully and grow depends on its ability to overcome the host's defense mechanisms that are antagonistic to colonization. Qualitative and quantitative differences are known to occur in a given site at different times, at different sites in an individual and between different individuals[26].

The development of the bacterial community on a clean tooth surface begins with the formation of the acquired pellicle. Bacteria colonize both pellicle and exposed enamel. Organisms such as S. sanguis, Veillonella and A. viscosus are early colonizers of the tooth surface[47]. S. salivarius which is found in abundance in saliva and the dorsum of the tongue is not able to adsorb well to tooth surface. The numbers of bacteria in saliva are important for adsorption as only a small percentage of available organisms colonize the pellicle. It has been shown that levels of 10^4 cells of S. mutans/mL of saliva are

necessary for colonization of a smooth surface[48]. The numbers are lower for colonization of a pit or fissure. S. sanguis, which has a greater affinity for colonization of hard tissue, needs a minimum of 10^3 cells/mL of saliva. S. mutans, therefore, has a lower affinity for the colonization of a smooth surface than does S. sanguis[48]. Such differences in adherence are an important ecological determinant in colonization[1,26,47,49]. "S. mitior" has been isolated from early plaque for a short period of time and is replaced by A. naeslundii[47]. As plaque continues to age there is a general shift from a dominant aerobic flora to a dominance by facultative and obligate anaerobes[26].

Different environmental conditions will exist at different times in each of the many habitats available (pits and fissures, smooth tooth surfaces, tongue, mucosal surfaces, saliva) due to the complex relationships between the tooth surface, saliva and soft tissue surfaces. The nutrient supply is normally intermittent but varied and rich. Because of these factors the mouth can really be regarded as a series of numerous microecosystems each with its own distinct microflora and environmental determinants[26]. The diversity in the nature of environments available is reflected in the wide variety of microorganisms present. They include Gram-positive and Gram-negative bacteria morphologically representing cocci, rods, spirochetes, filaments and vibrios. The microorganisms can range from aerobes to facultative organisms to obligate anaerobes. Just as the environment affects the microbial flora of a habitat, the metabolism of the microflora is constantly modifying the local environment and causing shifts in certain populations. This type of succession that occurs due to the microbial population changing the environment is termed autogenic succession. Depletion of the nutrient supply, alterations in pH or temperature and accumulation of metabolic end-products such as lactic acid are some of the factors that contribute to population succession[1,26].

Succession that is due to external, non-microbial factors is termed allogenic succession. Factors that tend to alter the oral environment include the presence or

absence of teeth, changes in the quality and/or frequency of nutrient intake, oral hygiene regimen, dental treatment, local or systemic antimicrobial use, hormonal factors and any condition that reduces salivary flow[26].

Whatever the environmental conditions, a climax community will become established for each distinct habitat. Within each of these habitats exist a number of unique ecological niches. The complexity of each of these microecosystems has made it difficult to isolate a single microorganism as the etiological agent in caries. The evidence tends to point to an imbalance in the microflora produced by environmental changes that favor acidogenic and aciduric bacteria rather than a single bacterial etiological factor[15,26,44,46,50].

2)CONCEPT OF INCIPIENT CARIES LESIONS

a)CLINICAL AND RADIOGRAPHIC FEATURES

The earliest clinical sign of caries is the incipient or "white spot" lesion. It can be seen most frequently on the facial or lingual surface of the tooth in the cervical region as a small, white, opaque area of enamel that has lost its normal translucency. Incipient lesions may also appear brownish in color due to the uptake of exogenous, organic pigmentation absorbed into the large pores of the lesion. The longer the lesion is present, the greater the amount of discoloration[1,49,51]. Habits such as smoking are also thought to contribute to the discoloration of these early lesion[51]. Incipient lesions are common on approximal areas of the tooth in the region just cervical to the contact area. These, however, are difficult to detect clinically but may be detected on radiographic examination as a cone-shaped radiolucency in enamel[1,49,51].

A study by Silverstone[52] in 1982 demonstrated the relationship between histological features of an incipient lesion and radiographic appearance. He found that, histologically, a lesion that penetrates two thirds of the enamel depth is not visible at all

radiographically. When a lesion appears to be confined to the outer one third of the enamel on radiographic examination, histologically it has progressed into dentin.

The inaccuracy of radiographic examination in determining the extent of demineralization in approximal caries lesions was also demonstrated in a study by Bille and Thylstrup[53]. The criterion for determining the indication for a restoration is macroscopic cavitation. They attempted to correlate radiographic findings with clinical changes to assess the accuracy of the radiographs in determining whether cavitation had occurred. A scoring system from 0-4 assessed the radiographic changes, with 0 indicating no radiographic change, 1 indicating a radiolucency in enamel only, 2 a radiolucency that has reached the amelodentinal junction, 3 a radiolucency midway through dentin and 4 indicating a radiolucency in close proximity to the pulp. Of 158 lesions that were examined, 13% (of 56 lesions) with a radiographic score of 0 or 1, 20% (of 35 lesions) with a score of 2 and 58% (of 67 lesions) with a score of 3 or 4 had clinical cavitation. Cavitation could only be assured where the radiographic score was 4 (indicating caries in close proximity to the pulp).

b)HISTOLOGY OF INCIPIENT LESIONS

The incipient lesion has been divided into a number of areas or zones when ground sections are examined by light microscopy. The number of zones found have ranged from three in a study by Darling[54] to the seven that were described by Gustafson[55] in 1957. Silverstone[51], however, has found that the early enamel lesion is most clearly divided into four zones. The deepest part of the advancing lesion is termed the translucent zone. Adjacent to this is the dark zone. The body of the lesion is the third zone and this occupies the bulk of the lesion. The most superficial zone is the surface zone.

Dental enamel consists of closely packed apatite crystals that are aligned along their long axis in an orderly arrangement. A property of this and other crystalline materials is birefringence. This is the property that enables a material to resolve plane polarized

light into two beams that travel at different velocities. Birefringence may be termed positive(+) or negative(-) according to the path taken by the slower and faster rays respectively. The birefringence in an enamel prism is referred to as negative 'intrinsic birefringence' because the slower ray has been found to vibrate perpendicular to the long axis of the prism.

In the caries process, spaces are created by demineralization of the enamel and when filled by a medium with a refractive index different from that of the apatite crystals (1.62) produces a type of birefringence called 'form birefringence' which is positive. With increased demineralization, the pore size of the crystals increases along with the positive 'form birefringence'. The observed birefringence as seen in a polarizing microscope is a result of a sum total of the negative 'intrinsic birefringence' of the mature enamel crystal and the positive 'form birefringence' produced by increased pore size of the crystal and the medium that they contain. The measurement of birefringence allows for the quantitative measurement of pore size and thus the extent of demineralization[49,51].

i)THE TRANSLUCENT ZONE

This was originally described as a 'light zone' by Williams[56] in 1897 who studied ground sections mounted in Canada balsam. Mummery[57] described it as a 'translucent zone' and postulated that it was an area of hypermineralization as a result of a vital response. Gustafson[55] also described the area as a zone of increased mineralization because of the observed higher negative birefringence compared to normal enamel. A study by Darling[58] in 1957, however, determined that the pore volume of enamel in the translucent zone was approximately 1% as compared to 0.1% in intact enamel. It was difficult to comprehend how a region of more porous enamel could be hypermineralized. It was not until 1967 when Crabb and Mortimer[59], using two-dimensional microdensitometry, established that the translucent zone is actually a zone of demineralization. The reason for the increase in negative birefringence in the

translucent zone is due to a loss of the positive form birefringence of intact enamel. In ground section, the small pores of intact enamel are filled with air because the large quinoline molecules are unable to penetrate them. Some positive form birefringence is produced because of the difference in refractive indices between air (1.0) and enamel (1.62). This reduces the overall negative birefringence. The pores of the enamel of the translucent zone are large enough for the quinoline molecules, therefore the loss of the positive form birefringence in intact enamel results in a greater negative birefingence[51].

This zone is not a consistent feature but has been found in approximately 50% of the carious permanent teeth as determined by Silverstone[60].

ii)THE DARK ZONE

The dark zone is a more consistent feature of caries lesions in that it has been found in 85-90% of lesions in permanent teeth[61]. Examination of this zone with polarizing light after imbibition with quinoline showed this to be a zone of positive birefringence as compared with the negative birefringence of intact enamel. It appears as a dark brown zone between the translucent zone and the body of the lesion due to the small pore size that excludes the large molecules of quinoline or Canada balsam so that the pores remain filled with air[51]. An explanation for the fact that the zone appeared positively birefringent while being more mineralized than the negatively birefringent body of the lesion was not forthcoming until a study was done by Darling and coworkers[62] in 1961. A previous study had determined the pore volume to be approximately 2-4%[58]. Using a variety of alcohols as imbibition media they determined that resultant birefringence was due to size and shape of the media rather than their refractive indices. They concluded that the dark zone was a system of micropores penetrable by aqueous media and methanol. The larger the molecular size of the media the less they were able to penetrate the pores. These inaccessible pores would thus remain filled with air producing positive form birefringence. In an aqueous medium the water molecules are

sufficiently small to penetrate the pores, thus the dark zone is not visible in ground section.

Silverstone[63] provided an explanation for the micropores in experiments on the remineralization of caries. He found that the appearance of the dark zone was actually due to a remineralization at the advancing front of the lesion. Some of the large pores of the body of the lesion were being remineralized and the dark zone is a combination of large pores and the remineralized micropores. This supports the concept of the caries lesion as a dynamic process of remineralization and demineralization.

The presence of the dark zone is dependent on rate of carious attack. *In vitro* studies demonstrated that a lesion produced over a period of 96 hours did not have a dark zone while a lesion that was slowly formed over a period of months had a dark zone indistinguishable from that of *in vivo* caries lesions[61,64].

iii)THE BODY OF THE LESION

The body is deep to the intact surface layer of enamel and is the largest part of the lesion. The area is translucent when ground sections are examined with transmitted light after quinoline imbibition. The Striae of Retzius are well demarcated and appear enhanced in this zone. Imbibition with water shows this region to have positive birefringence. Darling[58] determined that the pore volume in this area ranges from 5% at the periphery of the lesion to 25% at the centre of the lesion. He found alternate radiopaque and radiolucent lines in the subsurface region approximately 30 μm apart which he thought represented the Striae of Retzius. In the radiolucent area there was another set of alternating radiolucent and radiopaque lines approximately 6-8 μm apart which ran parallel to the long axis of the prisms. Some areas also showed a series of radiolucent lines approximately 4 μm apart at right angles to the prisms. These were thought to be cross striations of the prisms. Darling concluded that caries progressed along the Striae of Retzius with demineralization occurring by way of the interprismatic substance through the cross-striations of the prisms to the core of the prisms.

Crabb[65], however, disagreed with Darling's theory. He found that the Striae of Retzius demonstrated positive birefringence in ground sections imbibed with quinoline and reasoned that it was more likely that the Striae represented a path of resistance rather than a pathway to demineralization. In subsequent microradiographic studies Crabb[65,66,67] suggested that these alternate radiolucent and radiopaque bands were not due to demineralization but rather an 'unmasking' of an existing structural relationship. It was independent on the direction of the spread of demineralization but demineralization was necessary for it to appear.

Quantitative microradiographic studies by Bergman and Lind[68] in 1966 demonstrated a wide variation in the the mineral content which could not be correlated with the depth of the lesion. A lesion 300 μm deep had a mineral content of 29% whereas a lesion 600 μm in depth had a mineral content of 47% by volume.

Studies by Silverstone and others demonstrated well-mineralized bands through the body of the lesion that gave the lesion a 'laminated' appearance[65,69,70]. They appeared to follow the contour of the advancing lesion and are thought to arise from periods of arrest and remineralization in the caries process.

iv)THE SURFACE ZONE

The surface zone has been found to be relatively unaffected by the caries attack in the incipient lesion. Examination with a polarizing microscope after imbibition with water showed this area to be negatively birefringent in contrast to the positive birefringence of the highly demineralized body of the lesion. On microradiographs this region is a radiopaque area approximately 30 μm in depth[63].

In 1932, Applebaum[71] noted the presence of this intact surface layer by microradiographs. Its presence was confirmed by a number of other researchers[72,73,74,75] and generated a number of theories on its nature. Some of the suggested causes for the intact surface layer included a photographic artifact (Mackie Line)[72], an acquired organic protective layer[73] and a normally hypermineralized

surface[74]. Issac *et al.*[75] attributed the surface features to a higher fluoride and lead content. In a later study by Brudevold *et al.*[76] in 1965, the mineralized surface layer was attributed to the combination of a higher mineral and lower water content, higher concentrations of elements such as fluoride and low carbonate concentrations.

Several investigators have shown that the surface layer could still be produced in artificial caries experiments after removal of part of the outer enamel[63,64,77]. Silverstone[63] concluded that the surface zone remains well mineralized in the incipient lesion due to reprecipitation of calcium and phosphate ions from subsurface demineralization and from plaque overlying the lesion. As the surface begins to demineralize, the high fluoride concentration at the enamel/plaque interface would tend to favor precipitation. Continued progression of the lesion eventually results in loss of the surface integrity. The progression occurred from the surface of the enamel inwards and resulted in an increase in the rate of lesion progression once the surface integrity was lost.

Evidence on the pathway of caries attack differs when comparing the results from electron microscopic examination versus the polarizing light microscope. The observed preferrential sites of demineralization as determined by both techniques may be the result of damage occurring during section preparation. Current evidence suggests that demineralization affects both inter and intraprismatic enamel. The development of narrow channels between adjacent prisms suggest that prism junctions are especially susceptible to demineralization[51].

3)MICROBIOLOGY OF CARIES

Early studies of the oral microflora considered the bacterial community to be homogeneous[3,4]. In 1938, Bibby[78] was one of the first investigators to demonstrate that the microflora varied in different sites, but the study was limited by the culturing techniques and taxonomy of the day. A number of recent studies have shown

that there are actually many well defined, unique communities in the mouth[34,79,80,81]. Huis In't Veld *et al.*[80] demonstrated that fissure and approximal plaque from one individual could have greater heterogeneity than between plaque samples from comparable sites in different individuals. This study also found that while S. mutans could be isolated from both caries-active and caries-free subjects, S. mutans serotype d (later named S. sobrinus) was isolated only in caries-active individuals, thus implicating a specific serotype of S. mutans as a human odontopathogen. High levels of S. mutans were found in white spot lesions in a study by Duchin and van Houte[34] but could not be detected on clinically sound enamel 100 μm away.

It has become apparent that, unlike many bacterial diseases where a single pathogen causes disease, caries results from an ecological imbalance of the indigenous oral microflora caused by environmental pressures placed on the ecosystem[26,30,32,33,35].

Bowden *et al.*[27,30,32] have suggested that there is a "basic plaque" that exists in balance with the host. This basic plaque has a weak cariogenic potential and periods of demineralization may be offset by remineralization by host factors. By increasing the amount of sucrose in the diet, organisms such as S. mutans and Lactobacillus species are selected and become dominant. The ecological balance shifts in a direction that favors the development of a caries lesion.

a) BACTERIAL ROLE IN THE ETIOLOGY OF CARIES

Since Miller's early observations concerning the integral relationship of microorganisms and dental caries, there has been a search for specific bacteria as etiological agents[3]. The classic germ-free animal study by Orland and co-workers[11] established the critical involvement of microorganisms in the etiology of caries. Lactobacilli were the focus of considerable attention and were thought to be the etiological agent due to their acidogenicity and aciduricity[4,5]. Current research has

been mainly focused on S. mutans as the principal pathogen in dental caries, first identified by Clarke[7] in 1924. Fitzgerald and Keyes[17] rediscovered these "caries-inducing streptococci" in their studies on dental caries in hamsters in 1960. Although there is no direct evidence that S. mutans causes dental caries there is significant epidemiological evidence to support a close association between S. mutans and caries.

Krasse *et al.*[82] in 1968 found a positive correlation between S. mutans and caries activity in both an adult group and a group of preschool children. Each of these groups was subdivided into a caries-free and a caries-active group. They found that in both the adult and preschool group, all subjects that had S. mutans levels $\geq$20% of the total streptococcal counts were in the caries-active group. When S. mutans was $\leq$10% of the total streptococcal count, 12/14 of the preschool children and 17/21 of the adult group were caries-free.

A study by Duchin and van Houte[34] was undertaken to determine the relationship of S. mutans and Lactobacillus species to "white spot" or incipient smooth surface caries lesions, to lesions with cavitation and to sound enamel. They found that the plaque associated with incipient lesions and lesions exhibiting cavitation had significantly higher proportions of S. mutans than did sound enamel. Lactobacilli were not detected in the incipient lesions and were isolated from a small number of samples in very low numbers (0.02, 0.08 and 0.003 percent of the total cultivable flora) from sound enamel. Differences of over 100 fold were found in levels of S. mutans within the same incipient lesion. They were not able to correlate a specific level of S. mutans with caries-associated plaque because levels as low as 1% could be found associated with the development of a caries lesion. This study indicated that S. mutans is closely associated with early smooth surface lesions and that the lactobacilli are not.

S. sanguis, "S. mitior" and "S. milleri" have been found to be the most numerous of the streptococci in dental plaque[83,84]. Loesche *et al.*[41] in 1984 found that S. sanguis was negatively associated with the development of caries and Boyar and

Bowden[33] found that there was a negative correlation between "S. mitior" and caries. An association of "S. milleri" with caries has not been shown.

S. salivarius has been shown to be cariogenic in animal models[85,86] but due to its extremely low numbers in dental plaque its importance in the development of dental caries in humans is likely to be minimal[26,85,86,87].

Actinomyces species along with the streptococci have been found to be the dominant plaque organisms in mature dental plaque[81]. Hardie *et al.*[35] were unable to find a positive relationship between Actinomyces species and the development of caries but a later study by Milnes and Bowden[32] in 1985 showed a significant increase in numbers and isolation frequencies of A. viscosus in sites that developed caries lesions. Boyar and Bowden[33], however, found that A. odontolyticus increased in association with developing lesions.

Under appropriate conditions, it is probable that a number of plaque microorganisms could initiate caries considering the complexity of interactions between plaque microorganisms, tooth surface and dietary factors. There is a positive association between a number of acidogenic microorganisms and caries but the strength of the association varies between groups. Although S. sanguis, "S. mitior" and Actinomyces species are prominent plaque organisms their numbers do not generally increase with the development of caries. Their acidogenicity may be a contributing factor in caries development[1,50].

b)TRANSMISSIBILITY OF CARIES

In 1960, Keyes[88] demonstrated that caries was transmissible in hamsters and Osborne-Mendel rats. He also found that the cariogenic flora was sensitive to penicillin. The progeny of the animals that were treated with penicillin or erythromycin during pregnancy or lactation were found to have a very low caries activity as compared with the control group and this continued through several generations. It was felt that a high degree of microbial specificity must be involved in the caries process due to the fact that

that the animals treated with antibiotics still had a relatively complex microbial flora but one that had a low cariogenic potential.

c)CARIES SUSCEPTIBILITY OF DIFFERENT TOOTH SURFACES

The morphology and position of the teeth in relation to each other are the principal determinants in the colonization of the microflora. The crown of the tooth can be divided into a number of regions which, due to different environmental conditions, have a range of susceptibility to caries. The facial and lingual surfaces of the tooth are most exposed to masticatory trauma and are the most easily accessible to plaque-disrupting oral hygiene procedures[2,89]. The mesial and distal surfaces of the teeth that are in contact with adjacent teeth provide an environment more protected from mechanical trauma and the self-cleansing action of saliva. Food debris tends to collect, thus providing fermentable substrate for extended periods of time. The microflora therefore has an ecological advantage in this area and it is more caries-susceptible than the facial or lingual surfaces. The developmental pits and fissures that occur on the occlusal surfaces of posterior teeth, buccal of mandibular molars, lingual of maxillary molars and lingual of maxillary lateral incisors are the most caries-susceptible areas. The relative susceptibility of tooth surfaces to dental caries in decreasing order has been shown to be the occlusal fissures of molars, approximal of molars and occlusal of premolars, approximal of premolars followed by the approximal of incisors and canines. The facial and lingual surfaces of all teeth had the lowest susceptibility to caries[89].

d)CHARACTERISTICS AND VIRULENCE FACTORS

1)S. mutans

i)Characteristics

S. mutans was first described by Clarke[7] in 1924. He isolated microorganisms from caries lesions and because they appeared more ovoid than round, he thought that they were a mutant form of streptococcus, thus they were termed S. mutans. He noted that all strains were able to ferment glucose, lactose, raffinose, mannitol, inulin and salicin to acid and the acid production was rapid, producing a drop in pH from 7.0 to 4.2 in 24 hours. When grown on blood agar, no haemolysis or discoloration of the media was detected.

They are described as Gram-positive, catalase-negative, non-motile microorganisms[90]. The majority are either α-haemolytic or non-haemolytic when grown on sheep blood agar but there have been reports of ß-haemolytic strains[91]. Morphological features can vary from coccoid to ovoid to short rod shaped cells depending on growth conditions and the cells tend to grow in short chains. When grown on media containing sucrose the colonies are rough and hard.

ii)Classification

Based on DNA base composition and hybridization, S. mutans has been divided into five genetic groups designated I-V[92,93]. They have also been divided into eight serotypes 'a' through 'h'[94,95,96]. A summary of the relationship between the genetic and serotype groupings is illustrated in Table 1.1. The antigens for each serotype have been isolated and have been determined to be polysaccharides of the cell walls[90]. As can be seen from Table 1.1 the most common strain of S. mutans is genetic group I, serotype c. S. sobrinus has been isolated from humans[80] while S. rattus and S. cricetus have been isolated predominantly from hamsters and rats. It is not known whether S. ferus has any human strains but it has been found in wild rats.

Table 1.1

CHARACTERISTICS OF *S. mutans*[a)]

Subspecies of *S. mutans* group	Genetic group	DNA Base Content (mol % G+C)[b)]	Serotype	Approximate % of human isolates	Acid Prod from Raff[c)]	Starch	Inulin	Predominant Glucan
S. mutans	I	36-38%	c	80%	+	−	+	D>M
			e	6%	+	−	+	D>M
			f	4%	+	−	+	D>M
S. rattus	II	42-44%	b	<1%	+	−	+	D>M
S. sobrinus	III	44-46%	d,g,h	9%	−	−	−	M>D
S. cricetus	IV	42-44%	a	<0.1%	+	−	+	M>D
S. ferus	V	43-45%	c	?	−	+	+	

a)adapted from Nikiforuk(1985); Loesche(1986).

b)G + C, guanosine plus cytosine content

c)Raff, Raffinose

d)D, dextran(water soluble glucan); M, mutan(water insoluble glucan)

iii)Ecology

S. mutans does not begin to colonize the mouth, its natural habitat, until teeth begin to erupt. It also has been shown to colonize prostheses and disappears following removal of all teeth[46]. It has been isolated from human[97] and rat fecal material[98] but has a very limited distribution outside of humans. Dent *et al.*[99] reported that S. mutans had been isolated from the Indian fruit bat and the Patas monkey of the eighteen different animal species that were examined. Isolates from infants have been identified using serotyping and bacteriocin typing and found to be identical to those strains found as salivary isolates in the mother[100].

S. mutans has been shown to colonize pits and fissures and approximal areas of the tooth more readily than than the facial or lingual surfaces. These microorganisms do not colonize similar surfaces in the same mouth uniformly which indicates a relative inability to spread from one surface to another. Using streptomycin-resistant mutants of S. mutans, Gibbons and van Houte[101] found that when one side of the mouth was implanted with these strains they could not be isolated from the contralateral side. They suggested that this was due to low salivary concentrations and weak adsorptive properties.

Some strains of S. mutans have the ability to produce bacteriocins which are able to inhibit a wide range of Gram-positive microorganisms including S. mutans, S. salivarius, S. sanguis, S. pyogenes, mycobacteria, streptomyces and actinomyces. The specific bacteriocin of S. mutans has been appropriately termed "mutacin" by Hamada and Ooshima[102]. Van der Hoeven and Rogers[103] suggest that the production of mutacin confers an ecological advantage to a strain that is attempting to become established in a microenvironment.

iv)Metabolism

a)Sugar metabolism

Environmental factors have been shown to be a critical determinant in the metabolic pathway of glucose. Under conditions of glucose excess, the principal endproduct of fermentation by the glycolytic pathway is lactate. When glucose is limited, the endproducts are acetate, formate, and ethanol as well as lactate[104]. S. mutans has been shown to metabolize sucrose at a faster rate than can S. sanguis, "S. mitior" and A. viscosus[105]. In addition, using sucrose as an energy source, S. mutans has the ability to convert it into glucans and fructans by cell-bound and extracellular enzymes and also to form intracellular polysaccharides (glycogen-like glucans) as an energy reserve[106,107]. Compared with other streptococci, S. mutans was found to be more aciduric[108]. These properties are significant factors in the cariogenic potential of the organism and provide a selective advantage in an acidic environment of a developing caries lesion.

b)Intracellular polymer synthesis

The production of intracellular polysaccharides (IPS) by some strains of S. mutans is thought to contribute to their pathogenicity by providing an energy source during periods when exogenous sugar is limited or absent[109]. However, highly cariogenic serotype d and serotype g strains produce little IPS so that this property does not appear to be essential in the cariogenicity of S. mutans[110].

c)Extracellular polymer synthesis

Extracellular polysaccharides, namely glucans and fructans, are synthesized from sucrose by glucosyltransferase and fructosyltransferase respectively.

The glucans may be further divided into water-soluble dextrans and water-insoluble mutans. The dextrans consist of an α(1->6)-linked core glucose polymer with α(1->3), α(1->2) and α(1->4) branches. The insoluble mutans have a core with α(1->3) linkages with α(1->4) and α(1->6) branch linkages and are more resistant to α(1->

6) glucanase or dextranase. The insoluble nature of the mutans is due to the α(1->3) linkages and is an important factor in promoting S. mutans accumulation in plaque[49,90]. S. sobrinus (serotype d) has been shown by Trautner and colleagues[111] to produce more glucans and have a higher ratio of insoluble/soluble glucans than serotype c. This strain was earlier shown to be highly cariogenic and only isolated from caries-active sites[80].

The fructans occur in water-soluble and water-insoluble states with the former predominating. They are readily degraded by plaque bacteria and serve as a reservoir of fermentable substrate in plaque[80,90].

2)Lactobacillus species

i)Characteristics

These microorganisms have been described as Gram-positive, non-motile, non-sporing rods that commonly occur in chains. They can vary from long, slender rods to bent rods to coccobacilli depending on the oxygen tension, media composition and the age of the culture. With age, the cells may give variable results when Gram-stained. Lactobacilli are obligately saccharoclastic with the primary endproduct of fermentation being lactate. On agar media the colonies are smooth, convex and glistening with entire margins approximately 2-5 mm in diameter[112].

ii)Metabolism

The Lactobacilli are either homofermentative or heterofermentative. The homofermentative strains produce lactic acid as the major or exclusive endproduct of the fermentation of glucose by the Emden-Meyerhoff pathway. The most common oral homofermentative Lactobacillus species include L. casei, L. acidophilus, L. plantarum and L. salivarius. The heterofermentative strains metabolize glucose to CO_2, ethanol or acetic acid, and lactic acid by way of the 6-phosphogluconate pathway. The common oral heterofermentative strains include L. cellobiosus, L. fermentum, L. buchneri and L. brevis[112,113].

The most common of the oral lactobacilli are L. casei and L. fermentum[112,113].

Some strains have the capacity to produce IPS but this ability did not seem to be a critical factor in cariogenicity. A study by Fitzgerald *et al.*[21] in 1981 tested the cariogenic potential of 32 strains of lactobacilli as a monoinfection in gnotobiotic Sprague-Dawley rats. Only 7/32 Lactobacillus strains tested were capable of forming IPS. Six of the seven IPS-forming strains were associated with high caries scores but another 11/32 strains that were associated with high caries scores did not produce IPS.

iii)Pathogenicity

Lactobacillus species are highly aciduric and acidogenic but comprise a very small portion of the oral flora[112,113]. They only increase significantly and become predominant in plaque after caries lesions appear[32,33,85]. Their low isolation from intact smooth enamel surfaces may be attributed to their low affinity for enamel[114]. They are often the dominant microorganism from isolates of advanced dentinal caries which suggests that they are secondary contributors to the caries lesion[33,40,85]. In studies testing the cariogenicity of human strains of oral lactobacilli in gnotobiotic rats and conventional hamsters by Fitzgerald *et al.*[21,115], smooth surface lesions were rare. The predominant site for caries attack was molar fissures.

3)Other bacteria associated with caries

i)Actinomyces

This group of microorganisms shows considerable variation in both shape and size. Their morphological features include straight or slightly curved rods in short chains or clusters with or without branching, and slender filaments with varying degrees of branching. The short rods may or may not have clubbed ends. Although they are described as Gram-positive, staining is irregular. They are facultative anaerobes that are non-motile and non-sporeforming. Colonies grown on blood agar vary from 0.5-5.0 mm in diameter and may be dry and rough or mucoid, soft and smooth. The colonies are usually white or grayish-white except for A. denticolens which is pink when grown on horse

blood agar anaerobically and A. odontolyticus which has a reddish-brown pigment on blood agar.

They ferment carbohydrates producing formic, acetic, lactic and succinic acids from glucose with no gas formation[116].

The most reliable means of identifying Actinomyces to the species level is serology. Direct or indirect immunofluorescence techniques are most commonly employed. Attempts have been made to identify species by fermentation reactions but results have been inconsistent[116,117].

Actinomyces species have been shown to be early colonizers of plaque and, with Streptococcus, are the dominant group in established dental plaque[26]. A. viscosus has been shown to be able to aggregate with Veillonella[118] and A. naeslundii with Streptococcus species[119]. This property undoubtably contributes to increasing plaque thickness.

Animal studies have shown that A. naeslundii and A. viscosus can induce fissure caries[120], root caries[121] and periodontal disease[122,123] in gnotobiotic animals. Data from human studies have not suggested a major role in the initiation of caries lesions[35,106] except for the studies by Boyar and Bowden[33] which noted an increase in A. odontolyticus with the initiation of caries and Milnes and Bowden[32] which noted an increase in A. viscosus and a decrease in A. naeslundii. They have, however, been a consistent isolate from advanced caries lesions[124].

ii)S. sanguis/"S. mitior"

a)Characteristics-S. sanguis

S. sanguis strains are Gram-positive cocci approximately 0.8-1.2 μm in diameter and tend to grow in chains. When grown aerobically, pleomorphic or rod-shaped cells may be seen. On blood agar the colonies are usually α-haemolytic but ß-haemolytic and non- haemolytic strains have been found. The colonies have a smooth or matt surface. Hard, rough, adherent colonies are normally formed on sucrose plates from strains

capable of producing soluble extracellular polysaccharides (dextrans). A few strains, however, do not produce polysaccharides. They produce acid from glucose, maltose, salicin, sucrose and trehalose but do not ferment mannitol, xylose, glycerol or arabinose. Some strains are able to ferment sorbitol. The final pH in glucose broth is 4.6-5.2. Ammonia is produced from arginine, H_2O_2 is formed when grown aerobically and more than 50% hydrolyze esculin[125,126].

The principal habitat of S. sanguis is the oral cavity and it is found in dental plaque[127].

b)Characteristics-"S. mitior"

"S. mitior" strains are Gram-positive ovoid or spheroidal cocci that tend to grow singly, paired or in long chains. The majority of the strains are α-haemolytic. Colonies grown on sucrose agar may be hard and adherent if they produce extracellular polysaccharides or soft if they lack that capability. All strains ferment glucose, sucrose and maltose. Inconsistent results have been obtained from fermentation of raffinose, lactose, trehalose, inulin and cellobiose. They do not produce ammonia from arginine or hydrolyse esculin which is useful in differentiating "S. mitior" from S. sanguis[127].

c)Taxonomy of S. sanguis and "S. mitior"

These species have been poorly defined due to a lack of consensus on nomenclature and classification. This has led to confusion as some strains that have been used in studies are actually mislabeled (Table 1.2). The significant difference between the two species is the cell wall composition, with S. sanguis having glycerol teichoic acid and "S. mitior" having ribitol in the form of ribitol teichoic acid. Kilian *et al.*[125] propose the rejection of the designation S. mitis due to its type strain (NCTC 3165) being more accurately described as S. sanguis taxon 6 by his group.

Table 1.2
Synonyms and Differences between *S. sanguis* and "*S. mitior*"[a)]

	S. sanguis	"*S. mitior*"
Synonyms		
	S. sanguis I:B	*S. sanguis* I:A
	S. sanguis I	*S. sanguis* II
	S. sanguis biotype A	*S. sanguis* biotype B
	S. sanguis serotypes I, III, (I/II), IV	*S. sanguis* serotype II
	S. sanguis genospecies 1,3	*S. sanguis* genospecies 2
		S. mitis EPS-negative
Cell Wall components		
glycerol teichoic acid	+	−
ribitol	−	+
rhamnose	+	−

[a)]adapted from Kilian *et al.*(1986)

d)Ecology of S. sanguis and "S. mitior"

Both S. sanguis and "S. mitior" form a large proportion of the indigenous oral flora of man and constitute approximately 75% of the streptococci in supragingival plaque[30,33]. Different studies have noted inverse relationships between S. mutans and S. sanguis[41] and between S. mutans and "S. mitior"[15,33] in the developing caries lesion. This may be attributed to the inconsistencies in nomenclature among different researchers. Further clarification of each of the taxa with the two groups will be necessary to understand the ecology of these diverse groups of organisms.

iii)S. salivarius

S. salivarius strains are ovoid or spherical cocci approximately 0.8-1.0 μm in diameter and grow in short or long chains. Most are non-haemolytic. When grown on sucrose agar most strains produce soluble extracellular polysaccharides of the fructan type. There are some strains capable of producing insoluble glucans as well. The colonies are large, mucoid and may be smooth or rough. The final pH in glucose broth is 4.0-4.4 and they can produce acid from fermentation of glucose, sucrose, maltose, lactose, raffinose, inulin, salicin and trehalose. They are unable to ferment mannitol, sorbitol, xylose, glycerol or arabinose. Esculin and urea are hydrolyzed but arginine is not.[126].

S. salivarius is found predominantly on the tongue, soft tissue and saliva. Its numbers in plaque are generally low because it does not bind well to pellicle or hard tissue[26].

Although capable of inducing caries in gnotobiotic animals it is probably not a significant factor in human dental caries due to its low numbers in plaque[20,128,129].

iv)"S. milleri"

Named after W.D. Miller, this group of microorganisms are ovoid or spherical in shape, may be found singly, in pairs or in chains of variable lengths. Haemolysis is

variable with strains α-haemolytic, ß-haemolytic and non-haemolytic. Some strains are unable to grow in air alone. Growth either requires or is enhanced by the addition of CO_2. They are able to form acid from glucose, sucrose, lactose, maltose, salicin and trehalose but are unable to ferment mannitol, sorbitol, glycerol, inulin, xylose, arabinose and melezitose. Esculin and arginine are hydrolyzed[126].

"S. milleri" is found predominantly in the the gingival crevice and cervical plaque and is found infrequently in other sites in the oral cavity[26,49,84].

Strains of "S. milleri" are capable of producing caries in gnotobiotic rats but their significance, if any, in human dental caries is not known at this time[130].

v) Veillonella

This is a group of very small, non-motile, non-sporeforming, Gram-negative anaerobic cocci. Approximately 0.3-0.5 µm in diameter, they grow clumps, pairs or short chains. They are able to metabolize pyruvate, lactate, malate, fumarate and oxaloacetate but cannot ferment carbohydrates. Their nutritional needs are complex and they require CO_2 for growth. Colonies are grayish-white, smooth, soft, opaque and have an entire edge. The 1984 edition of Bergey's Manual of Systematic Bacteriology considers Veillonella alcalescens to be within Veillonella parvula due to high DNA homology and not to be a separate species[131,132,133].

Veillonella naturally occur in the oral cavity, intestinal and respiratory tract of man and some animals[132]. It is the most common Gram-negative coccus in dental plaque[49]. Numbers tend to increase as conditions become more favorable for anaerobes. The production of lactic acid by Streptococcus and Actinomyces species has been shown to favor colonization[134]. A study by McCabe and Donkersloot[135] demonstrated the ability of Veillonella to adhere by the extracellular streptococcal enzyme glucosyltransferase which adsorbed to the cells. Glucans were produced that entrapped the Veillonella cells. The ability of Veillonella to utilize lactic acid and form weaker acids such as acetic and propionic acids may reduce the potential for

demineralization of enamel. Their association with caries lesions[15,33,40,41] reflects a succession in the population due to changing environmental conditions (increase in lactic acid) rather than an etiological role. Co-infected gnotobiotic rats with S. mutans and Veillonella had fewer caries lesions than those that were mono-infected with S. mutans[23].

4)ANTIBACTERIALS IN THE CONTROL OF CARIES

The critical involvement of the plaque microorganisms in the etiology of dental caries has been well established. Elimination of all plaque has been shown to prevent both caries and periodontal disease[136]. Miller[3] recognized the potential of chemical antimicrobials in his classical experiments on the oral flora. He tested the effectiveness of over twenty antiseptics both *in vitro* and *in vivo* . Some were found to be effective but due to the caustic nature and bitter taste of the compounds their intraoral use was thought to be unsafe. Based on his theory on the nonspecific etiology of caries, treatment was focused on suppression or elimination of the entire oral flora. This has been termed the Nonspecific Plaque Hypothesis (NSPH), which assumes that caries results from the production of noxious substances from the entire oral flora. Disease results once the host's threshold of resistance has been exceeded. Chemotherapy according to this theory is difficult, because treatment is open-ended and prophylactic. Such an agent would have to be extremely safe and without adverse side-effects to be available in an over-the-counter form such as a toothpaste or mouthrinse. Suppression or elimination of the entire oral flora over a long period of time is not only unattainable but undesirable as well. Formation of dental plaque is a normal process and radically upsetting the ecological balance may result in superinfection and the development of resistant bacteria[2,8,137].

Current data, however, indicates that caries is a result of an imbalance of the indigenous oral flora. The Specific Plaque Hypothesis (SPH) states that only certain

plaques cause disease, and this is due to an increase in a certain indigenous organism(s) or because of the presence of a pathogen[137]. Shifts in the flora to such organisms as S. mutans, S. sobrinus and Lactobacillus species have been shown to be associated with the development of caries lesions[89].

a)ANTIBIOTICS

Antibiotics are natural antimicrobial substances produced by microorganisms. They act either by inhibiting (bacteriostatic) or killing (bactericidal) certain microorganisms. Many antibiotics in use today are synthetic or semi-synthetic derivatives of those produced naturally[136].

Experiments have shown that when penicillin, erythromycin or tetracycline is added to the diet or drinking water or is applied topically, both plaque formation and caries incidence is dramatically reduced in rodents[138-142]. Long-term prophylactic administration of penicillin in patients with a history of rheumatic fever has been shown in retrospective studies to cause a statistically significant decrease in dental caries[143,144]. Attempts to duplicate the anticaries effect of penicillin in humans followed the animal studies. Four studies assessed the effectiveness of penicillin-containing dentifrices in children[145-148]. Dosages ranged from 100-1000 units of penicillin with each brushing and the frequency of brushing in the studies was from 1-3 times/day. Three of the four studies did not find a significant reduction in caries. The study by Zander[148] demonstrated a caries reduction of greater than 50% in a two year study of 409 chidren. The lack of effectiveness in the studies by Hill and Kniesner[145], Walsh and Smart[146] and Hill *et al.*[147] may be attributed to insufficient concentration or contact time of the antibiotic in the oral cavity. Topical application of such a medically important antibiotic to treat dental caries was discontinued because of the potential for the development of resistant bacteria and sensitivity to the drug[2].

Vancomycin and kanamycin have been used in several studies to assess their anticaries effect due to some of their desirable properties. They are not medically important drugs and they are poorly absorbed through the gastrointestinal tract. Vancomycin inhibits cell wall synthesis and has a spectrum of activity limited to Gram-positive organisms while kanamycin inhibits protein synthesis and has a broader spectrum spectrum with activity against Gram-positive and Gram-negative organisms[136]. Topical use of vancomycin in hamsters eliminated S. mutans and inhibited caries[149]. A study by De Paola *et al.*[150] found that a 3% vancomycin gel self-administered daily for 5 minutes over a period of a year resulted in a decrease in caries increment of one surface compared with controls (4.42 DFS vs 3.43 DFS). The caries reduction was significant for fissure caries but not smooth surface caries and in newly erupted teeth rather than teeth that were present at baseline. A 5% kanamycin gel was applied twice a day for one week in children with rampant caries in a study by Loesche and colleagues[151]. All caries lesions were then restored and kanamycin was applied for one further week. At a 9 month recall the kanamycin-treated children were found to have seven times the number of new caries lesions as the control group but at the 14 and 37 month recalls there was a 46% reduction in new lesions compared with the control group. They attributed the increase in caries incidence at the 9 month recall in the kanamycin group to the selection of S. mutans from the depths of subclinical lesions. Once these reservoirs of odontopathic organisms were eliminated by restoration the subsequent decrease in caries incidence resulted. This study pointed out that chemotherapeutic agents used in the control of caries should be able to penetrate and kill odontopathic organisms in the depths of fissures and subclinical caries lesions. Restoration of overt lesions must be done prior to application of an antimicrobial agent to eliminate reservoirs of S. mutans and other odontopathic organisms.

A problem with the use of vancomycin is that resistant organisms such as Bacteroides can proliferate and its use results in a predominantly Gram-negative plaque. The long-term sequelae of a shift of this type is not known at this time[136].

Erythromycin, Spiramycin and Niddamycin are macrolide antibiotics that have a spectrum of activity similar to penicillin. Erythromycin was found to decrease the amount of plaque formed by 35% when administered as a liquid suspension over a period of 7 days in a study by Lobene *et al.*[152]. Of the 4 subjects tested, all suffered from diarrhea and 2/4 developed nausea and vomiting. Resistant strains increased markedly during the test period and remained high for 2-3 weeks post-antibiotic treatment. The percentage of streptococci of the total cultivable flora did not seem to be markedly affected. Erythromycin had the greatest effect on the spirochetes. All subjects had detectable levels of these organisms prior to the administration of the antibiotic. They disappeared and were not detected for 5-18 weeks after the administration of erythromycin. Niddamycin (also known as CC10232) was also found to cause a reduction in plaque (11-23%) and in gingivitis (55-72%)[153]. Spiramycin is unique in that it is concentrated in the salivary glands so that salivary levels exceed serum levels. It is excreted in gingival crevicular fluid for extended periods of time after systemic administration[154,155]. Systemic administration has produced a clinical improvement in periodontal disease by reducing pocket depth by approximately 30%[156].

The use of systemic antibiotics specifically for plaque control or inhibiting caries is inappropriate. Total or selective suppression of the entire indigenous oral flora may result in superinfection or an undesirable shift in the indigenous flora. The potential for allergic reactions, bacterial resistance to medically important antibiotics, systemic side effects are but a few of the disadvantages. The use of topical antibiotics under the SPH may be appropriate if an area is accessible to the agent and is able to remain in contact at

an appropriate concentration for a suitable period of time. It must have a spectrum of activity against the odontopathogens and produce a desirable population shift.

b)FLUORIDE

Fluoride has been shown to be a safe, effective chemotherapeutic agent in the prevention of caries. It is one of the few antimicrobial agents that can be used effectively according to the NSPH because it is well tolerated by the host and does not result in superinfection[137]. While there is well documented, extensive evidence that fluoride reduces caries, the mechanism by which it produces its clinical effect is still under investigation[136,137,157].

Fluoride has the ability to increase host resistance to demineralization by filling voids and stabilizing the apatite cystal with the addition of hydrogen bonds[158]. Posner[159] suggested that the exchange of the hydroxyl group with fluoride producing fluorapatite results in a less soluble crystal. These alone, however, are not enough to cause the dramatic reduction in decay that does occur[157]. Carbohydrate metabolism of streptococci is affected by fluoride by inhibiting enolase, thereby preventing the formation of lactic acid[160]. The inhibition of enolase results in decreased levels of phosphoenolpyruvate (PEP) and inhibition of the PEP-dependent sugar transport mechanism. The net result of this is decreased ICP formation. Fluoride has also been found to lower the surface energy of enamel thus making plaque formation and bacterial attachment more difficult[161] and desorb proteins which would adversely affect pellicle formation[162].

i)Water Fluoridation

Four independent clinical trials began between 1945 and 1947 in the United States and Canada to assess the effect of supplementing the communal water supply with fluoride to a level of 1.0 ppm. Neighboring communities with low fluoride (<0.2 ppm) were used as controls. The decrease in DMFT in the studies ranged from 55.5% to 70.1%. As a result, the number of people receiving fluoride in the water supply in the

United States increased rapidly between 1950 and 1955. Approximately 53% of the population of the United States and 35% of Canadians currently have fluoride-supplemented water supplies[157].

Plaque has been shown to concentrate fluoride and in areas of low fluoridation the levels ranged from 6-180 ppm[163]. Dawes *et al.*[164] found that the levels of fluoride were higher in plaques from individuals in fluoridated communities compared with nonfluoridated communities. The plaques from fluoridated communities produced less acid[165] and had fewer numbers of S. mutans[166].

ii)Fluoride Dentifrices

A study by Muhler and colleagues[167] in 1950 was undertaken to determine which fluoride salt was most effective in decreasing enamel solubility. Stannous fluoride was found to be the most effective and was incorporated into a dentifrice by Proctor and Gamble (Crest® toothpaste). A 20-25% reduction in caries (DMFS) was noted over a three year period[168]. Presently all ADA and CDA accepted toothpastes have sodium monofluorophosphate (MFP) or sodium fluoride (NaF) as the active ingredient. Stannous fluoride was replaced due to its labile nature and the staining it caused with prolonged use[157].

iii)Topical Fluoride

A study by Knutson and Scholtz[169] in 1949 demonstrated that topical applications of concentrated fluoride solutions resulted in a 40% reduction in caries. An extensive longitudinal clinical study by Englander *et al.*[170,171] treated groups of schoolchildren with a 0.5% APF, 0.5% neutral NaF or a placebo gel daily for a period of 21 months. They found a caries reduction of 75-80% (DMFS) in both fluoride groups immediately after the test period as well as a profound effect two years later. There was still a 55-63% reduction in DMFS in the fluoride groups compared with the control group. This group recommended APF over NaF due to greater amounts of fluoride incorporated into the enamel of the APF group which represented a reserve for slow

fluoride release[171]. Axelson and Lindhe[172] studied the effect of a 10 minute professionally administered tooth polishing with a 5% MFP paste performed once every two weeks for 20 weeks. The test group had 0.1 new carious surfaces compared to 3.0 new carious surfaces in the control group after one year and this was maintained for a second year.

Loesche *et al.*[173,174] studied a group of institutionalized teenagers that was selected on the basis of the presence of S. mutans in the approximal plaque from the mesial of the mandibular first molar or the pooled plaque from several fissures. The test group received daily 5-minute applications of 1.23% APF gel for two weeks while the control was given a placebo gel. S. mutans decreased in the test group by 70% after one week, 58% after six weeks and 73% after twelve weeks. The S. mutans level in the control group was found to increase at each sampling period. Although *in vitro* tests showed S. mutans, S. sanguis and "S. mitior" equally sensitive to fluoride, *in vivo* S. sanguis levels did not decrease in the test group. An explanation may be due to the ability of S. sanguis to colonize both hard and soft tissue while S. mutans colonizes hard tissue only. S. sanguis would selectively recolonize the teeth first due to higher levels in saliva and soft tissue reservoirs.

A study by Maltz and Emilson[175] assessed the sensitivity of Streptococcus, Lactobacillus and Actinomyces to various fluoride salts. Lactobacilli were the most resistant organisms to all of the fluoride salts tested. Stannous fluoride (SnF_2) and cupric fluoride (CuF_2) were found to have a bactericidal effect at lower concentrations than sodium fluoride (NaF) and ammonium fluoride (NH_4F). At low pH levels, the bactericidal effect of NaF was enhanced while no difference was noted with SnF_2 or CuF_2. Stannous fluoride was found to have a greater antimicrobial effect than the other salts tested.

c)CHLORHEXIDINE

Chlorhexidine is a cationic bisbiguanide that was first synthesized by ICI England in a search for an antimalarial agent. It was found to have low toxicity, high antibacterial activity and it bound strongly to skin[176]. Due to its high therapeutic index, it is one of the safest of antiseptics[177]. Anions such as nitrate, phosphate and chloride readily inactivate chlorhexidine. It is most active in the pH range between 5.5 and 7.0[178] and it exerts its antibacterial effect by disorganizing the cytoplasmic membrane and coagulating intracellular macromolecules[179].

At concentrations of 100 µg/mL, chlorhexidine is bactericidal and has bacteriostatic activity at levels as low as 0.11 µg/mL. Streptococci are the most sensitive of the plaque microflora[180]. The cationic chlorhexidine adsorbs well to negatively charged bacteria with binding occurring at the phosphate groups of lipopolysaccharides and carboxyl groups in protein[181]. The anionic groups of the mucosa and the phosphate, sulphate and carboxyl groups of the salivary glycoproteins and pellicle also provide strong binding sites for chlorhexidine[182] and serve as a reservoir for the slow release of chlorhexidine[183].

A study by Löe and Rindom Schiøtt[184] demonstrated the effectiveness of chlorhexidine in preventing gingivitis and plaque accumulation in the absence of mechanical oral hygiene. A 0.2% mouthrinse used twice daily or a 2.0% mouthrinse daily was found to inhibit plaque formation completely. All subjects using the 2.0% solution developed a yellow-brown stain on their teeth after the 5th day of use. Stain removal was accomplished by routine flossing and brushing. When the mouthrinses were discontinued, plaque began to form at a normal rate after 24 hours.

Löe and colleagues[185] also found that chlorhexidine mouthrinses were able to prevent the formation of white spot lesions. Twenty-four students with healthy gingivae and clean teeth were divided into three groups as follows:

GROUP 1: 8 students who ceased all oral hygiene and rinsed with a 50% sucrose mouthrinse 9 times a day for 22 days.

GROUP 2: 8 students who ceased all oral hygiene and rinsed with 50% sucrose mouthrinse 9 times a day and 2 times a day with 10 mL of 0.2% chlorhexidine gluconate for 22 days.

GROUP 3a): 4 students who ceased all oral hygiene and rinsed with 0.2% chlorhexidine 2 times a day for 22 days.

3b): 4 students who maintained oral hygiene by brushing twice a day for 22 days.

The group that rinsed with sucrose for the experimental period had a significant increase in their Caries Index(186) and developed white spot lesions (facial or lingual smooth surfaces only) while the other groups showed little change. The level of cleanliness and gingivitis in the group that used 0.2% chlorhexidine without oral hygiene procedures was found to be comparable to that in the group that performed meticulous hygiene procedures.

A four month study on the effect of chlorhexidine mouthrinses confirmed the effectiveness of chlorhexidine in preventing the formation of plaque(187). The Plaque Index(188) decreased 66% and the Gingival Index(189) was 24% lower than in the placebo group.

In a 6 month study, children were given 0.2% chlorhexidine mouthrinses 6 times a week. The mean Plaque Indices were 1.24 and 1.54 in the chlorhexidine and placebo groups respectively. They found a more significant difference in gingivitis, with the Gingival Indices 0.15 in the chlorhexidine group and 0.74 in the placebo group(190).

Chlorhexidine has not been very effective when used in a dentifrice[191,192]. Substantivity may have been lost when incorporated into a dentifrice or brushing might have eliminated the reservoir for prolonged release[136].

The long term effects of daily chlorhexidine was examined in an extensive study by Löe, Rindom Schiøtt and colleagues[193-195]. The subjects were medical and dental students. The experimental group of 61 students performed normal hygiene procedures and rinsed daily with a 10 mL of 0.2% aqueous solution of chlorhexidine gluconate during the two year study period. The control group of 59 students used a placebo rinse daily and performed normal hygiene procedures during the period of study. Their results may be summarized as follows:

1)The chlorhexidine group exhibited less plaque and gingivitis but more supragingival calculus during the test period.

2)There was a significantly higher amount of tooth staining in the chlorhexidine group.

3)Levels of salivary bacteria were reduced 30-50% without a detectable shift to Gram-negative organisms.

4)The number of students that had levels of S. mutans in their saliva decreased significantly in the chlorhexidine group.

5)Gram-positive bacteria were found to be more sensitive than Gram-negative bacteria and streptococci more sensitive than staphylococci.

6)Chlorhexidine exerted a selective pressure on the salivary flora resulting in a slight increase in organisms less sensitive to chlorhexidine.

7)No local or systemic effects could be attributed to the use of long-term chlorhexidine treatment. Parameters measured included hemoglobin, methemoglobin, erythrocytes, white blood cells, sedimentation rate, liver and kidney function, urinary glucose and protein.

8)No detectable changes were found in the structure of the oral epithelia between test and placebo groups.

9)There was no significant differences in enzyme activity (lactic dehydrogenase, malic dehydrogenase and glucose-6-phosphate dehydrogenase) between test and placebo groups and it was concluded that long-term exposure to chlorhexidine did not result in a chronic inflammmatory process.

The plaque-inhibiting ability of chlorhexidine after long-term use was assessed by Gjermo and Eriksen[196]. Subjects had used a chlorhexidine dentifrice for 2 years and subsequently discontinued all oral hygiene procedures and rinsed with 10 mL of 0.2% chlorhexidine mouthrinses twice daily for 1 week. They found that previous use of chlorhexidine dentifrices for the 2 year period had not diminished the plaque-inhibiting capacity of chlorhexidine.

Flötra and colleagues[187] found significantly more side effects in their previously-mentioned study. Of the 40 subjects using a daily chlorhexidine mouthrinse in the 4 month study 10 of them developed mucosal lesions and 3 of these were severe. Other side effects included discoloration of the tongue (12/40 test subjects), discoloration of teeth (12% of tooth surfaces) and discoloration of silicate restorations

(62% of restorations). They recommended use of chlorhexidine rinses for short-term use only and that its use should be closely monitored[197].

There is a wide range of sensitivity of microorganisms to chlorhexidine. Using an *in vitro* agar diffusion method, Emilson[198] found that S. mutans, S. salivarius, Propionibacterium, Selenomonas, E. coli and Staphylococcus had low minimum inhibitory concentration (MIC) values, S. sanguis and Lactobacillus had intermediate values while Veillonella, Pseudomonas and Klebsiella had the highest MIC values. A number of clinical studies have demonstrated the sensitivity and long-term suppression of S. mutans after chlorhexidine treatment[199-203]. S. sanguis could be suppressed but returned to pre-treatment levels within 7 days of cessation of treatment[199,202]. In a study that utilized chlorhexidine gel in custom fitted trays, Emilson[200] found that levels of S. sanguis actually increased immediately after chlorhexidine treatment then levelled off. Lactobacillus counts were not changed in those studies that measured their numbers[200,203]. A. viscosus/A. naeslundii were strongly suppressed in clinical studies but returned to pretreatment levels within 7 days[199,202].

The studies that demonstrated that chlorhexidine was not effective against Lactobacillus[200,203] measured salivary levels of lactobacilli in subjects that were treated with a localized application of a chlorhexidine gel. Salivary levels were unaffected due to the fact that all caries lesions were restored prior to chlorhexidine treatment and the gel application method did not eliminate lactobacilli that normally colonize the dorsum of the tongue, vestibular mucosa and the hard palate.

d)CHLORHEXIDINE/FLUORIDE

The compatibility and effectiveness of chlorhexidine and fluoride has been assessed in a number of studies[204-209]. Kirkegaard *et al.*[204] tested a combination of 0.2% chlorhexidine gluconate and 0.2% sodium fluoride that was found to be compatible and did not result in a precipitate of a chlorhexidine-fluoride salt. The presence of

chlorhexidine did not decrease the incorporation of fluoride into enamel and was found to be effective in reducing plaque. A study by Luoma *et al.*[205] combined 0.05% sodium fluoride and 0.022% chlorhexidine acetate into a solution. The Plaque Index was reduced by 44% and the total plaque mass by 38%. Rinses with sodium fluoride solution alone decreased the plaque mass by 41% but had no effect on the Plaque Index. Daily application of a gel containing 0.5% chlorhexidine, 0.2% sodium fluoride and 2.0% methylcellulose completely eliminated S. mutans from plaque after a 3 month test period. In those subjects that initially had S. mutans levels greater than 1% in the plaque samples, S. sanguis levels tended to increase during the test period[206]. Radiation caries was found to be eliminated in patients that received topical applications chlorhexidine/fluoride solutions[207]. In a study on schoolchildren with high DMS scores, a twice daily chlorhexidine-fluoride solution rinse for a 3 day period reduced salivary S. mutans levels by 75%[208]. These levels remained below pretreatment levels for approximately 18 days.

The combination of chlorhexidine and sodium monofluorophosphate was found to be incompatible[209]. An inactive chlorhexidine-monofluorophosphate salt was formed when combined in concentrations that would be effective clinically (0.2% chlorhexidine digluconate and 0.8% sodium monofluorophosphate).

CHAPTER 2

RATIONALE FOR PRESENT STUDY

a)THE EFFECT OF pH ON THE SENSITIVITY OF *LACTOBACILLUS* TO CHLORHEXIDINE AND THE ANTIBIOTICS MINOCYCLINE AND SPIRAMYCIN

Both *S. mutans* and *Lactobacillus* species have been shown to have a close association with the formation and presence of dental caries in humans[1,2,8,15,27,30-37,41,80,90]. Longitudinal studies of the microflora of developing lesions have suggested that there is a succession of *S. mutans* to *Lactobacillus* species[31-33, 35]. In studies of incipient lesions[33] and nursing bottle caries[32] *Lactobacillus* species have been shown to be associated with progression of the incipient lesion to cavitation.

Chlorhexidine has been used extensively in controlling plaque [182,184,185,187,191,193,199,200] and the colonisation of the mouth by *S. mutans* [198,200,201,203,208]. Isolates of the *S. mutans* group are particularly sensitive to chlorhexidine [198]. Apparently, *Lactobacillus* species are much less so [203] and will survive levels of chlorhexidine which are bacteriocidal to *S. mutans*. If it is proposed to use chlorhexidine in attempts to control or eliminate *Lactobacillus* , the concentrations employed may have to be relatively high. Other factors to be considered include the physical parameters of the environment of the organism. If it is intended to eliminate *Lactobacillus* within a specific approximal area with an incipient caries lesion, or prevent colonisation of tooth surfaces in young children at risk of nursing caries, environmental pH could be a significant factor. In both of these instances the pH is likely to be low. Caries lesions can have a pH of 4.0 [9] while nursing caries results, in part, from the almost constant presence of carbohydrate-containing or low pH soft drinks in the child's mouth [32]. The present study will determine the sensitivity of a

range of Lactobacillus species (Type culture strains and fresh isolates) to chlorhexidine at different pH levels. Topical antibiotics could also be considered suitable for localized therapeutic application to eliminate bacteria associated with incipient caries lesions, and two antimicrobials, Minocycline (Lederle Labs, ON) and Spiramycin (Rhône Poulénc, QB) will also be tested for their activity against Lactobacillus.

Thus, the data on the chlorhexidine sensitivity of Lactobacillus species *in vitro* at different pH levels will be used as a basis for determining levels of chlorhexidine employed *in vivo*. Comparisons of the effectiveness of chlorhexidine relative to antibiotics will be used to confirm the superiority of one agent over another.

In the second study b), the effect of the application of the most effective agent *in vivo* will be measured in a small number of human volunteers.

b)THE EFFECT OF CHLORHEXIDINE AND SUPERFLOSS AND SUPERFLOSS ALONE ON APPROXIMAL MICROFLORA

The aim of this clinical trial is to determine the effect of a single local application of Superfloss® (European Health Products, Inc., New Canaan, CT) impregnated with a 2% chlorhexidine solution on the approximal microflora. This will be compared with the effect of Superfloss alone on the approximal microflora. Population shifts among the genera Streptococcus, Veillonella, Actinomyces and Lactobacillus and within the genus Streptococcus will be monitored at baseline, 5 minutes after Superfloss application, on day 3 and day 7. The effect on Lactobacillus species (if detected) and S. mutans in particular will be observed for suppression and subsequent recolonization.

CHAPTER 3

MATERIALS AND METHODS

a)ORIGIN OF SPECIES TESTED

A total of 29 strains of Lactobacillus were tested. Five strains were from the American Type Culture Collection and 24 were freeze-dried isolates from a nursing caries study by Milnes and Bowden[32]. Nineteen strains of Streptococcus isolated from patients with bacterial endocarditis were also included in some of the tests at pH 6.7 in order to provide a basis for comparison of the relative sensitivity of the two genera to chlorhexidine. Four strains of S. mutans (Biotype 1) were included in the tests of chlorhexidine sensitivity at a range of pH from 5.0 to 7.0. Three of the isolates were from the study by Milnes and Bowden[32] and one was a Culture Collection strain (INGBRITT). Their species designation and origins are shown in Table 3.1.

b)BROTH DILUTION METHOD FOR CHLORHEXIDINE SENSITIVITY

The freeze-dried strains of Lactobacillus and Streptococcus were plated on Rogosa[210,211] and blood agar plates (Oxoid CM 271 Blood Agar Base No. 2) supplemented with Vitamin K_1, haemin and 5% defibrinated sheep blood (Atlas Lab., Winnipeg) respectively. After 96 hours growth under anaerobic conditions the strains were subcultured to assure a pure culture. Pure colonies were picked off the plates and a suspension was prepared in 1.0 mL of MRS broth[212] in a Falcon tube (Becton Dickinson, NJ).

The broth dilution method used was based on that of Stalons and Thornsberry[213] and the test medium was MRS broth . This broth gave excellent growth of all of the isolates of Lactobacillus and 1% W/V agar (Difco Lab., MI) was added to this medium for the preparation of plates. The Streptococcus isolates were also tested in MRS broth and all strains grew heavily in the broth without chlorhexidine.

Table 3.1

Species	Number of Strains	Source	Origin
L. casei	3	ATCC[a]	
	4	Own isolates[b]	Nursing caries
L. fermentum	13	Own isolates	Nursing caries
L. acidophilus	2	ATCC	
	1	Own isolates	Nursing caries
L. plantarum	5	Own isolates	Nursing caries
L. brevis	1	Own isolates	Nursing caries
"S. mitior"	6	Dr. T. Louie[c]	endocarditis isolates
S. bovis	3	Dr. T. Louie	endocarditis isolates
S. salivarius	1	Dr. T. Louie	endocarditis isolates
S. faecalis	2	Dr. T. Louie	endocarditis isolates
S. viridans	7	Dr. T. Louie	endocarditis isolates
S. mutans	1	Culture Collection	
	3	Own isolates	Nursing caries

a)American Type Culture Collection
b)Milnes and Bowden, 1985
c)Louie, 1984 unpublished data

MRS broths prepared at pH 7.0, 6.5, 6.0, 5.5, 5.0 and 6.7 had filter-sterilized chlorhexidine gluconate (Ayerst, Winnipeg, MB) added to them to give a range of 1 to 100 µg/mL of chlorhexidine. The broths were inoculated with a standardised inoculum of each test isolate. The inocula were prepared from a 48 hour culture on Rogosa agar (Difco Lab., MI). Cells from the plate were suspended in MRS broth to a density of 150 Klett units, and 20 µL of this suspension was inoculated into 5 mL of test broth. Viable counts of the inocula were also made on MRS agar using a Spiral Plater (Spiral System Inc., Cincinatti, OH). When all of the tests had been run, the count data were examined to see whether the variation in viable counts obviously affected the test results. Plotting the viable counts of the inoculum against chlorhexidine sensitivity for all isolates and individual species showed no relationships. Inoculated tubes were incubated under anaerobic conditions (N_2 80%, CO_2 10%, H_2 10% V/V) in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, MI) for 48 hours. The tubes of broth were examined for growth and the last tube showing visible growth and the first negative tube was recorded. Cultures were made from both tubes and the pH and optical density of the last tube showing growth was measured.

c) TIME FOR KILLING

Chlorhexidine is bactericidal, and it was important to know the time necessary for chlorhexidine to kill a given number of Lactobacillus cells. Cells for the test were taken from a 48 hour culture on MRS agar and suspended in MRS broth at pH 6.7 to give a reading of 50 Klett units. The low optical density inoculum in this experiment was utilized to reduce the total amount of protein present in the media, thereby increasing the effectiveness of chlorhexidine. Counts were made of the original suspension and sterile chlorhexidine was added to produce the minimum inhibitory concentration effective for that organism. The suspension was mixed and a sample was taken immediately (time 0), diluted and plated using a Spiral Plater. Sequential samples were

taken at 2, 4, 6, 8 and 10 minutes thereafter, and plated as before and counted. The number of surviving cells was plotted against time of exposure.

d)AGAR DILUTION METHOD FOR ASSAY OF SENSITIVITY TO ANTIBIOTICS

The method used followed that of Lennette *et al.*(214). Dilutions of two antibiotics Minocycline (Lederle Labs, ON) and Spiramycin (Rhône Poulénc, QB) in a range of 0.25-128 µg/mL were prepared in DST agar (CM. 261 Oxoid Canada). The pH of the medium was adjusted to pH 5.0, 6.0 and 7.4 before autoclaving and the antibiotics were added after autoclaving as filter-sterilized solutions. Minocycline was dissolved in distilled water and Spiramycin in 0.75% dimethyl sulfoxide and distilled water(215). The inocula for the tests were prepared in the same way as those for the broth dilution tests except that the suspensions were adjusted to 250 Klett units for an adequate lawn seed. A series of plates of DST agar of pH 5.0, 6.0 and 7.4 containing dilutions of the antibiotics were inoculated with 20 test isolates using a Steers replicator (216). Inoculated plates were allowed to dry and then incubated in an anaerobic chamber for 48 hours. The lowest concentration of antibiotic which prevented growth was recorded as the minimum inhibitory concentration (MIC). As with the inocula for the broth dilution test, viable counts were made on the inocula for the plate dilution test.

e)PILOT STUDY OF THE EFFECT OF CHLORHEXIDINE ON APPROXIMAL MICROFLORA

i)INTRODUCTION

Both incipient caries lesions and caries-free sites in children and adults were included in the study in order to assess the effect of chlorhexidine on a range of microflora. The object was to determine the effect of a single local application of 2% chlorhexidine when applied by means of Superfloss® (European Health Products, Inc., New Canaan, CT) on the approximal microflora and monitor the microbiology over a period of 7 days. This was compared with the effect of Superfloss alone on the

approximal microflora. If Lactobacillus species and S. mutans could be sufficiently suppressed or eliminated and the sites recolonized by a non-cariogenic flora, demineralization of the site might be prevented.

ii)SUBJECT GROUP

A total of 10 subjects were included in this study. Five subjects were adults between the ages of 25 and 33 years of age. Five subjects were children between the ages of 4 and 16 years of age. The subjects were accepted into the study if

1)no antibiotics had been taken in the 6 months prior to the study.

2)they had unrestored approximal sites without cavitation on the same surface of the same posterior teeth on opposite sides of the mouth.

Four of the 5 children in the study were patients in the Pediatric Dentistry program at the Faculty of Dentistry, University of Manitoba. These patients were chosen to test the effectiveness of chlorhexidine on the microflora of incipient caries lesions. The nature of the study was explained to the subjects and parents of the subjects where appropriate and an informed consent was obtained (Appendix A).

iii)METHODS

Clinical studies have shown concentrations of 5% chlorhexidine to be safe and effective in reducing plaque microflora[199,202]. Due to the effectiveness of lower concentrations of chlorhexidine in suppressing Lactobacillus species and Streptococcus species (Chapter 3), and clinical studies that have shown suppression of plaque microflora[184,185,187,191-196,200,201,206-208,217-219], a 2% solution of chlorhexidine was used for this study. The 2% aqueous solution of chlorhexidine gluconate was prepared within 24 hours of its use on the test site for each subject.

The subjects were instructed not to perform any oral hygiene procedures for the 24 hour period preceeding the first samples. During the 7 day test period, subjects brushed their teeth but did not floss.

a)SAMPLING DEVICE

The method for taking approximal plaque samples from the test and control sites was that used by Bowden *et al.*[81]. Abrasive metal strips (Lightning Strips, Moyco Industries, Philadelphia, PA) were tapered to a point with the working end approximately 1 mm by 8 mm in size. The abrasive strips, gauze and scissors were sterilized prior to sampling in a Harvey's Chemiclave (MDT Corp., Torrance, CA) at 132^{o} C for 20 minutes at 20 p.s.i. The test and control sites were isolated and dried with sterile gauze prior to sampling. The abrasive strip was placed interproximally just cervical to the contact point with the abrasive side towards the site to be sampled. The strip was moved back and forth against the tooth 6 times and then removed. The smooth side of the strip was wiped on sterile gauze to assure that the plaque was from the test site and not from the adjacent tooth. The working tip of the strip was cut off with sterile scissors and immediately placed into 1 mL of sterile reduced transport fluid (RTF) [220] to prevent loss of anaerobes.

b)APPLICATION OF CHLORHEXIDINE

The chlorhexidine was applied to the test sites by means of Superfloss. The sponge-like nylon section of Superfloss was soaked in the 2% aqueous chlorhexidine solution and left in contact with the test site for a period of 5 minutes. The control site was treated with plain Superfloss which was left in place for the same 5 minute period. Superfloss was used because the chlorhexidine could be applied specifically to the test site and it is effective in removing plaque accumulations[221].

Samples were taken from the test and control sites at the following times:

1)Day 0 at 9:00 a.m.

5 minute application of Superfloss/chlorhexidine to the test site and plain Superfloss to the control site.

2)Day 0 immediately after Superfloss application.

3)Day 0 at 5:00 p.m.

4)Day 3 at 9:00 a.m.

5)Day 7 at 9:00 a.m.

c)MICROBIOLOGICAL ANALYSIS

i)CULTURING OF SAMPLES

The samples in RTF were sonicated for 30 seconds using a Vortex Jr. Mixer (Scientific Ind. Inc., NY) within one hour of taking the samples. Serial dilutions of 1:10, 1:100, 1:1000 and 1:5000 were prepared in RTF for each and plated by means of the Spiral Plater. The samples were plated on blood agar (in duplicate for aerobic and anaerobic growth); TYC[222] with 0.2 units/mL Bacitracin[33,223]; Rogosa agar[210,211] (Difco Lab.) and Maconkey agar[224] (BBL, Div. Becton, Dickinson & Co., MD). The cultures grown aerobically (Maconkey and blood agar plates) were incubated at 37° C for 72 hours while the cultures grown anaerobically (TYB, Rogosa and blood agar plates) were incubated at 37° for 96 hours.

ii)COUNTING COLONIES

Counts were made on those plates where the dilutions afforded 50-200 colonies using a "cookie cutter" (Spiral System Inc., Cinncinnati, OH) to delineate defined areas of the media[31]. The total cultivable flora was estimated from the blood agar plates incubated anaerobically. The number of colony-forming units (CFU) for each identified organism was then expressed as a percentage of the total cultivable flora.

iii)GROUPING OF ISOLATES

The strains were initially divided into groups based on cellular morphology, atmospheric requirements, catalase production and Gram-staining[220](Table 3.2).

1)CELLULAR MORPHOLOGY

The isolates were identified as cocci or rods/filaments using light microscopy.

2)ATMOSPHERIC REQUIREMENTS

Aerobic growth was assessed on blood agar plates incubated at 37° for 72 hours and anaerobic growth on blood agar plates in an anaerobic chamber at 37° for 96 hours.

3)GRAM-STAIN

Lillie's or Hucker's modification of this stain was used to divide the isolates into Gram-positive and Gram-negative groups [225, 226].

4)CATALASE PRODUCTION

The method used for assessing the production of catalase was that of Cowan and Steel[227]. Bubbling of the sample within 5 seconds of applying a drop of 30% H_2O_2 (Fisher Sci. Co., NJ) was indicative of a positive result.

iv)IDENTIFICATION OF SPECIES

Further tests were limited to the genera Streptococcus, Lactobacillus, Actinomyces and Veillonella.

a)Streptococcus

The tests were based on those used by Kilian *et al.*[125] (Table 3.3 and 3.4).

i)Carbohydrate fermentation

The medium used to assess carbohydrate fermentation consisted of 2% proteose peptone, 0.5% yeast extact, 0.5% sodium chloride, 0.1% disodium phosphate, 1.5% Bacto-Difco agar, and 0.002% bromo-cresol purple[227]. The test carbohydrates were added as filter-sterilized solutions to this in a concentration of 0.5% w/v. The carbohydrates tested were mannitol, sorbitol, raffinose, melibiose, trehalose, amygdalin and inulin.

Table 3.2

GROUPING OF ISOLATES

MORPHOLOGY	GRAM STAIN	CATALASE	AEROBIC GROWTH	ANAEROBIC GROWTH	GENUS
COCCUS	+	-	+	+	Streptococcus
	+	+	+	+	Staphylococcus Micrococcus
	-	+	+	+ / -	Neisseria
	-	+ / -	-	+	Veillonella
ROD/FILAMENT	+	-	+	+	Lactobacillus Actinomyces
	+	+	+	+	Actinomyces Rothia Corynebacterium
	+	+	-	+	Actinomyces Eubacterium
	-	-	-	+	Bacteroides Capnocytophaga Fusobacterium

Table 3.3

Identification of *Streptococcus* isolates[a)]

SPECIES	GRAM	CATALASE	ARGININE	ESCULIN	MANNITOL	SORBITOL	RAFFINOSE	MELIBIOSE	TREHALOSE	INULIN	TYC AGAR	H2O2 PROD
S. salivarius	+	-	-	+	-	-	-	-	+ / -	+ / -	H/S	-
"S. milleri"	+	-	+	+	-	-	-	-	+	+ / -	S[b)]	-
S. sanguis												
Taxon												
1	+	-	+	+	-	-	+	+	+	+	H[c)]	+
2	+	-	+	-	-	-	+ / -	-	+	+	H	+
3	+	-	+	+	-	-	-	-	+	+ / -	H	+
4	+	-	+	+	-	-	+	+	+	+	H	+
5	+	-	+	+	-	-	-	-	+	+	H	+
6	+	-	+	+	-	-	-	-	+	+	H	+
"S. mitior"												
Taxon												
1	+	-	-	-	-	-	+ / -	-	-	-	H/S	+
2	+	-	-	-	-	-	+ / -	-	-	-	S	+
3	+	-	+	-	-	-	+	+	-	-	S	+
S. mutans												
S. mutans	+	-	-	+	+	+	+	+	+	+	H	-
S. rattus	+	-	+	+	+	+	+	+	+	+	H	-
S. ferus	+	-	-	+	+	+	-	NT[d)]	NT	NT	H	-
S. cricetus	+	-	-	+ / -	+	+	+	+ / -	+	+ / -	H	-
S. sobrinus	+	-	-	+ / -	+	+	+ / -	+ / -	+ / -	+ / -	H	+

a) adapted from Kilian *et al.*(1985) and Hardie (1986)
b) S= soft colony on TYC agar
c) H= hard colony on TYC agar
d) NT= not tested

Fermentation was evident where the plates changed color from purple to yellow which indicated a pH change from 7.6 to 5.5.

ii)Esculin Hydrolysis

Isolates were tested for the hydrolysis of esculin on esculin agar plates[227]. A positive test was indicated by a blackening of the medium around the colonies.

iii)Production of H_2O_2

Colonies were picked off the blood agar plates and placed onto a moistened reagent strip (Peroxid-Test, Merckoquant strips, Merck, Darmstadt Germany). A positive test result was indicated by the strip turning blue.

iv)Arginine Hydrolysis

Hydrolysis of arginine to ammonia was tested by growing each strain in arginine broth[228] which was followed by the addition of Nessler's reagent (BDH Chemical, England). A positive test was indicated by an immediate bright orange color which disappeared in a few seconds after the addition of Nessler's reagent.

v)Production of Extracellular Polysaccharides

The Streptococcus isolates were grown on TYC plates[222]. Those strains capable of producing extracellular glucosyl transferases, which result in the formation of glucans and levans from sucrose, form characteristic colonies on sucrose plates. The colonies that are formed are adherent and are hard and rubbery in consistency. The strains were grown under anaerobic conditions for 48 hours at 37° C and examined under a dissecting microscope.

Isolates were grouped into different patterns based on the above tests and freeze-dried. Representatives from each of these patterns had all of the above characterization tests repeated to assure accuracy and were further identified by the following tests.

vi)Carbohydrate fermentation

The same basal medium described earlier was used and the carbohydrates tested were salicin, arbutin, dulcitol, D-xylose, α-methyl-D-glucose and inositol (Table 3.4).

vii)Growth and acid production on NaF agar plates

The medium consisted of 3.0% tryptone soy broth, 0.50% yeast extract, 0.1% glucose, 0.002% bromo-cresol purple and 1.5% Bacto-Difco agar. The pH was adjusted to 6.5 before autoclaving. After separate autoclaving of the agar in 200 mL of distilled water and the remainder of the components in 800 mL of distilled water they were combined. 4.0 mL of laked blood was added to the medium with either 10 or 50 ppm of sterile NaF. Growth was assessed by examination under a dissecting microscope and acid production by a yellowing of the medium surrounding the colonies.

viii)Enzyme activity tests

API ZYM tests (API Laboratory Products Ltd.,St. Laurent, QB) were used to assess activity of a group of glycoside hydrolases, aminopeptidases and proteases to characterize S. sanguis and "S. mitior". The enzymes tested include alkaline phosphatase, esterase, esterase lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, acid phosphatase, α-galactosidase, ß-galactosidase, ß-glucuronidase, α-glucuronidase, ß-glucosidase, N-acetyl-ß-glucosidase, α-mannosidase and α-fucosidase (Table 3.4).

b)Lactobacillus

Organisms which were Gram-positive, catalase-negative rods were subcultured onto Rogosa plates. This is a selective media for the genus Lactobacillus. The cultures were grown anaerobically for 48 hours. Those organisms able to grow aerobically and anaerobically were identified as belonging to the genus Lactobacillus and a further series of tests as described by Bowden *et al.*[30] (Table 3.5) were used for identification to the level of species. The strains were tested for fermentation of cellobiose, mannitol,

Table 3.4

Characterization of S. sanguis and "S. mitior"[a)]

	S. sanguis Taxon						"S. mitior" Taxon		
	1	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**
Salicin	+	+/-	+	+	+	+	-	-	+/-
α-L-fucosidase	-	-	-	+	+	+	-	-	-
ß-D-lactosidase	-	-	-	+	+	+	-	-	+
Alkaline Phosphatase	-	-	-	+	+	+	+	+	+
α-D-galactosidase	+	+	-	+	+	-	+/-	+/-	+
Acid Phosphatase	+	-	+	+	+	+	+	+	+
ß-D-fucosidase	-	-	-	-	-	-	+	+	+

a) adapted from Kilian *et al.*(1985).

Table 3.5

Simple Screening System for the identification of Lactobacillus Isolates a)

STRAIN	GAS PRODUCTION		ACID FROM	HYDROLYSIS OF	ACID FROM				
	GLUCOSE	GLUCONATE	RIBOSE	ESCULIN	CELLO	MANN	MELIB	RAFF	MELIZ
THERMOBACTERIUM									
L. acidophilus	−	−	−	+	+	−			
L. salivarius	−	−	−	−	−	+			
STREPTOBACTERIUM									
L. casei	−	+	+				−	−	
L. plantarum	−	+	+				+	+	
BETABACTERIUM									
L. fermentum	+	+	+	−	−				−
L. cellobiose	+	+	+	+	+				−
L. buchneri	+	+	+	+	−				+
L. brevis	+	+	+	+	−				−

a)from Bowden *et al.* (1976).

melibiose, raffinose and melezitose. The basal media was a lactobacilli sugar base to which 2% (w/v) of the carbohydrate was added. Bromocresol purple was added as an indicator of fermentation. Production of acid turned the cultures yellow. Ribose and esculin broths were filter sterilized. The other carbohydrates were autoclaved for 20 minutes at 115° C (Barnstead Lab Sterilizer, Sybron Corp., Manitoba) and added to sterile broth. Sodium gluconate and glucose broths were used to assess gas production. All cultures were incubated anaerobically for 48 hours at 37° C. Hydrolysis of esculin occurred when the broths turned black upon the addition of 1% w/v ferric chloride.

c)Actinomyces

Gram-positive, catalase-positive rods and Gram-positive, catalase-negative rods that did not grow on Rogosa media were included in whole cell agglutination tests[229-235]. Antisera to A. odontolyticus (serotype I, NCTC 9335), A. viscosus (serotype II, WVU 627) and A. naeslundii (serotype I, ATCC 12104) were prepared in rabbits and used in the agglutination tests[229,235]. Actinomyces viscosus and Actinomyces naeslundii were differentiated by means of the catalase test and whole cell agglutination (Table 3.6) A suspension of cells were prepared on a sterile, glass slide in saline. One drop of a 1/10 dilution of the antiserum was added and the slide was rocked back and forth. Examination of the slide by means of a dissecting microscope determined whether or not agglutination had occurred.

d)Veillonella

Gram-negative anaerobic cocci were tested for the production of acid in 1% glucose broth. The pH was checked after incubation for 48 hours under anaerobic conditions at 37° C. Lack of acid production indicated that these isolates were in the genus Veillonella. End-product analysis was performed by gas-liquid chromatography after culturing in peptone broth with 0.5% sodium lactate at 37° C for 72 hours[236]. Veillonella produced acetate and propionate from lactate.

Table 3.6

Characterization of Actinomyces Isolates[a)]

Strain	Catalase	A. viscosus (WVU 627)	A. naeslundii (ATCC 12104)	A. odontolyticus (NCTC 9335)
A. viscosus				
1)Typical	+	+	-	-
2)Atypical	-	+	-	-
A. naeslundii				
1)Typical	-	-	+	-
2)Atypical	+	-	+	-
Intermediate Strains				
	-	+	+	-
	+	+	+	-
A. odontolyticus	+ / -	-	-	+

a) from Bowden (personal communication)

CHAPTER 4

RESULTS

a)SENSITIVITY OF LACTOBACILLUS AND STREPTOCOCCUS ISOLATES TO CHLORHEXIDINE AT pH 6.7

In all cases, the broth dilution test gave unequivocal results. Repeat tests on the same organism showed that the reproducability was 90% or better with only two isolates of the twenty-seven showing a variation of one tube dilution. The bactericidal effect of chlorhexidine on Lactobacillus species was evident. Cultures from the first tube without growth were negative in 57/62 tubes tested. The pH levels of the last tubes with growth are shown in Table 4.1. In general, the pH was low, ranging from 4.6-5.9; however, one L. casei and two L. plantarum isolates did not lower the pH beyond 6.4. The numbers of viable cells in 1 mL of inoculum ranged from $3.5x10^2$ to $2.6x10^8$.

Nineteen strains of Streptococcus were tested for their sensitivity to chlorhexidine at pH 6.7 by the broth dilution test (Table 4.2). Thirteen of the strains were sensitive to levels of chlorhexidine between 1-4 µg/mL. These included isolates of S. bovis, "S. mitior", S. faecalis and S. salivarius. Three strains were sensitive to levels of chlorhexidine between 4-10 µg/mL, two strains between 10-20 µg/mL and only one strain survived 20 µg/mL. The latter strain was identified as an enterococcus.

Figure 4.1 shows the levels of chlorhexidine necessary to prevent growth of different Lactobacillus strains at a pH of 6.7. Strains of L. casei and L. plantarum and the single fresh isolate of L. acidophilus were the most resistant while isolates of L. fermentum and L. brevis were equivalent in their sensitivity. Some intraspecies variation was evident, for example, with strains of L. fermentum varying in sensitivity from 2-20 µg/mL of chlorhexidine.

Table 4.1

In vitro sensitivity of Lactobacillus species to chlorhexidine at pH 6.7

SPECIES	CHLORHEXIDINE SENSITIVITY µg/mL	FINAL pH	KLETT O.D.[a]	SPIRAL PLATE COUNT
L. fermentum				
DS 366	2.0	5.4	105	4.80[b]
CH 268	4.0	5.5	73	11.00
CH 370	10.0	5.5	78	TNTC[c]
CH 324	10.0	5.5	252	0.04
DS 274	2.0	5.6	104	5.40
KT 276	4.0	5.8	166	11.90
DSM 6	2.0	5.4	219	0.70
CH 273	10.0	4.7	79	0.10
DS 264	2.0	5.3	68	1.85
KT 287	20.0	5.9	85	1000.00
LB 273	20.0	5.5	77	2.40
JG 193	10.0	5.5	52	1.44
DS 338	2.0	5.4	85	TNTC
L. casei				
CH 418	40.0	4.8	154	735.00
CH 444	20.0	4.8	266	3.99
CH 290	10.0	5.3	47	4.80
ATCC 15008	20.0	4.6	218	25,900.00
ATCC 4646	40.0	4.6	137	10,100.00
ATCC 393	60.0	6.5	103	TNTC
L. acidophilus				
CH 267	60.0	4.8	72	5.29
ATCC 521	10.0	4.6	43	0.15
ATCC 4356	20.0	4.6	45	1.04
L. plantarum				
JG 188	40.0	4.8	210	2,700.00
CH 320	40.0	6.4	163	4,400.00
CH 464	40.0	6.4	124	46.00
CH 374	40.0	4.8	288	TNTC
L. brevis				
KT 239	10.0	5.5	165	12.80

a)O.D.,Optical Density of last tube showing growth
b)$\times 10^4$
c)TNTC, too numerous to count

Table 4.2

In vitro sensitivity of Streptococcus species to chlorhexidine at pH 6.7

SPECIES	CHLORHEXIDINE SENSITIVITY µg/mL[a)]
"S. mitior"	
SF 924	4.0
SF 939	4.0
G 27	1.0
G 534	20.0
G 721	1.0
BM 205	4.0
S. bovis	
PB 236(82)	1.0
346	1.0
G 162	4.0
S. faecalis	
347	NI[b)]
470	4.0
S. salivarius	
666	1.0
S. viridans	
8937	10.0
544	10.0
PB 219	20.0
PB 236(83)	10.0
PB 237	1.0
G 1163	1.0

a)standardized at an absorbance of 150 Klett Units
b)the maximum concentration of chlorhexidine that was used to test the Streptococcus strains was 20 µg/mL

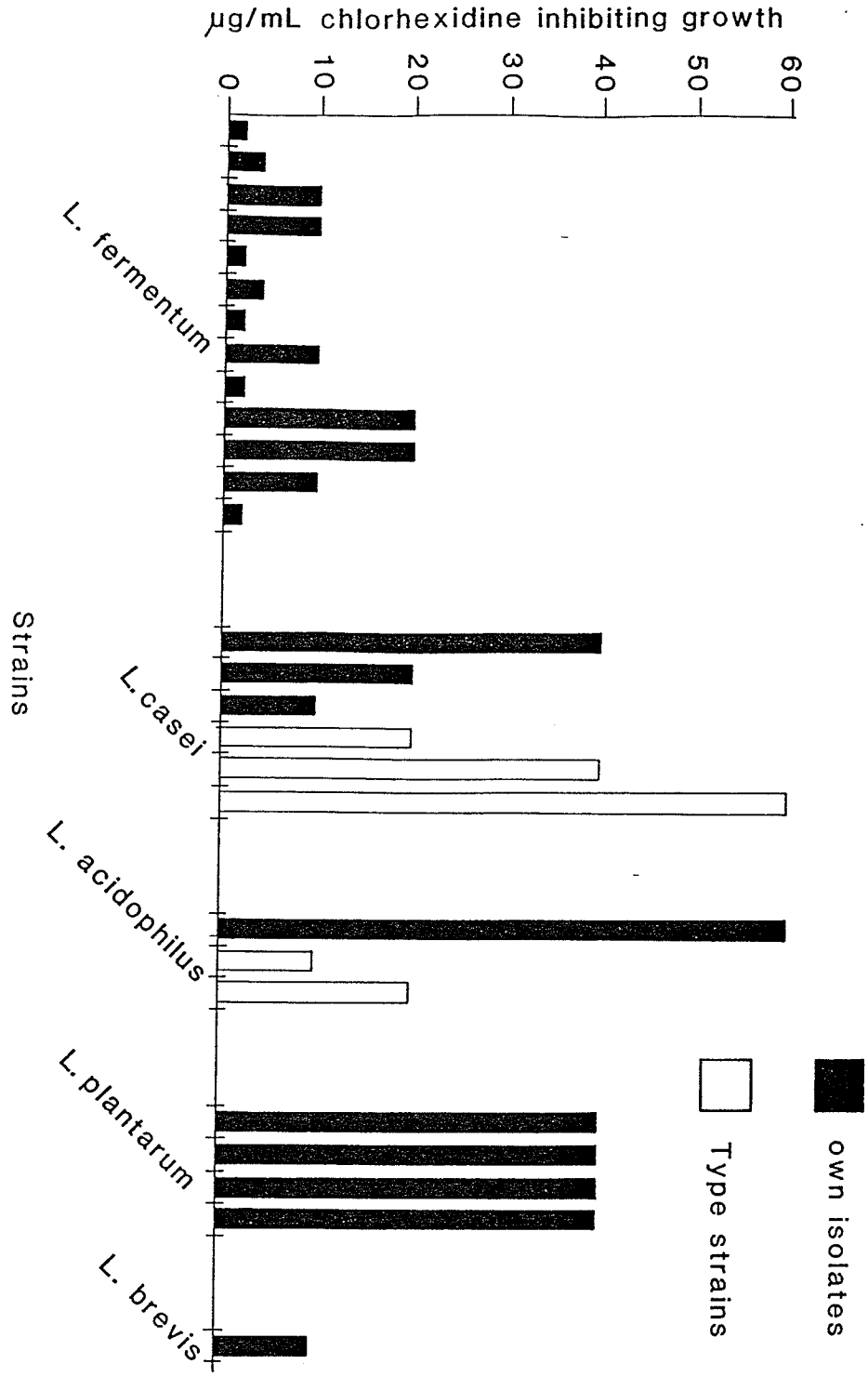


Figure 4.1

In vitro sensitivity of Lactobacillus species to chlorhexidine at pH 6.7

b)EFFECT OF pH ON THE SENSITIVITY OF *LACTOBACILLUS* AND *STREPTOCOCCUS* ISOLATES TO CHLORHEXIDINE

The mean values of chlorhexidine necessary to inhibit growth and kill selected *Lactobacillus* and *Streptococcus* strains in media of different pH are shown in Figure 4.2. In all cases chlorhexidine became less effective as the pH was lowered. Although strains of *L. casei* were initially more resistant, only one strain (ATCC 393) was able to survive beyond 60 µg/mL of chlorhexidine at pH 5.0. However, 4 of 5 strains of *L. plantarum*, which showed a similar resistance to *L. casei* at pH 7.0, survived 80 µg/mL of chlorhexidine at pH 5.5. Strangely, *L. plantarum* isolates (JG 188, CH 320 and CH 464) were more sensitive at pH 5.0 when they resembled *L. casei* and were inhibited by 60 µg/mL of chlorhexidine. The most dramatic effect of the environmental pH was seen with the single strain of *L. brevis*, which was originally in the more sensitive group. At pH 5.0 this strain required 80 µg/mL of chlorhexidine to inhibit growth.

Six strains of *L. fermentum* were sensitive to less than 4 µg/mL of chlorhexidine at pH 7.0, and in all but two strains this level was raised to a minimum of 10 µg/mL at pH 6.0 (Table 4.3). In contrast to the *Lactobacillus* strains, the four isolates of *S. mutans* tested at different pH levels showed little change in sensitivity with the mean MIC's between 3.5 µg/mL (pH 7.0) to 1.5 µg/mL (pH 5.0). It is possible that *S. mutans* became more sensitive as the pH was lowered.

c)KILLING TIME EXPERIMENT

Table 4.4 shows a loss of numbers of viable cells upon exposure to the MIC of chlorhexidine. It was apparent that cells varied in their resistance over the short time period tested. The effectiveness of chlorhexidine was obviously dependent on the number of cells present. For several strains, the MIC of chlorhexidine did not have a dramatic effect on the number of viable cells (*L. casei* KT 238, CH 444, *L. plantarum* CH320, CH 374, *L. brevis* KT239). The highest numbers of viable cells that were added in the broth dilution tests at pH 6.7 was 5.2×10^6 cells/mL.

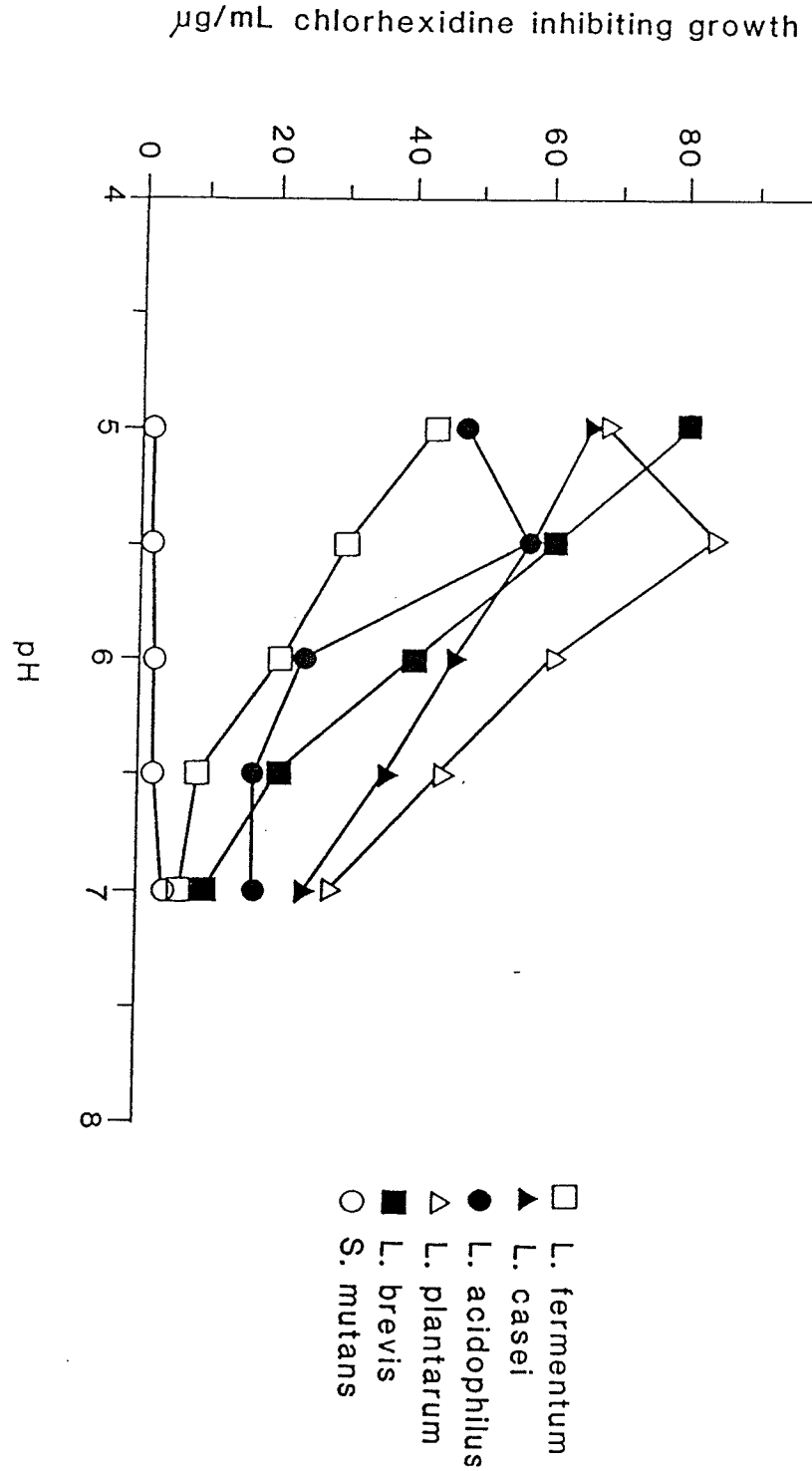


Figure 4.2

Effect of environmental pH on the sensitivity of Lactobacillus and Streptococcus to chlorhexidine

Table 4.3

The effect of environmental pH on the sensitivity of Lactobacillus and Streptococcus to chlorhexidine

SPECIES	CONCENTRATION OF CHLORHEXIDINE µg/mL INHIBITING GROWTH				
pH	7.0	6.5	6.0	5.5	5.0
L. fermentum					
DS 366	2.0	4.0	20.0	10.0	80.0
CH 268	4.0	10.0	10.0	20.0	20.0
CH 370	10.0	20.0	40.0	60.0	80.0
CH 324	10.0	10.0	20.0	40.0	40.0
DS 274	2.0	4.0	10.0	10.0	20.0
KT 276	10.0	20.0	40.0	60.0	60.0
DSM 6	2.0	2.0	2.0	10.0	10.0
CH 273	2.0	10.0	10.0	20.0	60.0
DS 264	2.0	2.0	4.0	20.0	10.0
KT 287	10.0	20.0	40.0	60.0	80.0
LB 273	2.0	10.0	10.0	40.0	20.0
JG 193	20.0	20.0	60.0	40.0	60.0
DS 338	4.0	2.0	2.0	4.0	20.0
L. casei					
CH 418	20.0	60.0	60.0	80.0	60.0
KT 238	20.0	40.0	40.0	40.0	60.0
CH 444	20.0	40.0	60.0	80.0	60.0
CH 290	10.0	10.0	20.0	20.0	40.0
ATCC 15008	40.0	40.0	60.0	60.0	60.0
ATCC 4646	40.0	40.0	40.0	60.0	60.0
ATCC 393	20.0	20.0	40.0	60.0	100.0
L. acidophilus					
CH 267	40.0	40.0	60.0	NI[a)]	NI
ATCC 521	2.0	2.0	2.0	10.0	2.0
ATCC 4356	10.0	10.0	10.0	60.0	40.0
L. plantarum					
JG 188	40.0	40.0	60.0	80.0	60.0
CH 320	20.0	40.0	60.0	80.0	40.0
CH 464	20.0	40.0	40.0	60.0	40.0
CH 374	20.0	40.0	60.0	100.0	100.0
LB 282	40.0	60.0	80.0	100.0	100.0
L. brevis					
KT 239	10.0	20.0	40.0	60.0	80.0
S. mutans					
INGBRITT	2.0	2.0	2.0	2.0	2.0
BM 52	4.0	2.0	2.0	2.0	2.0
BM 127	4.0	2.0	2.0	2.0	2.0
BM 94	4.0	2.0	2.0	2.0	2.0

a)NI, not inhibited

Table 4.4

Time for killing of selected Lactobacillus by chlorhexidine

Species	Chlorhexidine µg/mL	Initial count	Time of exposure (minutes) 0	2	4	6	8	10
L. casei								
KT 238	20.00	0.60[a)]	0.56	0.46	0.28	0.64	0.52	0.40
ATCC 393	60.00	16.80	6.00	5.70	4.20	5.50	4.00	2.70
CH 444	20.00	2.00	2.60	2.70	2.90	2.70	2.50	2.00
L. fermentum								
JG 193	10.00	8.50	1.70	1.60	3.40	2.90	4.00	4.40
CH 273	10.00	1.40	0.01	0.00	0.00	0.00	0.00	0.00
CH 268	4.00	200.00	6.10	5.80	2.50	2.50	1.90	1.20
L. plantarum								
CH 320	10.00	6.00	5.40	7.60	7.00	1.60	2.20	1.70
CH 374	40.00	8.00	5.80	4.80	5.60	6.40	4.60	3.60
L. acidophilus								
CH 267	60.00	0.08	0.03	0.02	0.04	0.01	0.01	0.00
L. brevis								
KT 239	10.00	0.70	0.25	0.23	0.10	0.05	0.10	0.05

a)viable counts $x10^6$/mL

Consequently, only with L. fermentum (CH 268) did the number of cells added in the killing test greatly exceed that used for the measurement of the MIC.

d)SENSITIVITY OF LACTOBACILLUS ISOLATES TO ROXAMYCIN AND MINOCYCLINE

Tables 4.5 and 4.6 shows the sensitivity of twenty isolates of Lactobacillus to Spiramycin and Minocycline at pH 5.0, 6.0 and 7.4. Spiramycin was more affected by low pH than Minocycline, as all strains were resistant to up to 128 µg/mL of Spiramycin at pH 5.0. In general, Minocycline was not significantly affected by a lowering of the environmental pH. However, some strains were relatively resistant to this antibiotic (one strain of L. casei and one strain of L. plantarum). At pH 7.4 Spiramycin was generally more effective than Minocycline.

Table 4.5

CONCENTRATION OF SPIRAMYCIN µg/mL INHIBITING GROWTH

SPECIES	pH 5.0	pH 6.0	pH 7.4
L. fermentum			
DS 366	NI[a)]	16	0.25
CH 324	NI	16	0.25
DS 274	NI	16	0.25
DSM 6	NI	32	0.25
DS 264	NI	32	1
LB 273	NI	16	0.25
JG 193	NI	32	2
DS 338	NI	32	1
L. casei			
KT 238	NI	32	2
CH 290	NI	16	2
ATCC 393	NI	32	4
L. acidophilus			
CH 267	NI	32	2
ATCC4356	NI	16	0.25
L. plantarum			
JG 188	NI	32	0.25
CH 320	NI	32	2
CH 464	NI	32	2
CH 374	NI	8	0.25
LB 282	NI	16	2
CH 270	NI	32	1
L. brevis			
KT 239	NI	16	0.25

a)NI, no inhibition

Table 4.6

CONCENTRATION OF MINOCYCLINE µg/mL INHIBITING GROWTH

SPECIES	pH 5.0	pH 6.0	pH 7.4
L. fermentum			
DS 366	16	8	16
CH 324	8	8	16
DS 274	16	16	16
DSM 6	8	8	16
DS 264	16	8	8
LB 273	16	8	16
JG 193	16	16	16
DS 338	16	16	16
L. casei			
KT 238	2	1	2
CH 290	64	32	16
ATCC 393	8	4	8
L. acidophilus			
CH 267	4	4	4
ATCC4356	2	2	2
L. plantarum			
JG 188	64	64	32
CH 320	8	4	8
CH 464	2	1	2
CH 374	2	0.5	2
LB 282	2	1	2
CH 270	8	8	8
L. brevis			
KT 239	16	16	16

e)EFFECT OF CHLORHEXIDINE/SUPERFLOSS AND SUPERFLOSS ON APPROXIMAL MICROFLORA

i)SAMPLES RETURNING TO PRETREATMENT LEVELS

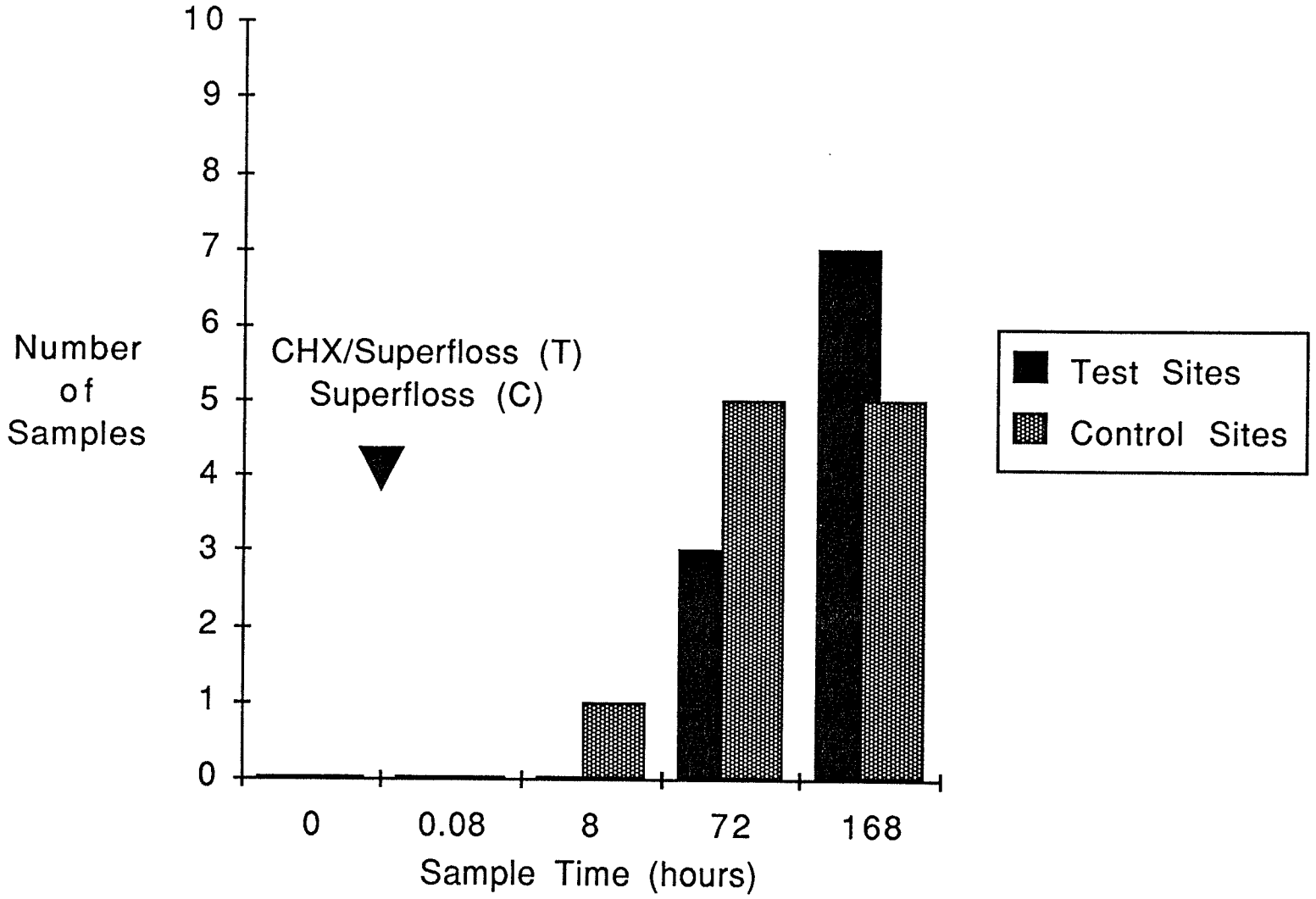
The flora of the control sites returned to pretreatment levels earlier than that of the test sites at 72 hours after treatment with Superfloss or chlorhexidine/Superfloss respectively (Figure 4.3). However, Figures 4.4 and 4.5 show the data indicating recovery to pretreatment levels of each of the control and test sites up to 168 hours. At this time 8/10 test sites and 5/10 control sites had recovered to pretreatment levels. Thus, the recovery of the test sites exceeded that of the control sites at 168 hours contrasting with the results at 72 hours. This difference may be explained by the bactericidal effect of chlorhexidine on the microflora of the test sites. Actinomyces were suppressed by chlorhexidine and did not return to pretreatment levels even after 168 hours. Without competition from Actinomyces, Streptococcus species were able to recolonize and become a dominant member of the microflora (Figures 4.6 and 4.7).

ii)RATIO OF GENERA AT THE TEST AND CONTROL SITES

Figures 4.6 and 4.7 show the distribution of the identified genera at the control and test sites respectively. The most notable difference between the control and test sites is that at control sites Actinomyces, Veillonella and Gram-negative anaerobic rods persisted in approximately the same proportion to the other genera throughout each of the five sampling times. As previously mentioned, Actinomyces species were almost completely eliminated from the test sites immediately after treatment with chlorhexidine/Superfloss. They recovered very slowly and were replaced by Streptococcus species. Although Veillonella and Gram-negative anaerobic rods comprise a small percentage of the total cultivable flora they were eliminated from the test sites and were not isolated until the 72 hour and 168 hour sample respectively. The Streptococcus species recolonized more readily and were the dominant genera throughout the test period. No lactobacilli were detected.

Figure 4.3

Number of Samples Returning to Pretreatment levels



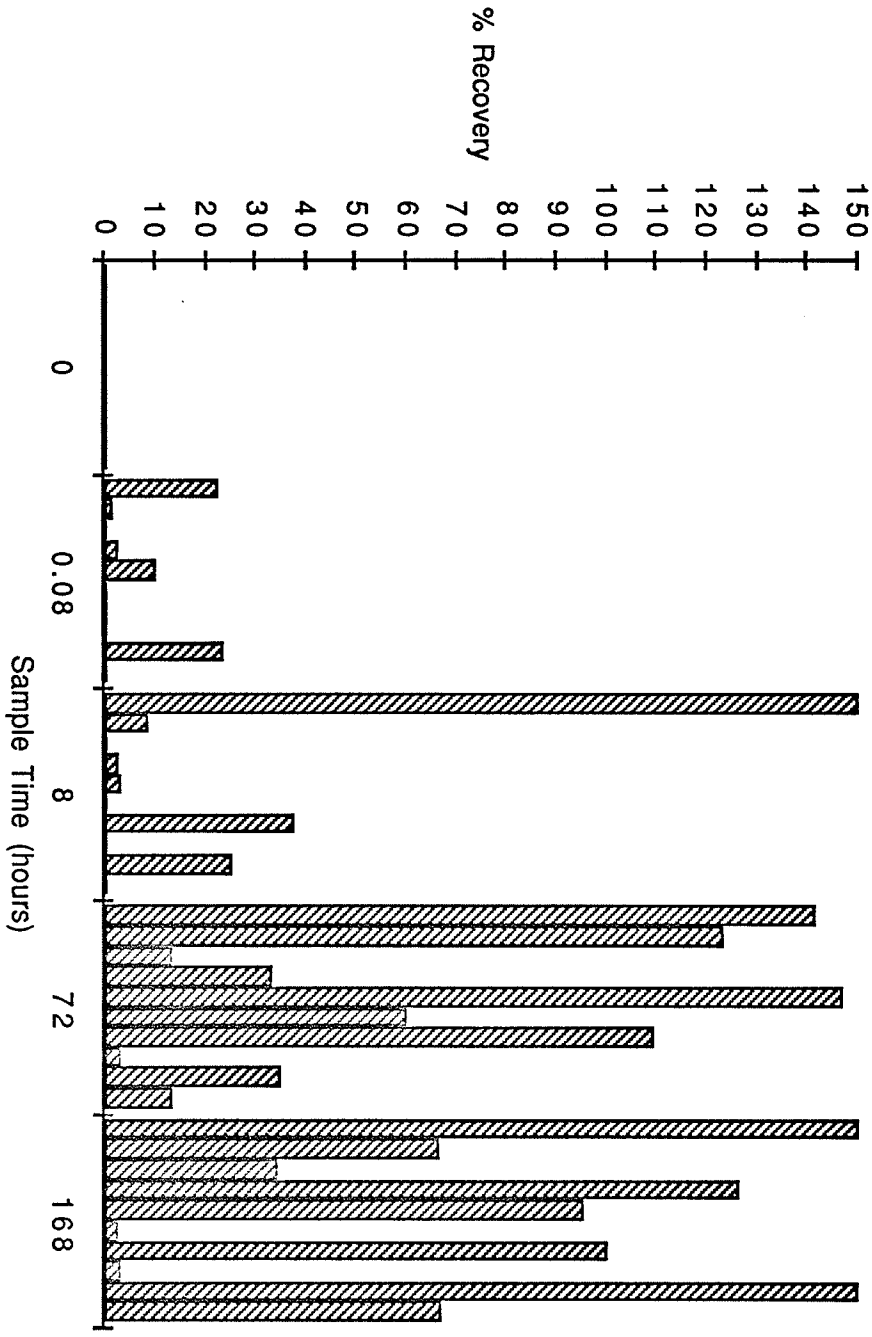


Figure 4.4

% Recovery of Total Cultivable Flora (Control Sites)

Figure 4.5

% Recovery of Total Cultivable Flora (Test Sites)

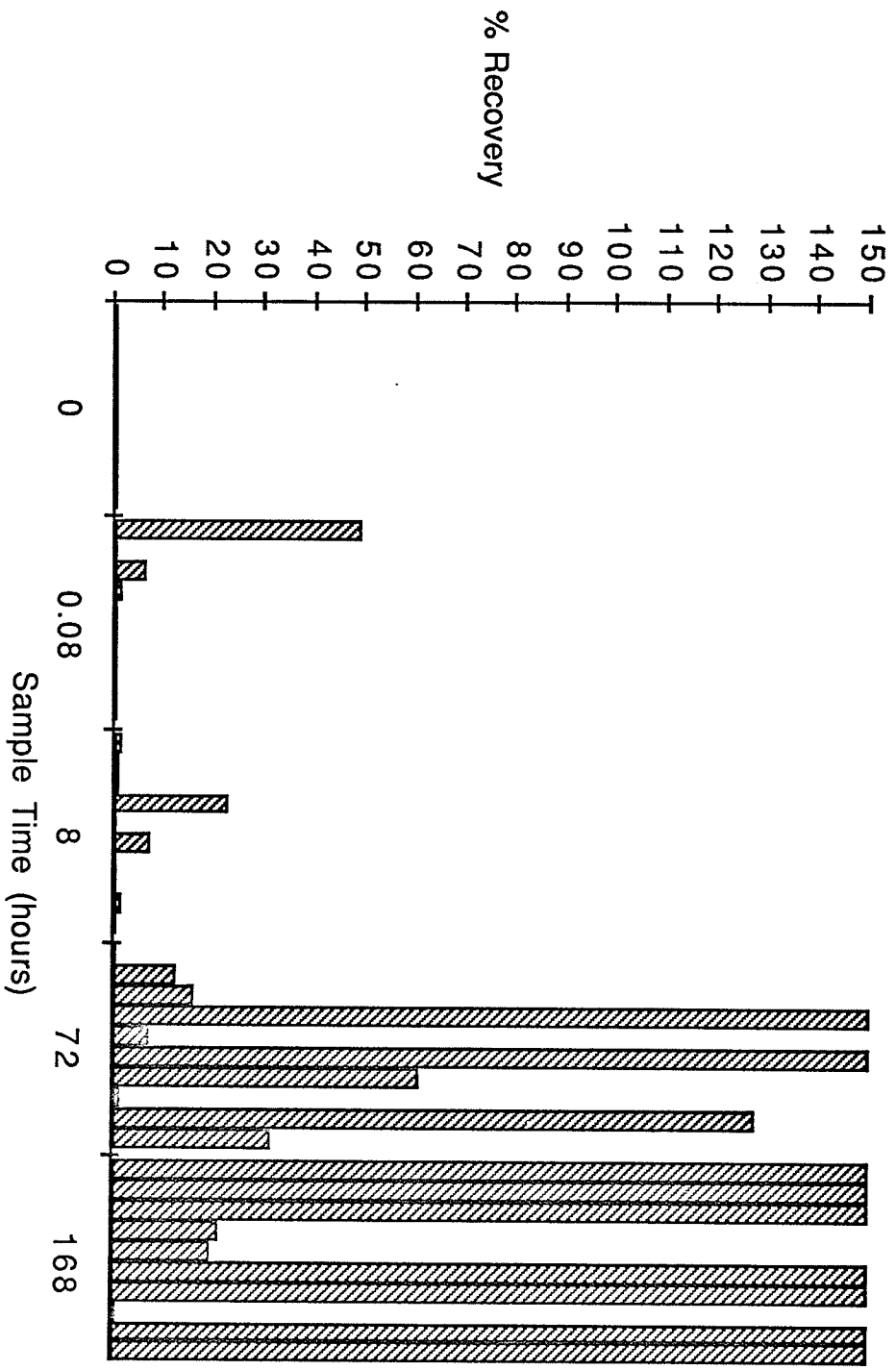


Figure 4.6

Ratio of Genera within a Sample (Control Sites)

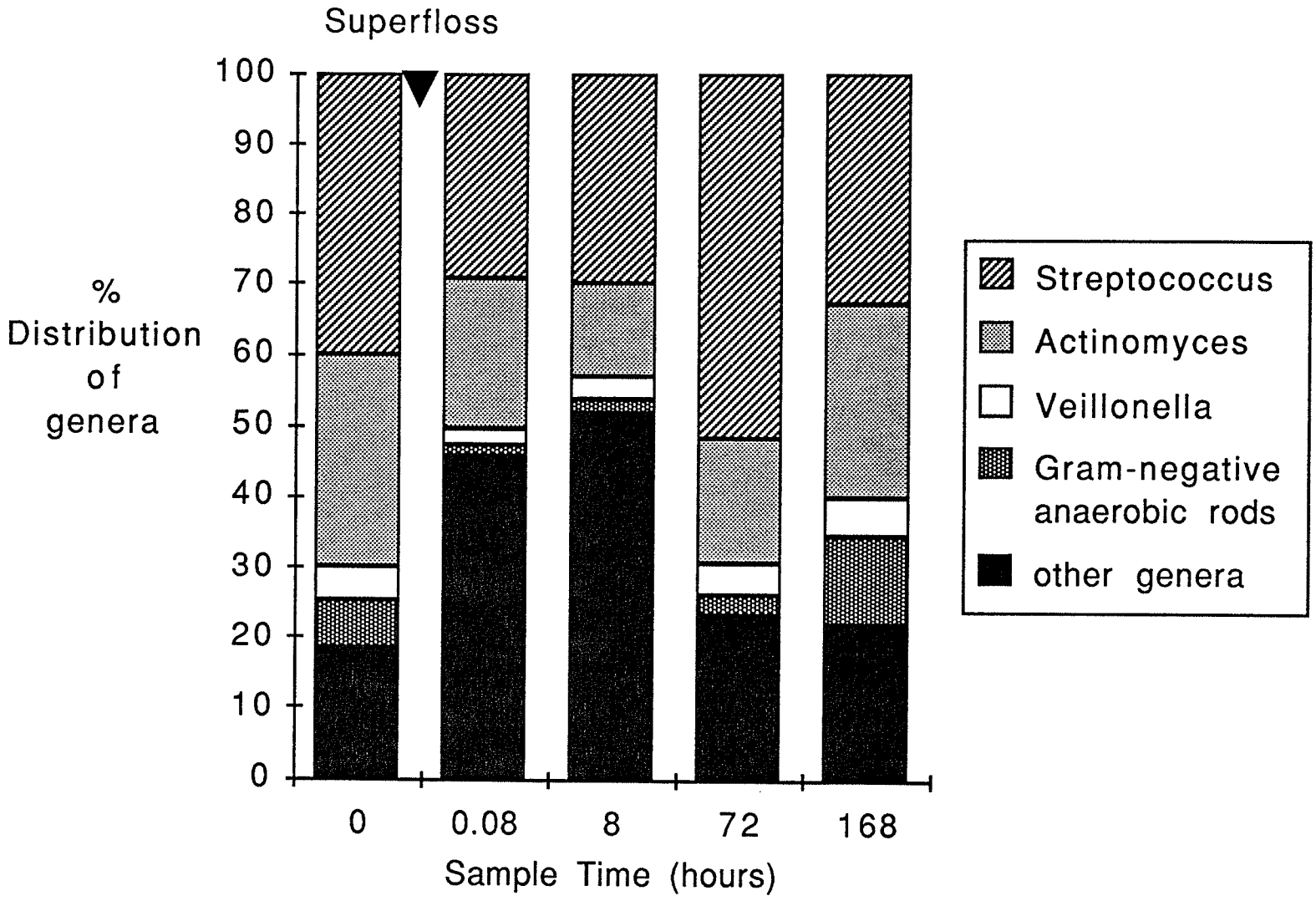
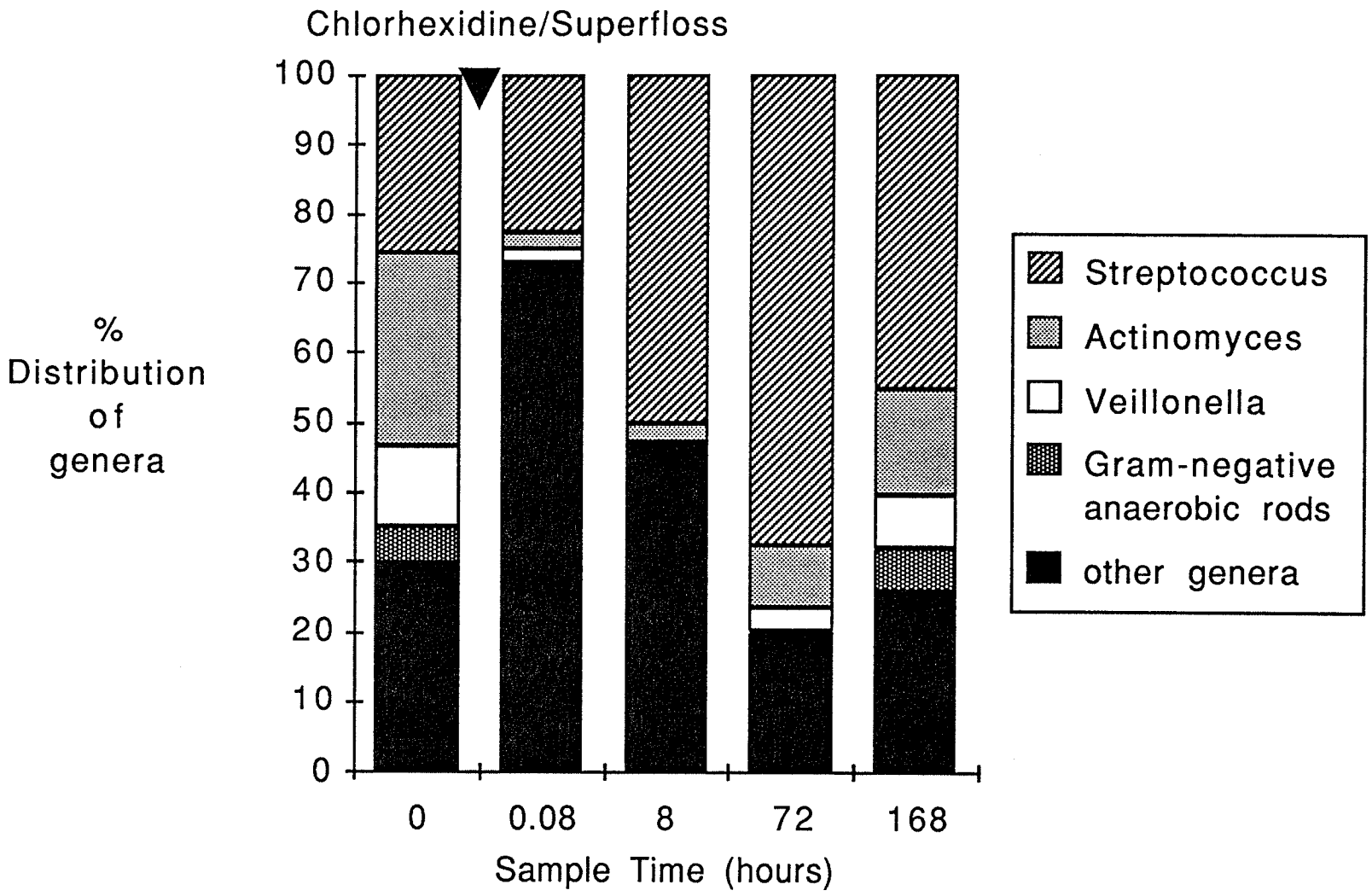


Figure 4.7

Ratio of Genera within a Sample (Test Sites)



iii)RECOVERY OF STREPTOCOCCUS SPECIES/TAXA

A total of 563 isolates of Streptococcus were subjected to the set of tests as described in Chapter 3. These were then sorted into groups by their ability to ferment the test carbohydrates, hydrolyze arginine and esculin, produce H_2O_2 and type of colony formation on TYC plates. The sorting was done on the Amdahl computer at the University of Manitoba. Once the strains were grouped, they were given a "Pattern number".Twenty-eight different reaction patterns were initially found (Table 4.7). Where possible, the patterns were identified to species and taxa level. Data from the test and control sites for the individual subjects are listed in Appendix B. The combined data from the ten subjects are illustrated in Figures 4.8 and 4.9. Due to the complexity of the data, the patterns were then identified in species and taxa where possible in order to analyze population shifts within Streptococcus. Using the Kilian *et al.*(125) classification of S. sanguis and "S. mitior" and Hardie and Bowden's(228) classification of oral streptococci, the following groups were formed:

1)"S. mitior" Taxon ?	Patterns 1, 19, 22
2)"S. mitior" Taxon 3	Pattern 14
3)S. sanguis Taxon 2	Pattern 4
4)S. sanguis Taxon 5	Pattern 20
5)S. mutans (not sobrinus)	Pattern 18
6)Sorbitol fermenters	Patterns 6, 13, 15
7)S. salivarius	Pattern 10
8)"S. milleri"	Pattern 12

The most frequently isolated patterns were then identified by their isolation frequencies (Tables 4.8 and 4.9). Patterns 1, 4, 6, 10, 12, 13, 14, 15, 18, 19, 20 and 22 were found to be the predominant patterns. The combined patterns designated as species or taxa are shown in in Figures 4.10 and 4.11. The actual percentage recovery of the total flora from the sites is illustrated by the line graph in Figures 4.10 and 4.11. The

Table 4.7

Characterization Patterns of Streptococcal Isolates

	M	S	F	O								
PATTERN	Mann	Sorb	Raff	Melib	Tre	Amyg	Inulin	Arg	Esc	H2O2	TYC	SPECIES
1	-	-	+	+	-	-	-	-	-	w	H	"mitior" Taxon 1
2	-	-	+	+	+	+	+	+	-	-	H	sanguis Taxon 2
3	-	-	-	-	+	-	-	-	-	-	S	?
4	-	-	-	-	+	+	+	+	-	+/-	H	sanguis Taxon 2
5	-	-	-	+	+	+	-	-	-	?	S	"mitior" Taxon 2
6	-	+	+	-	+	+	+	+	-	-	H	?
7	-	+	-	-	+	-	+	-	-	-	S	?
8	-	-	-	-	-	-	-	-	-	+	S	salivarius
9	-	-	-	+	-	-	-	-	-	-	S	salivarius
10	-	-	-	-	-	-	-	-	-	-	-	salivarius
11	-	-	-	-	+	-	-	+	-	-	?	?
12	-	-	-	-	+	+	-	+	-	-	S	milleri
13	-	+	+	+	+	-	+	+	-	-	H	?
14	-	-	+	+	-	-	-	+	-	-	S	"mitior" Taxon 3
15	-	+	-	-	+	-	+	-	-	-	H	?
16	-	-	+	+	-	-	+	-	-	-	H	"mitior" Taxon 1
17	-	+	+	+	+	+	+	-	-	-	H	?
18	+	+	+	+	+	+	+	-	-	-	H	mutans
19	-	-	+	+	-	-	+	-	-	+	S	"mitior" Taxon ?
20	-	-	-	+	+	+	+	+	-	-	H	sanguis Taxon 5
21	-	-	-	-	-	-	+	-	+	-	S	salivarius
22	-	-	+	+	+	+	+	-	-	-	S	"mitior" Taxon ?
23	+	-	+	+	+	+	+	-	-	+	S	?
24	-	-	+	+	+	-	+	-	-	+	S	"mitior" Taxon ?
25	-	+	+	+	-	-	+	-	-	-	S	?
26	+	-	+	+	-	-	+	-	+	-	S	?
27	-	-	-	+	+	-	+	-	-	+	H	"mitior" Taxon 1
28	-	-	-	+	+	-	+	-	-	+	S	"mitior" Taxon ?

Figure 4.8

Streptococcal Patterns (Control Sites)

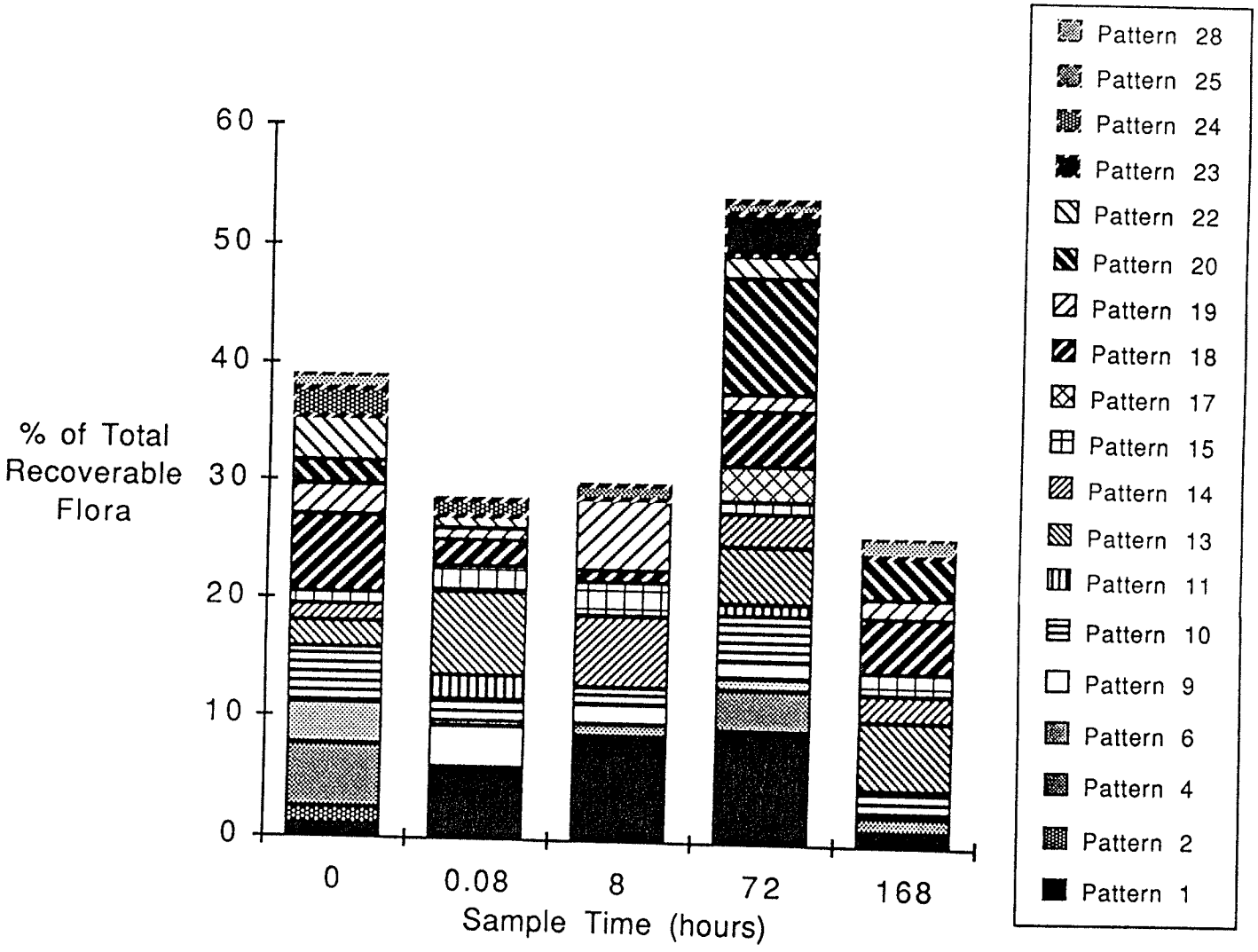


Figure 4.9

Streptococcal Patterns (Test Sites)

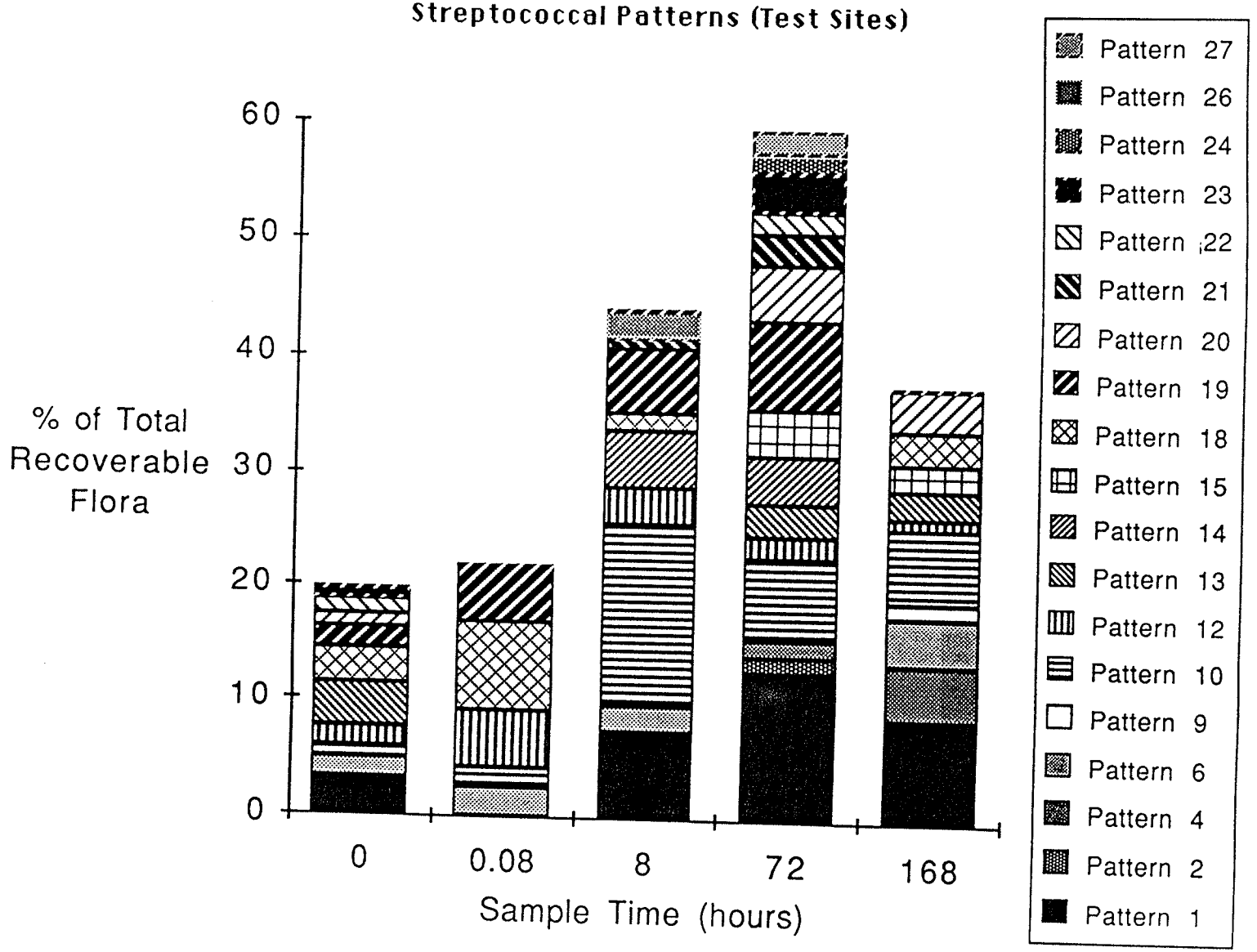


Table 4.8

ISOLATION FREQUENCY OF STREPTOCOCCAL PATTERNS (CONTROL SITES/10)

	SAMPLE TIME (HOURS)				
PATTERN	0	0.08	8	72	168
1	1	3	4	5	2
2	2	0	0	0	0
3	1	0	0	0	1
4	1	0	2	2	2
5	0	0	0	0	1
6	2	1	1	2	0
9	1	2	1	2	0
10	5	2	2	3	2
11	1	1	0	2	0
12	3	2	1	1	1
13	3	2	0	5	3
14	2	0	2	1	2
15	2	3	2	1	3
17	0	0	0	2	0
18	2	1	1	4	3
19	1	1	3	1	2
20	2	0	0	6	5
21	1	0	0	0	1
22	2	1	1	2	1
23	1	0	0	1	0
24	2	1	0	0	0
25	0	1	1	1	0
26	0	0	1	1	0
27	1	0	0	1	0
28	1	0	0	0	1
Unidentified Patterns	0	0	1	0	1

Table 4.9

ISOLATION FREQUENCY OF STREPTOCOCCAL PATTERNS (TEST SITES/10)

	SAMPLE TIME (HOURS)				
PATTERN	0	0.08	8	72	168
1	4	0	2	5	5
2	1	0	0	2	1
4	1	1	1	2	4
6	1	1	2	0	4
9	1	0	0	0	2
10	2	1	3	3	4
11	0	0	0	1	1
12	3	1	1	2	2
13	2	0	0	3	3
14	2	0	1	2	1
15	2	0	0	2	3
17	2	0	0	1	0
18	2	1	1	1	1
19	3	1	1	2	0
20	3	0	0	7	4
21	0	0	1	1	0
22	2	0	0	2	1
23	1	0	1	1	0
24	0	0	0	2	1
25	0	0	0	1	0
26	0	0	1	0	0
27	1	0	0	2	1
28	1	0	0	1	1
Unidentified Patterns	1	0	1	2	3

Figure 4.10
Streptococcal species/Control Sites (n=10)

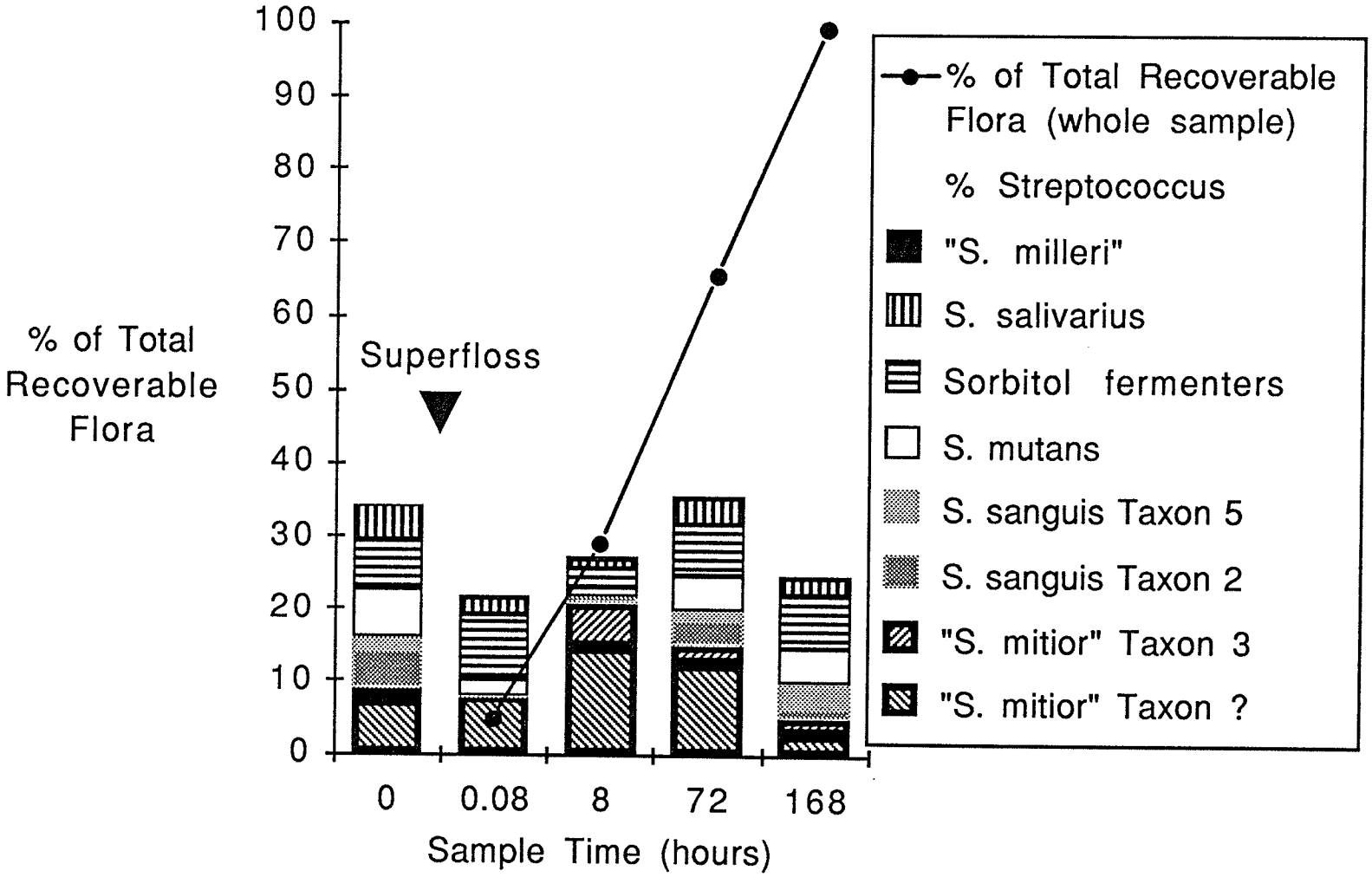
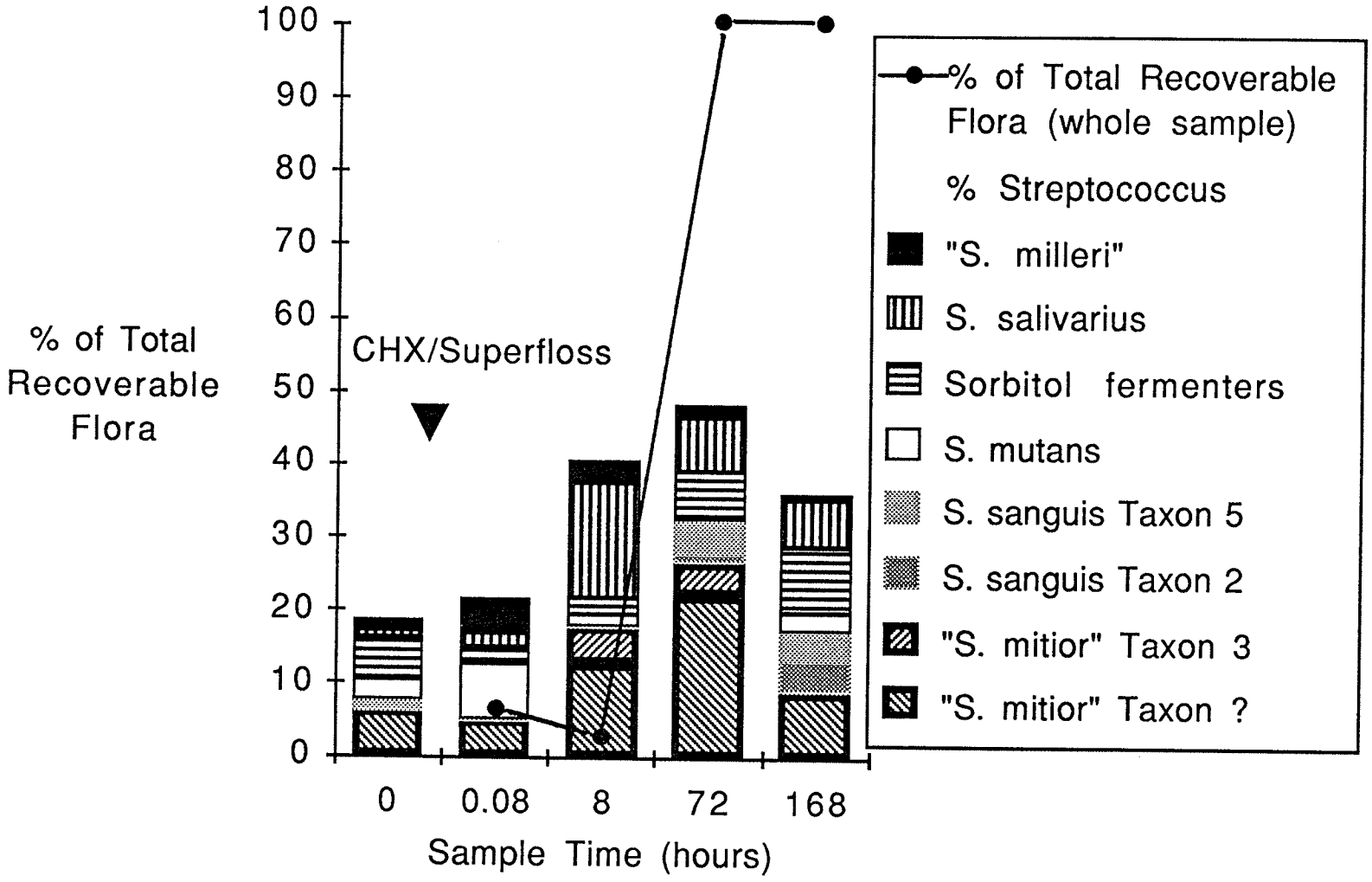


Figure 4.11
Streptococcal Species/Test Sites (N=10)



Streptococcus species shown in the bars actually represent a percentage of this total recoverable flora. The data in Figures 4.10 and 4.11 show that "S. mitior" increased at test sites up to 72 hours and decreased at 168 hours. This contrasts to the control sites where a decrease in "S. mitior" occurred earlier at 72 hours. It is apparent that "S. mitior" colonized the treated sites after 8 hours in both test and control sites. However, the growth of "S. mitior" appeared to be enhanced after chlorhexidine which suppresses Actinomyces species. S. sanguis appeared to be affected similarly by Superfloss and chlorhexidine/Superfloss. At both the control and test sites S. sanguis was eliminated and there was little recovery until the 72 hour sample. The group designated "sorbitol fermenters" was eliminated at the test sites and did not recover until the 72 hour sample. They persisted at the control sites virtually unchanged except for the 8 hour sample. "S. milleri" persisted throughout the sampling at the test sites and was not detected at the control sites. The strains designated as S. salivarius persisted at the control sites and increased at the test sites after chlorhexidine/Superfloss was applied. S. mutans persisted at the control sites but formed a smaller percentage of the streptococci until the 72 hour sample. At the test sites S. mutans persisted immediately following chlorhexidine/Superfloss treatment but was virtually eliminated at 8 hours and not detected at 72 hours. Recovery to pretreatment levels occurred at 168 hours.

iv)COMPARISON OF THE GROUPED PATTERNS OF S. sanguis and "S. mitior" WITH THE KILIAN *et al.* CLASSIFICATION

Sixty-one freeze-dried strains from the current study, representative of specific patterns, were subjected to the extensive series of tests described in Chapter 3. The additional tests included carbohydrate fermentation, growth and acid production on sodium fluoride plates and enzyme activity tests. Five type strains (including ATCC 10556, ATCC 10557 AND ATCC 10558 which were reference strains in the Kilian *et al.* classification) and 3 freeze-dried isolates that had previously been subjected to an

extensive series of tests and identified were also tested (see Appendix C for examples of the reactions of selected patterns).

Our results differed from those of Kilian *et al.* in that ATCC 10556 (S. sanguis Taxon 1) did not ferment raffinose or salicin, did not hydrolyze arginine or esculin and α-D-galactosidase was negative. ATCC 10558 (S. sanguis Taxon 5) differed from Kilian's strains by fermenting melibiose and in lacking activity of α-L-fucosidase and α-D-galactosidase. Kilian's results for ATCC 10557 ("S. mitior" Taxon 1)were reproduced for the characteristics that were tested.

The group designated "S. mitior" Taxon ? included 6/55 strains in pattern 1 that fermented trehalose and 2/55 strains that fermented mannitol. Strains in pattern 19 included 14/23 strains that fermented inulin and 2/23 strains hydrolyzed esculin. Pattern 23 differed from Kilian's group in that 14/34 strain fermented trehalose, amygdalin and inulin and18/34 fermented trehalose and amydalin.

Pattern 14 was most closely related to "S. mitior" Taxon 3. However, only 6/20 hydrolyzed arginine.

Twenty-four isolates were grouped into pattern 4 and named S. sanguis Taxon 2. All of them fermented amygdalin, 6/24 did not hydrolyze arginine and 6/24 did not produce extracellular polysaccharide.

Pattern 20 was designated S. sanguis Taxon 5. Of 58 strains, 18 did not produce extracellular polysaccharide and only 17 strains hydrolyzed both arginine and esculin.

v)RATIO OF ACTINOMYCES SPECIES AT TEST AND CONTROL SITES

The ratio of the Actinomyces genera as a percentage of the total cultivable flora was not affected by Superfloss alone on the control sites (Figures 4.6) as was previously mentioned. A. naeslundii was first detected at the 8 hour sample and persisted throughout the test period (Figure 4.12). There was an increase in the proportion of strains designated "Intermediate strains" during the 8, 72 and 168 hour samples.

Figure 4.7 demonstrates a dramatic decrease in the proportion of Actinomyces species following chlorhexidine/Superfloss treatment at the test sites. The Actinomyces that were able to survive were identified as A. odontolyticus.(Figure 4.13) They comprised approximately 90% of the Actinomyces species at 0.08 and the 8 hour samples.

Figure 4.12

Ratio of Actinomyces species within a Sample (Control Sites)

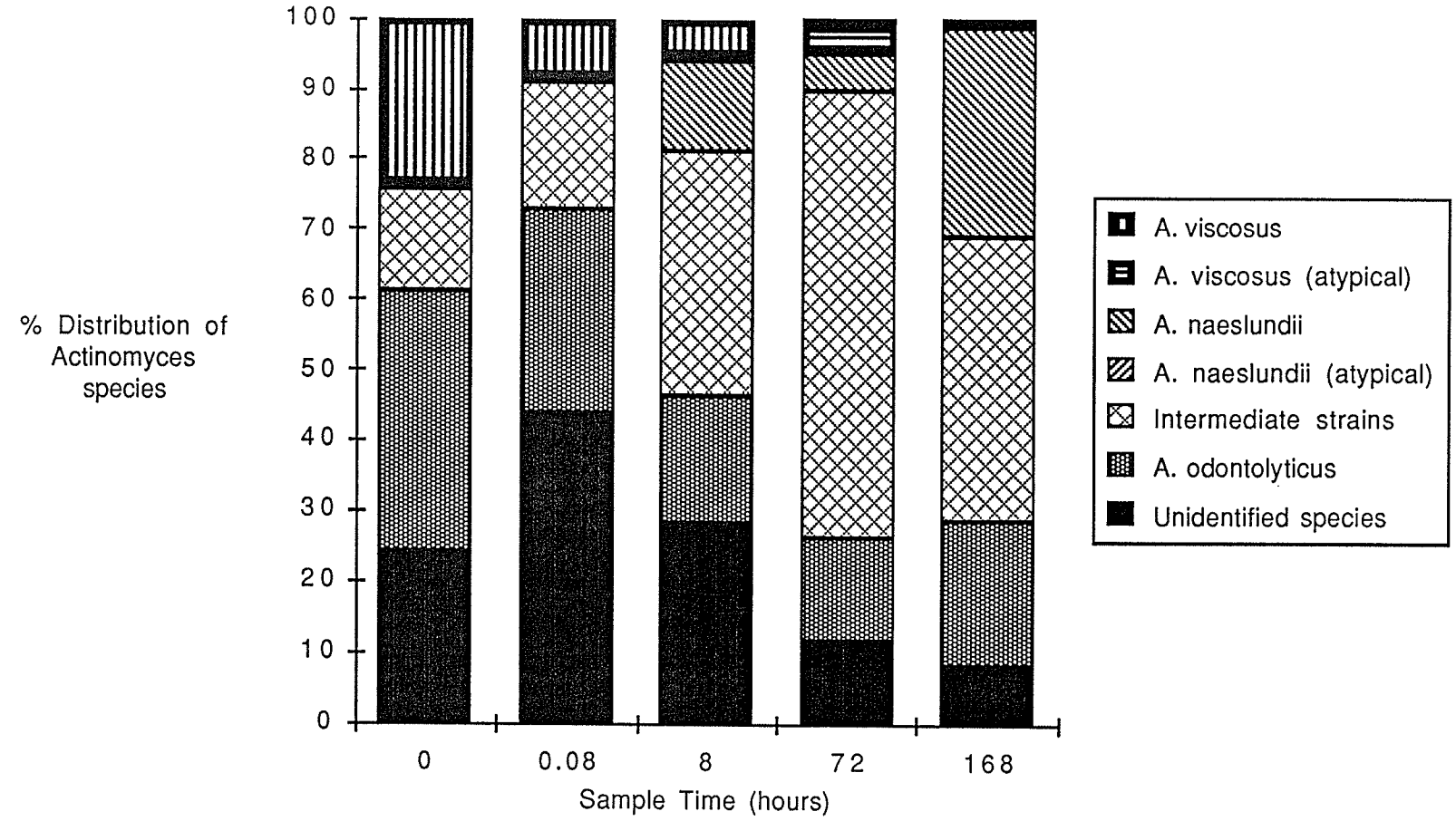
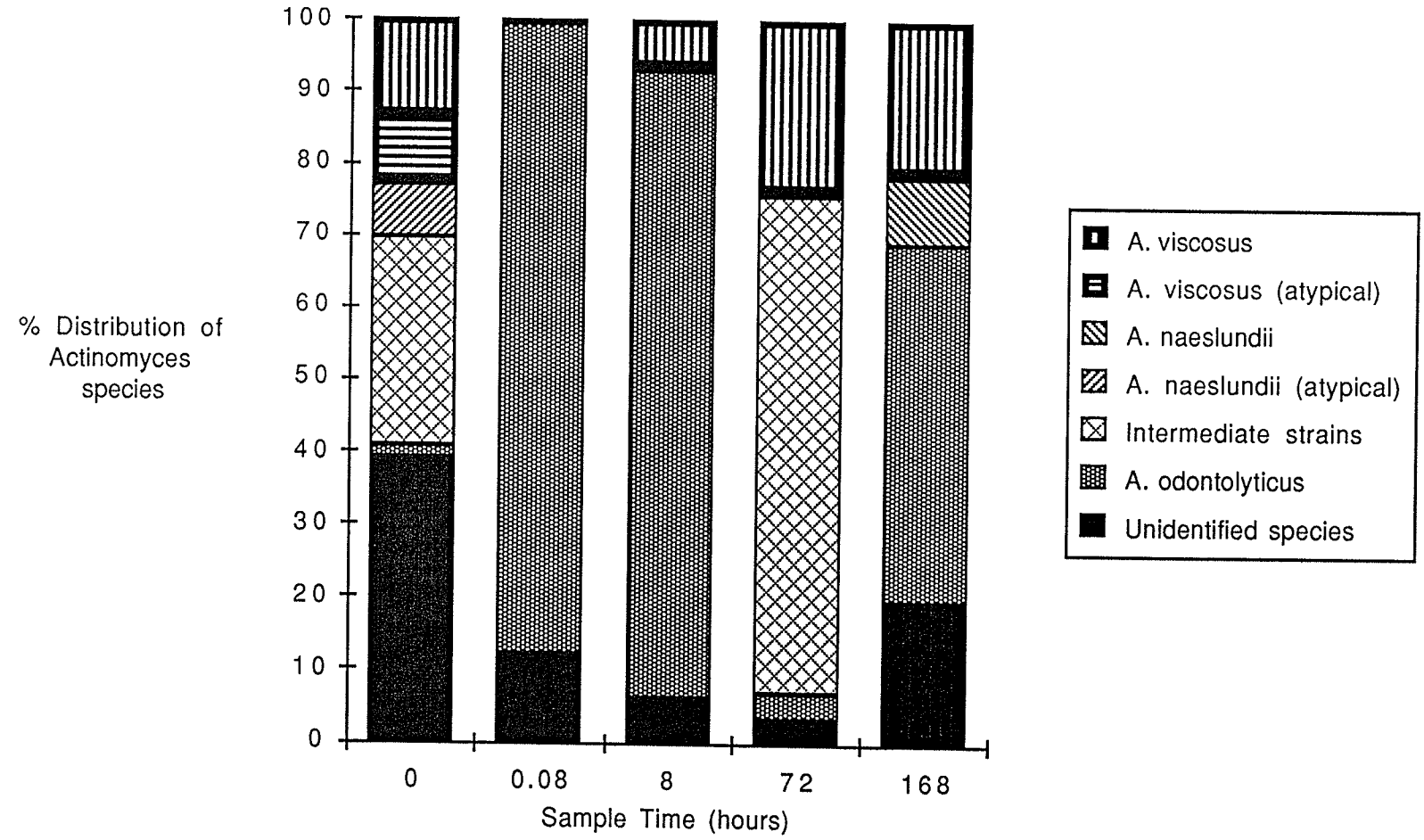


Figure 4.13

Ratio of Actinomyces species within a Sample (Test Sites)



CHAPTER 5

DISCUSSION

a)CONTROL OF THE ORAL MICROFLORA WITH CHLORHEXIDINE

The present study was directed at the control of the microflora associated with incipient caries lesions. Lactobacillus species and S. mutans have both been shown to have a close association with dental caries in humans[8,15,27,31-36,39,41,80-82,85-87,89,90]. Longitudinal studies on the microflora of developing caries lesions have shown a succession from S. mutans to Lactobacillus[27,32,33,35,90] and that Lactobacillus is associated with the progression of caries lesions to cavitation[32,33]. The use of chlorhexidine in preventing caries has mainly been directed towards the suppression of S. mutans [199-203]. Streptococcus species have been shown to be the most sensitive members of the plaque microflora[180]. although there is a range of sensitivity of strains within the genus[198]. S. mutans is known to be particularly sensitive to chlorhexidine[199-203] while S. sanguis is relatively resistant [198,200]. Emilson's study[198] found that the MIC for S. mutans was 1-4 µg/mL and 8-128 µg/mL of chlorhexidine for S. sanguis. The results from the present study are in agreement with his findings (Tables 4.2 and 4.3). Emilson[198] tested 10 strains of Lactobacillus and found the MIC ranged from 2-32 µg/mL but little other data is available on the sensitivity of Lactobacillus to chlorhexidine. Lundström and Krasse[203] found that chlorhexidine had no effect on salivary Lactobacillus counts in their study. The focus of our initial study was to determine the level of chlorhexidine necessary to suppress Lactobacillus. That level would then also effectively suppress S. mutans.

The results of the *in vitro* study indicate a wide range of sensitivity of Lactobacillus to chlorhexidine with both inter and intraspecies variation. The MIC range for the

strains tested at pH 6.7 was 2-60 µg/mL of chlorhexidine and up to 100µg/mL at pH 5.0. The results also indicated that at levels of chlorhexidine just below the MIC, the Lactobacillus strains were still producing acid (Table 4.1). Because the environmental pH of a developing caries lesion may be low[5] , and chlorhexidine is not as effective at low pH, sufficient concentration must be used *in vivo* to be effective. As the activity of chlorhexidine is non-specific, its action will also be severely limited by the presence of extraneous protein. The non-specific action reducing the level of available chlorhexidine may be the reason that S. sanguis appears to survive or recover from even the most concentrated gel treatments [199,202] as the strains of the species tested here did not survive 10 µg/mL of chlorhexidine. The limited *in vitro* killing studies suggest that protein may protect the cells, as with a relatively high number of cells in the inoculum used for the tests the numbers surviving may be high. However, it should be noted that in the killing experiments recorded here only viable cell counts were made. It may be that many dead cells were also present and these would serve to bind chlorhexidine and protect the viable cells. Future *in vitro* studies of sensitivity should consider the presence of dead cells and an analysis of total protein should be made.

In order to attempt to control Lactobacillus in association with incipient approximal caries lesions, it is necessary to utilize a high concentration of chlorhexidine and allow it to interact with the target area for a sufficient period of time. The data from the present study make it clear that many strains of Lactobacillus are resistant to up to 80 µg/mL of chlorhexidine (0.8 mg.%) at pH 5.0. The various levels of chlorhexidine that have been used for mouthrinses and gels (0.2 gm.%-5.0 gm.%) would easily kill Lactobacillus *in vitro*. However, the limited *in vivo* studies[203] suggest that a direct correlation from *in vitro* studies to the *in vivo* situation may not be valid.

In comparison to chlorhexidine, the antibiotics tested were bacteriostatic. Minocycline was effective at low pH compared to Spiramycin, which in the test system used here, was ineffective at pH 5.0. Given the limited data available from the present

study, Minocycline might be a better choice than Spiramycin in environments of low pH. However, as these agents are not bacterocidal, their impact on the flora might be short-lived relative to chlorhexidine and acid production may not be inhibited. Therefore, chlorhexidine was used in our pilot study to assess its *in vivo* effect on approximal microflora.

The effect of chlorhexidine on the plaque community may be seen as a two-stage process. Initially there is an ecological catastrophe where the plaque flora is reduced to approximately 5% of the pretreatment levels. Recovery of the plaque flora is in turn affected by their differential sensitivity to chlorhexidine. "Normal" recolonization is affected in that Actinomyces is not a significant part of the early community[26]. Suppression of specific genera such as Actinomyces allowed Streptococcus to compete more effectively and become dominant in the plaque community. Within Streptococcus the "S. mitior" group of organisms selectively recolonized more readily at the test sites rather than the control sites.

b)DELIVERY OF CHLORHEXIDINE TO SPECIFIC SITES

Various methods of delivery of chlorhexidine have been used clinically to suppress plaque formation. Mouthrinses with chlorhexidine ranging from 0.2-2.0% have been effective in inhibiting plaque[187,190,193-197] though not totally without side-effects such as staining of restorations and teeth and mucosal ulceration. Chlorhexidine dentifrices were not found to be as effective in inhibiting plaque[191,192]. Chlorhexidine gels[199,200,202,203] have been used in concentrations up to 5%[199,202]. At this latter concentration S. mutans was suppressed for 21 days and plaque inhibition was achieved[199]. Varnishes have also been shown to be effective[237].

A localized application was chosen for this study so that a high concentration might be utilized and to minimize side-effects of chlorhexidine. A concentration of 2.0% chlorhexidine was determined to be appropriate based on the results of our *in vitro*

sensitivity tests on Lactobacillus and Streptococcus. Superfloss® was the vehicle of choice because of its ability to carry the chlorhexidine to the site and its superior mechanical effectiveness over dental floss in plaque removal[221]. Our pilot study was undertaken to assess the effectiveness of Superfloss versus Superfloss with 2.0% chlorhexidine on approximal microflora.

Superfloss alone (control sites) was found to be as effective as Superfloss/chlorhexidine (test sites) in the reduction of the microflora. At the 0.08 hour sample (immediately after application of Superfloss or Superfloss/chlorhexidine) the control and test sites were 6.06 and 5.76% of the pretreatment counts. It could be suggested that an ecological catastrophe occurred at both control and test sites. The physical effect of Superfloss alone can be differentiated from the combined physical and chemical effect of Superfloss/chlorhexidine. There were qualitative differences between the test and control sites as mentioned previously. Recolonization of the control and test sites differed in that the control sites recovered earlier than the test sites. By the 168 hour sample more of the test sites had recovered to pretreatment levels. This may be explained by the differences in competition at each of the sites as the community develops. Actinomyces was effectively eliminated from the test site and did not return to pretreatment levels at the 168 hour sample. Streptococcus was able to colonize effectively and dominate the test sites during the test period without competition from Actinomyces. The proportions of the genera at the control sites were not greatly affected by Superfloss alone.

Within the genera Streptococcus and Actinomyces, the test and control sites did demonstrate differences in recolonization. "S. mitior" increased at the test sites up to 72 hours while they decreased at the control sites from the 8 hour sample. Again, this may be attributed to lack of competition from Actinomyces. S. mutans persisted throughout all of the control site samples and was detected at the 0.08 hour sample at the test sites. However, S. mutans levels decreased and were not detected at the 72 hour sample of the

test sites. It appeared better able to compete and colonize at the control sites. Although Actinomyces were virtually eliminated from the test sites, A. odontolyticus comprised approximately 90% of the few Actinomyces that were present at 0.08 and 8.0 hours. No data are available on the sensitivity of A. odontolyticus to chlorhexidine. However, it is known that this species is associated with carious dentin[238].

The treatment of the interproximal area demonstrates the effect of an ecological catastrophe (physical and chemical versus physical alone) on a bacterial community both in terms of organisms able to survive as well as selective recolonization of the site. The slower growth rate of Actinomyces compared to that of Streptococcus further prevents Actinomyces from becoming a prominent member of the early plaque community[239]. The colonization pattern of Streptococcus on the control sites in the present study is comparable to the colonization pattern found by Nyvad and Kilian[239] in studies of the growth of bacteria on enamel and cementum *in vivo*.

c)CHARACTERIZATION OF THE MICROORGANISMS

Extensive tests were done to characterize Streptococcus to a species and taxon level where possible. With S. sanguis/"S. mitior"/"S. mitis" currently undergoing reclassification, it is important to know which of the streptococci are actually responsible for initial plaque formation. Kilian *et al*[125] found that although both S. sanguis and "S. mitior" are pioneers in plaque formation, there were differences in colonization between caries-active and caries-inactive children. "S. mitior" Taxon 1b (EPS-negative strains) and Taxon 2 were the principal colonizers in the caries-active group while S. sanguis Taxon 2 was dominant in the caries-inactive group. Such differences in plaque formation may play an important role in determining the cariogenic potential of the plaque. Recent studies by Nyvad and Kilian[239] and Gilmour *et al.*[240] have recommended a reclassification of this group of microorganisms into S. sanguis (2 groups), S. oralis ("mitior") and S. mitis (arginine + and arginine - groups).

The range of characterization tests used in this study included sensitivity to fluoride. Such tests have not been used by others studying the oral streptococci. Within the groups S. salivarius (pattern 10), S. sanguis Taxon 5 (pattern 20) and "S. mitior" Taxon ? (pattern 22) there were 7 strains (1, 3 and 3 respectively) that were able to grow and produce acid on 50 ppm fluoride plates at pH 6.5. All 7 strains were isolated from the 72 hour sample and 6/7 were from the control sites. The ability to survive and produce acid at this level of fluoride may confer a selective advantage for these strains after a major ecological catastrophe has occurred. A role for fluoride resistance in plaque development has not really been examined to date. However, the presence of such bacteria suggest that this phenomenon may have some significance.

Although Superfloss alone and Superfloss/chlorhexidine both produced a profound ecological impact, the qualitative differences at the genus and species level achieved by Superfloss/chlorhexidine warrant further study. In future studies on the effect of chlorhexidine on the microflora of incipient caries lesions it may be appropriate to modify the method. A 2.0% chlorhexidine mouthrinse prior to the localized application of chlorhexidine could be used to eliminate reservoirs of potentially cariogenic microorganisms such as S. mutans and Lactobacillus species. A mouthrinse may be more appropriate than a gel or varnish because it will contact buccal mucosa. "S. mitior" Taxon 1b has previously been described as an early colonizer in caries-active children and the majority of streptococcal isolates from buccal mucosa is of this type[125]. A single application of Superfloss soaked in 5% chlorhexidine would then be used at the site of the incipient caries lesion.

d)CONCLUSIONS

1)Lactobacillus are more resistant to chlorhexidine than Streptococcus.

2)There are inter and intraspecies variations in Lactobacillus sensitivity to chlorhexidine with L. fermentum and L. brevis the most sensitive and L. casei and L. acidophilus the most resistant.

3)Environmental pH is a major factor in the effectiveness of chlorhexidine such that it is less effective at low pH.

4)The amount of protein at the site may be a factor in the effectiveness of chlorhexidine (although not examined in the present study) and should be considered in future chlorhexidine sensitivity studies.

5)The method of application of chlorhexidine to a specific site by Superfloss is effective in producing qualitative and quantitative changes to the approximal microflora.

6)Superfloss alone was as effective as chlorhexidine/Superfloss in introducing an ecological catastrophe.

7)Chlorhexidine/Superfloss also had an effect on the recolonization pattern of the sites by the virtual elimination of Actinomyces. Streptococcus dominated the recovery of the test sites.

8)"S. mitior" was the predominant early colonizer of both test and control sites.

9)Chlorhexidine was effective in inhibiting the recolonization of the sites by S. mutans.

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Appendix A

Consent Form

Consent Form

I,_____ do hereby consent to participate, as a subject, in the experimental study conducted by Dr. B. Cleghorn and Dr. G. Bowden.

I understand that dental plaque samples will be taken from my teeth five times over a period of seven days for purposes of bacterial analysis and that an antiseptic will be applied once between my teeth for a period of five minutes.

The nature and purpose of this study have been explained to me to my satisfaction.

I understand that I have the right to revoke this consent and withdraw from the study at any time.

Signed:

Date:

Witness:

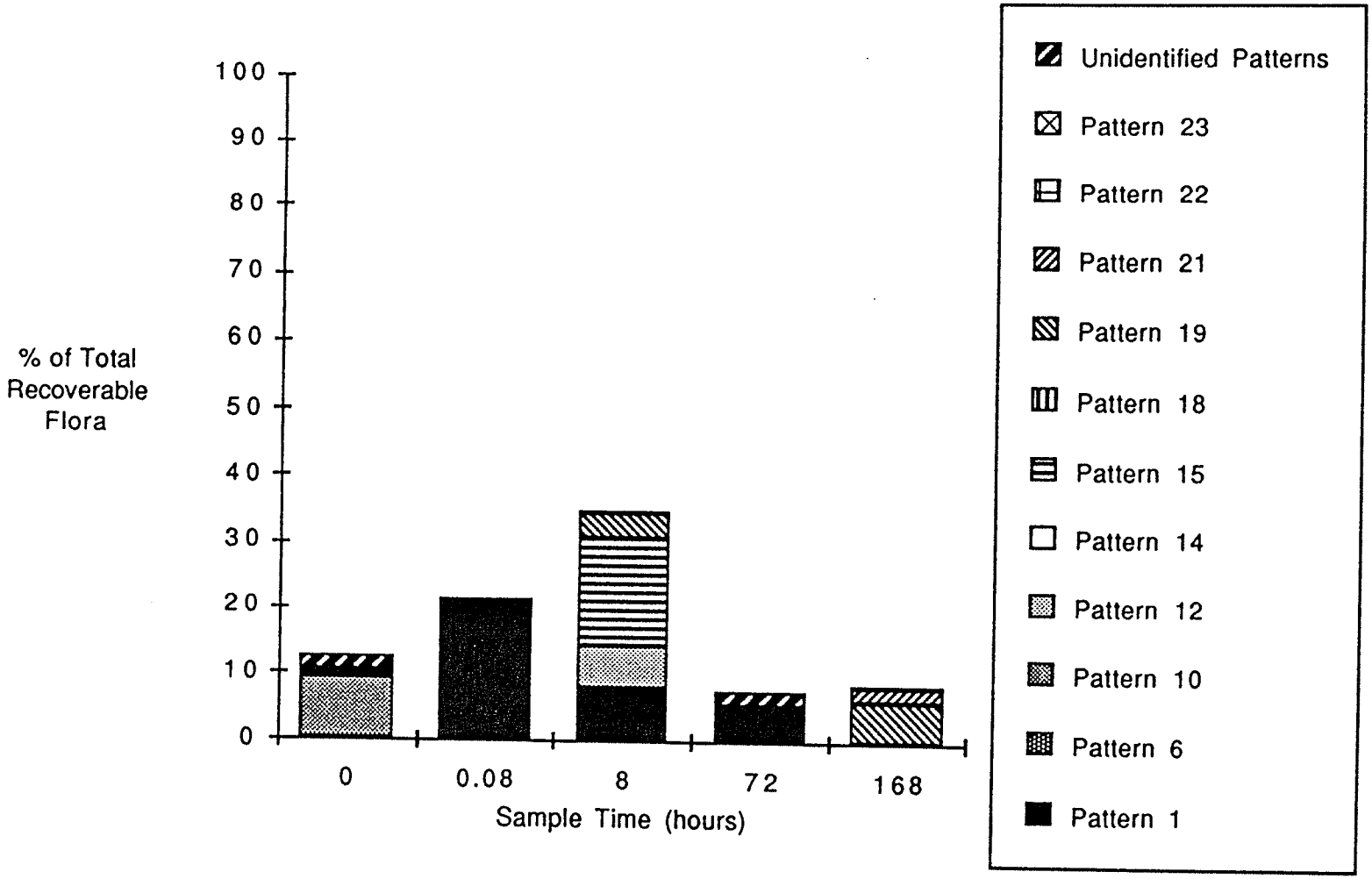
Birthdate:

Address:

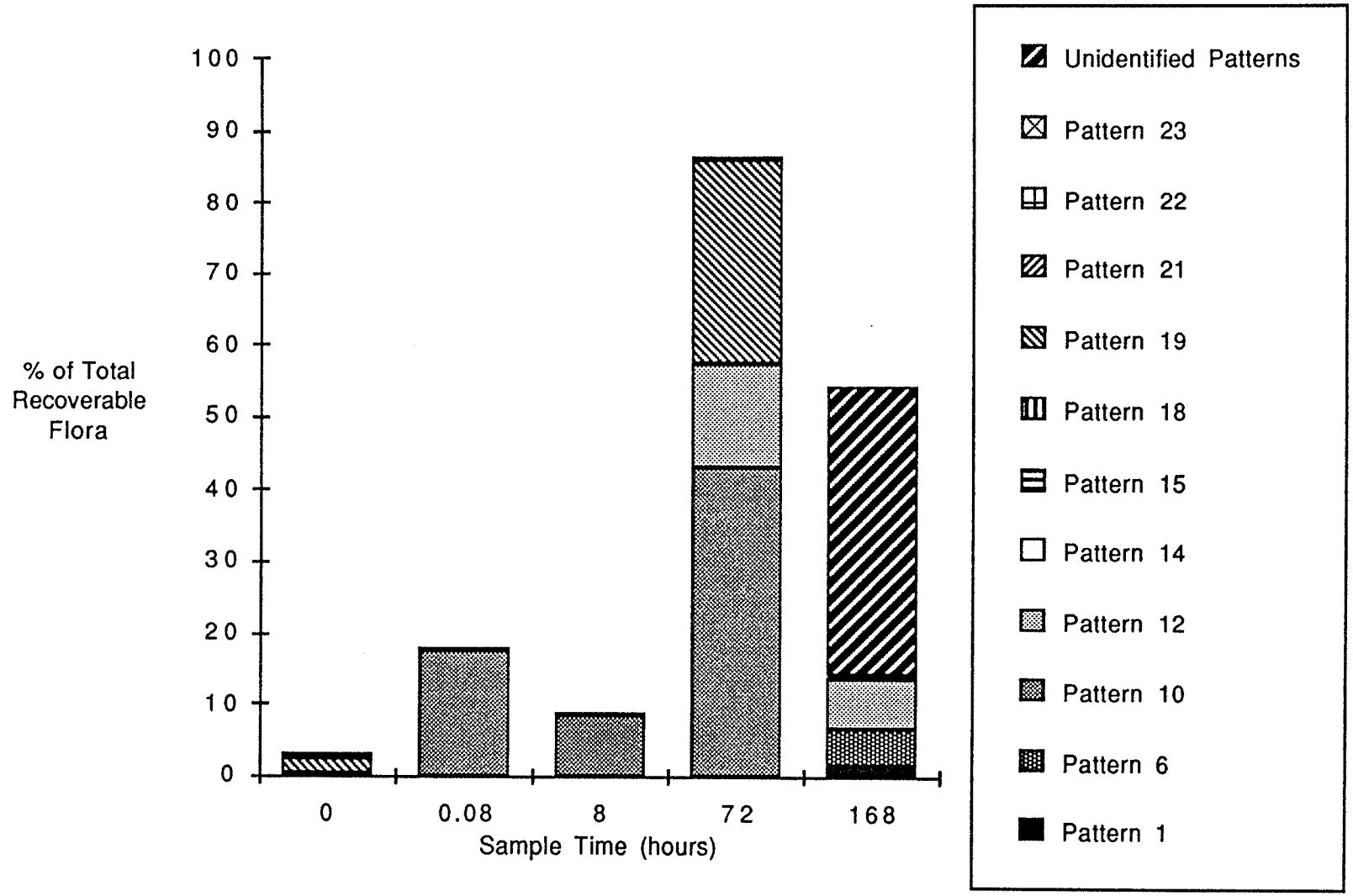
Appendix B

Streptococcal Patterns/Subject

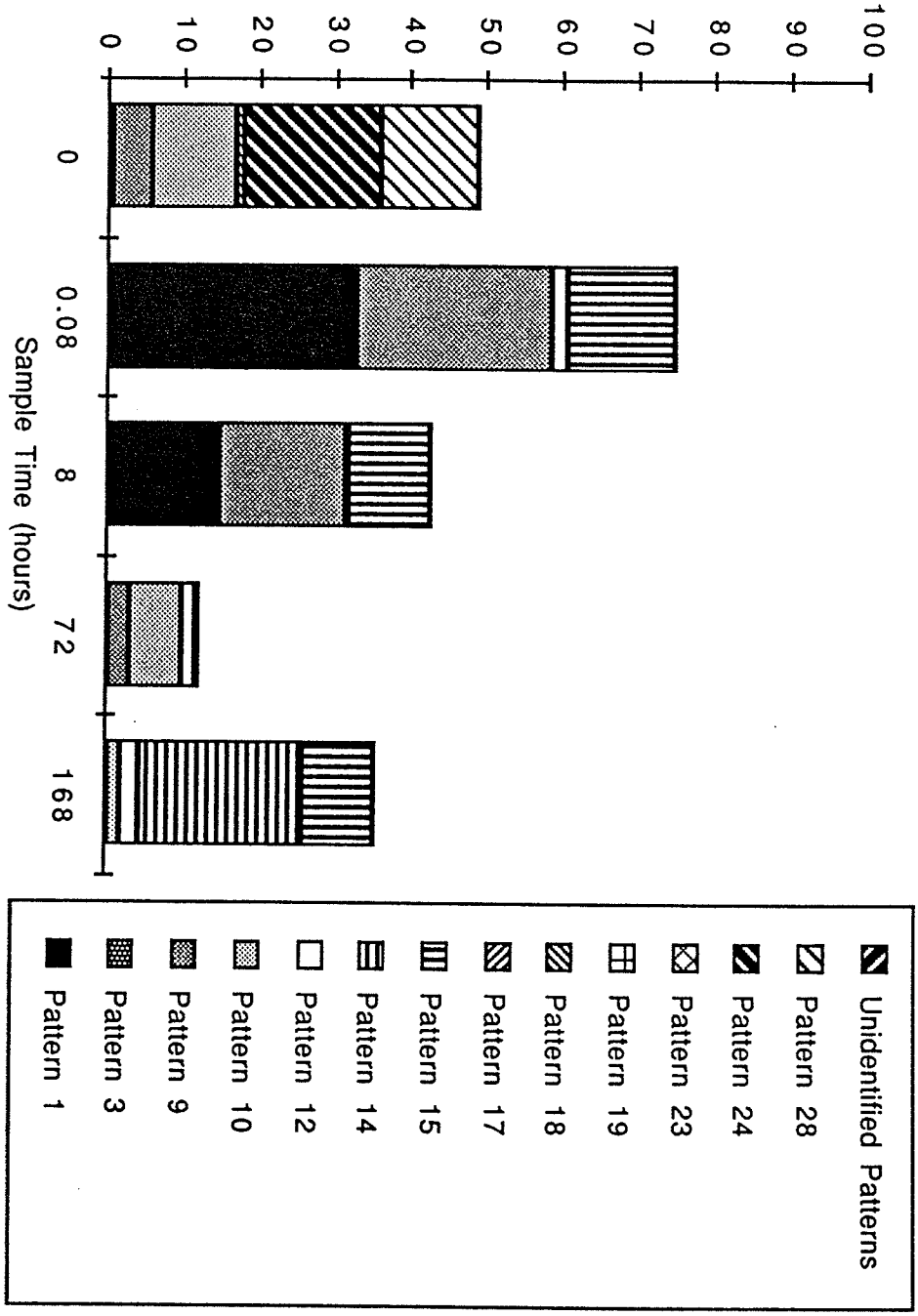
Streptococcal Patterns/Control Sites/Patient-NB



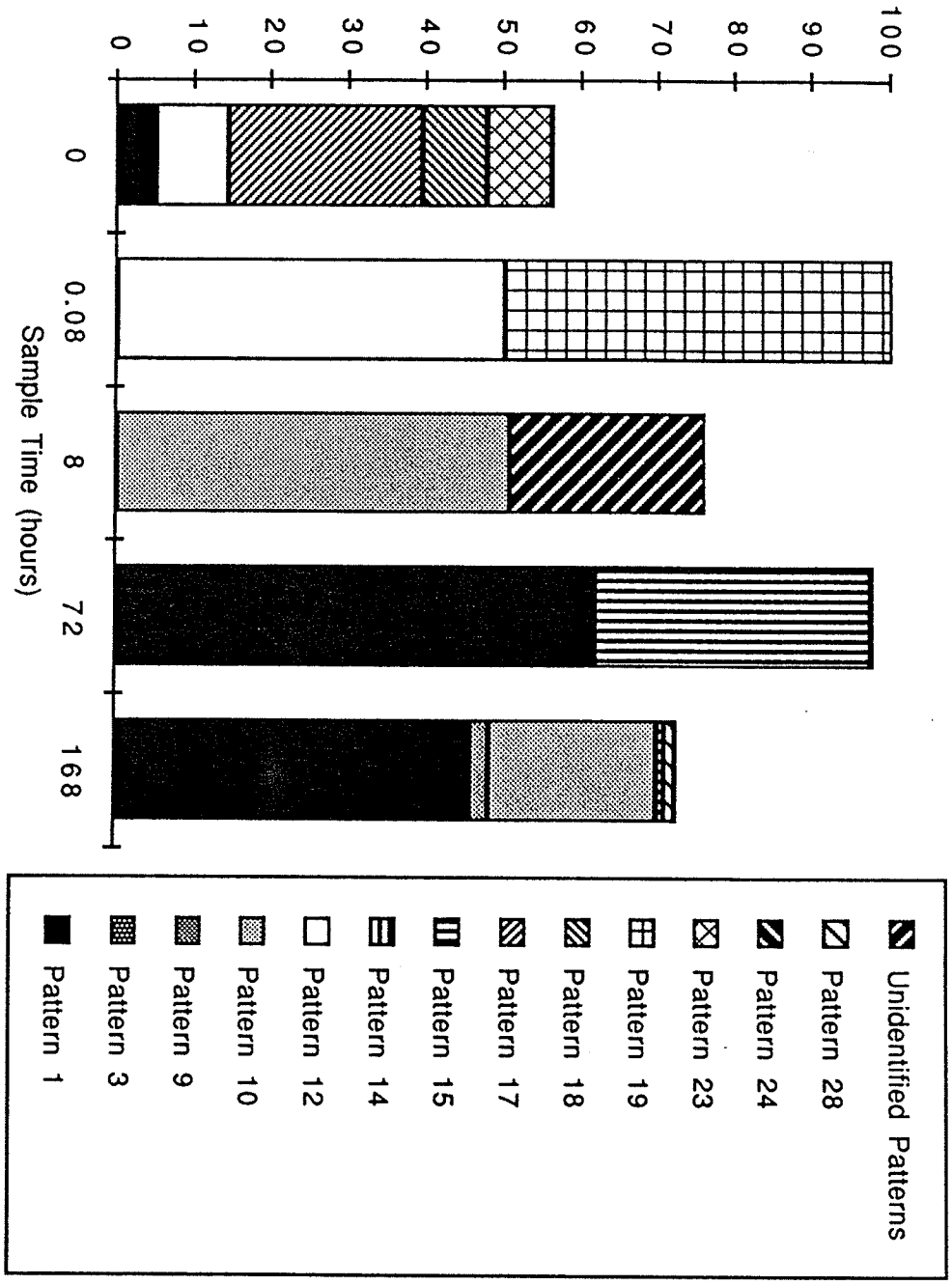
Streptococcal Patterns/Test Sites/Patient-NB



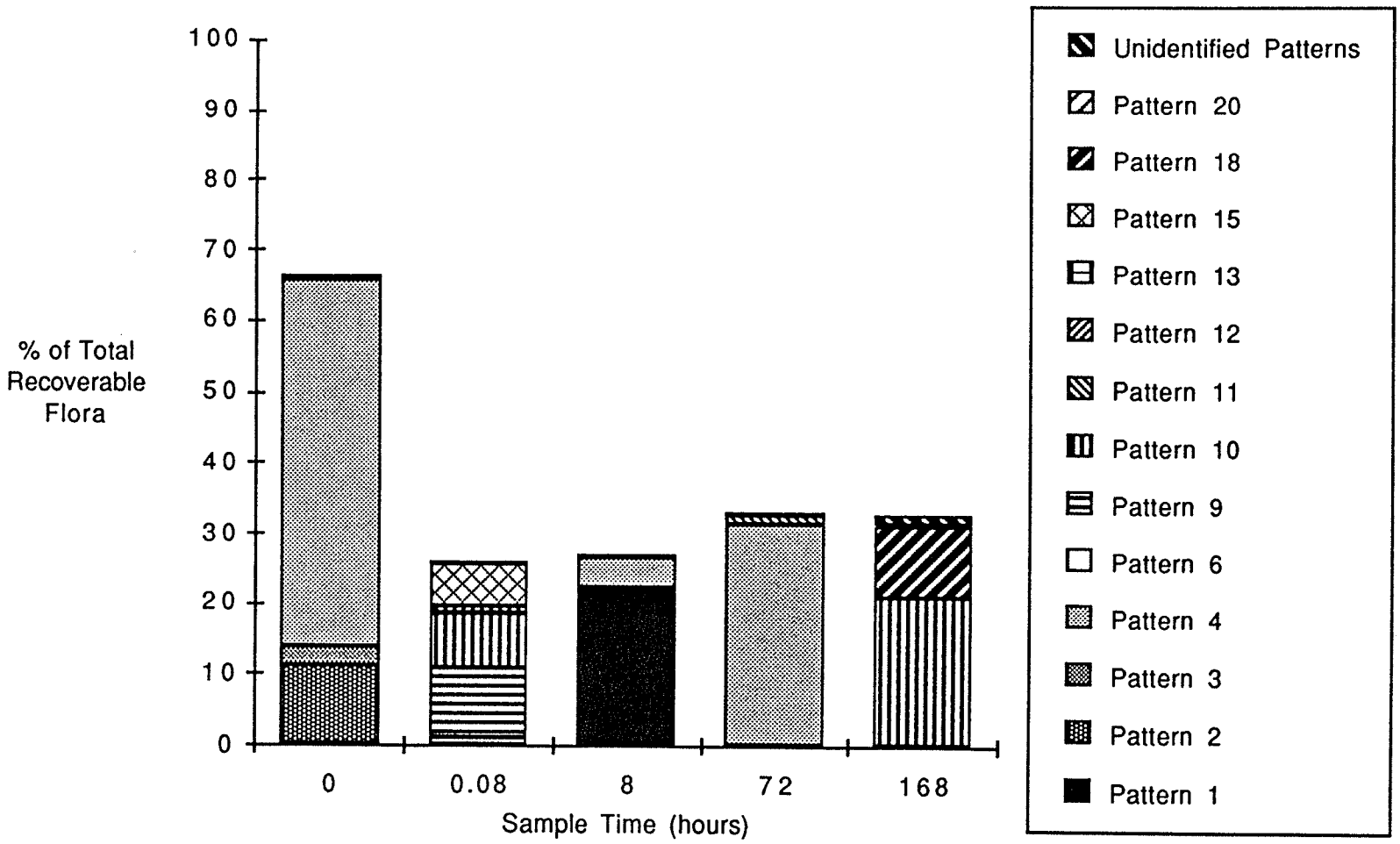
Streptococcal Patterns/Control Sites/Patient-MS



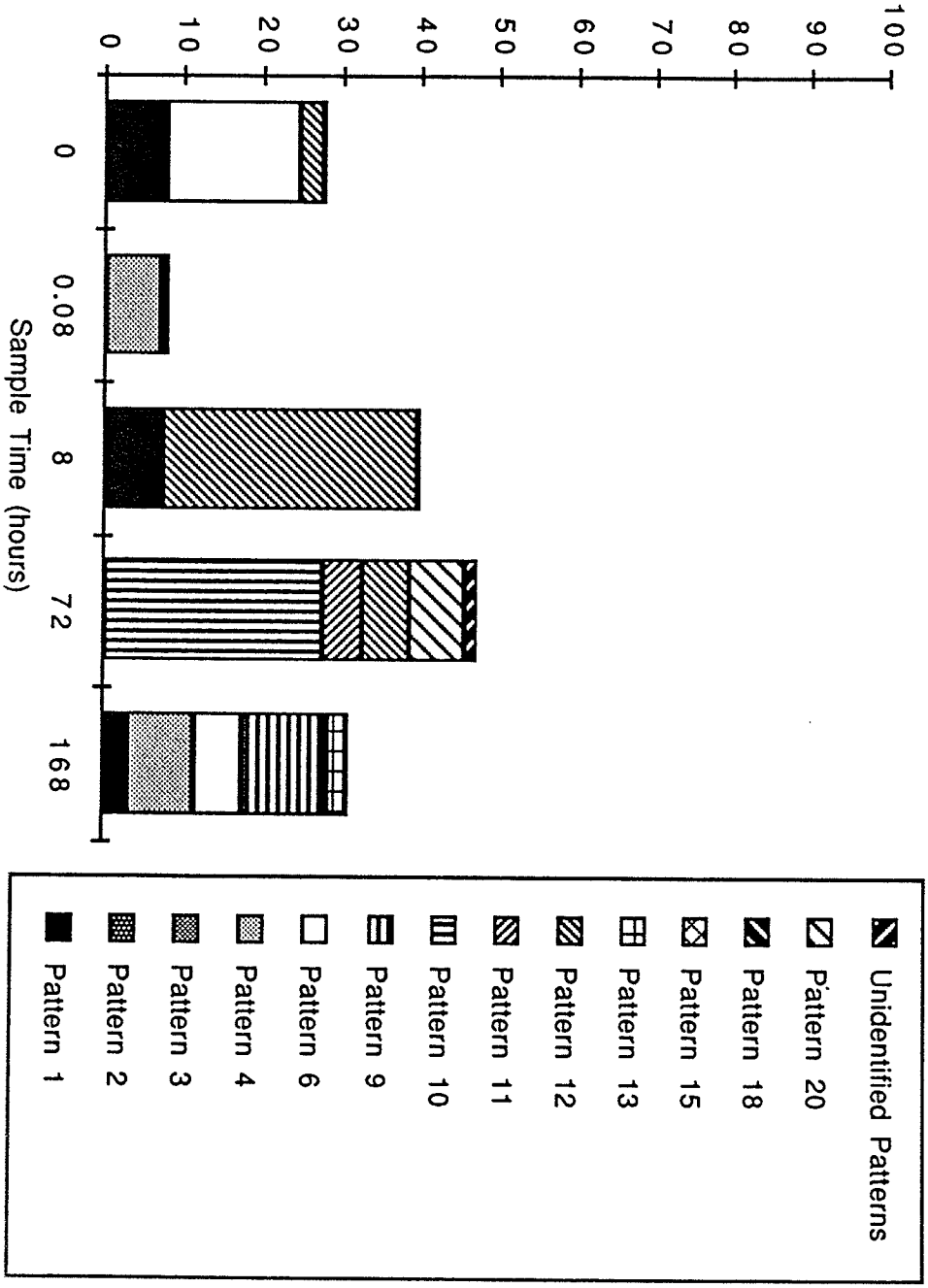
Streptococcal Patterns/Test Sites/Patient MS



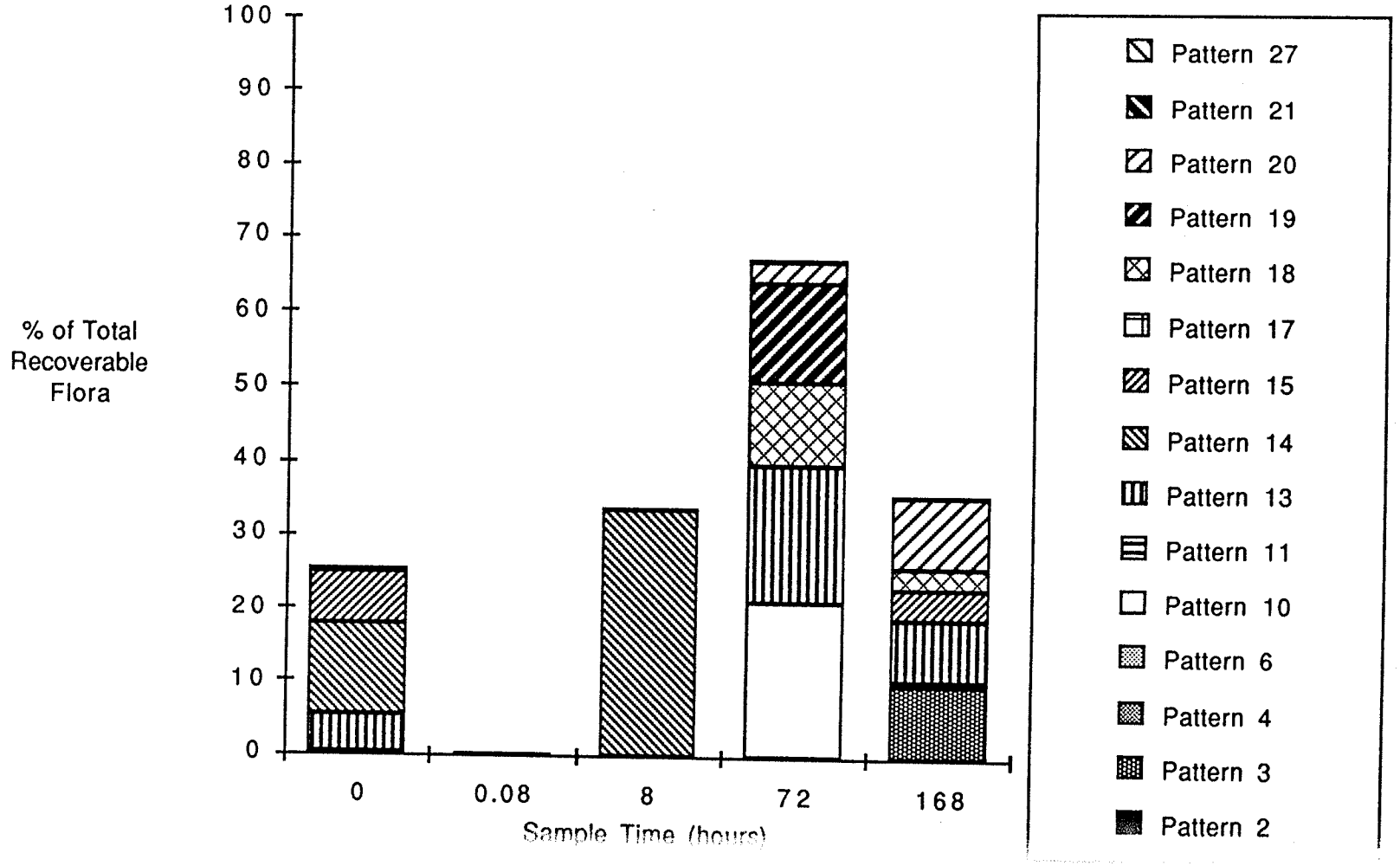
Streptococcal Patterns/Control Sites/Patient-DC



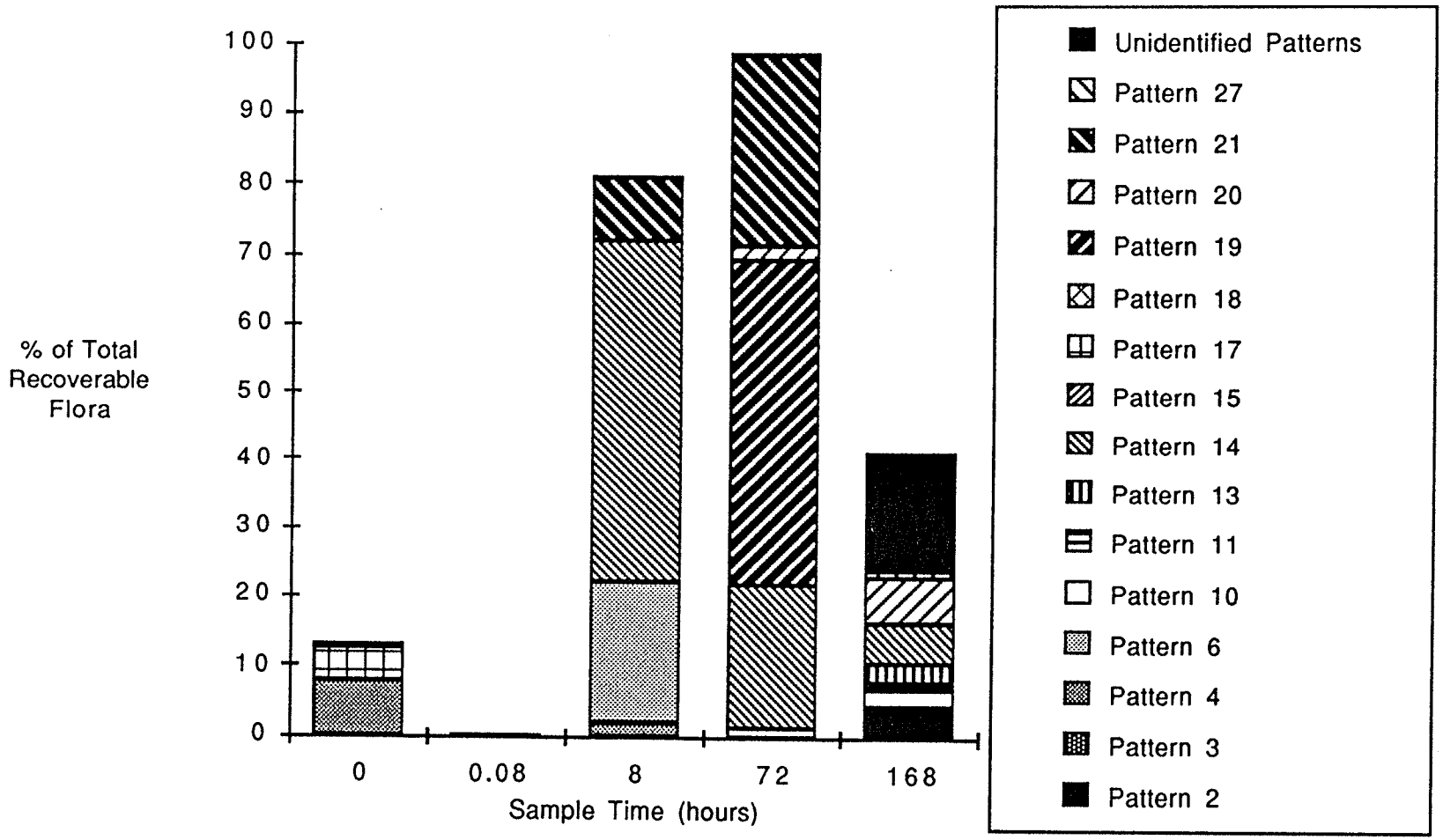
Streptococcal Patterns/Test Sites/Patient-DC



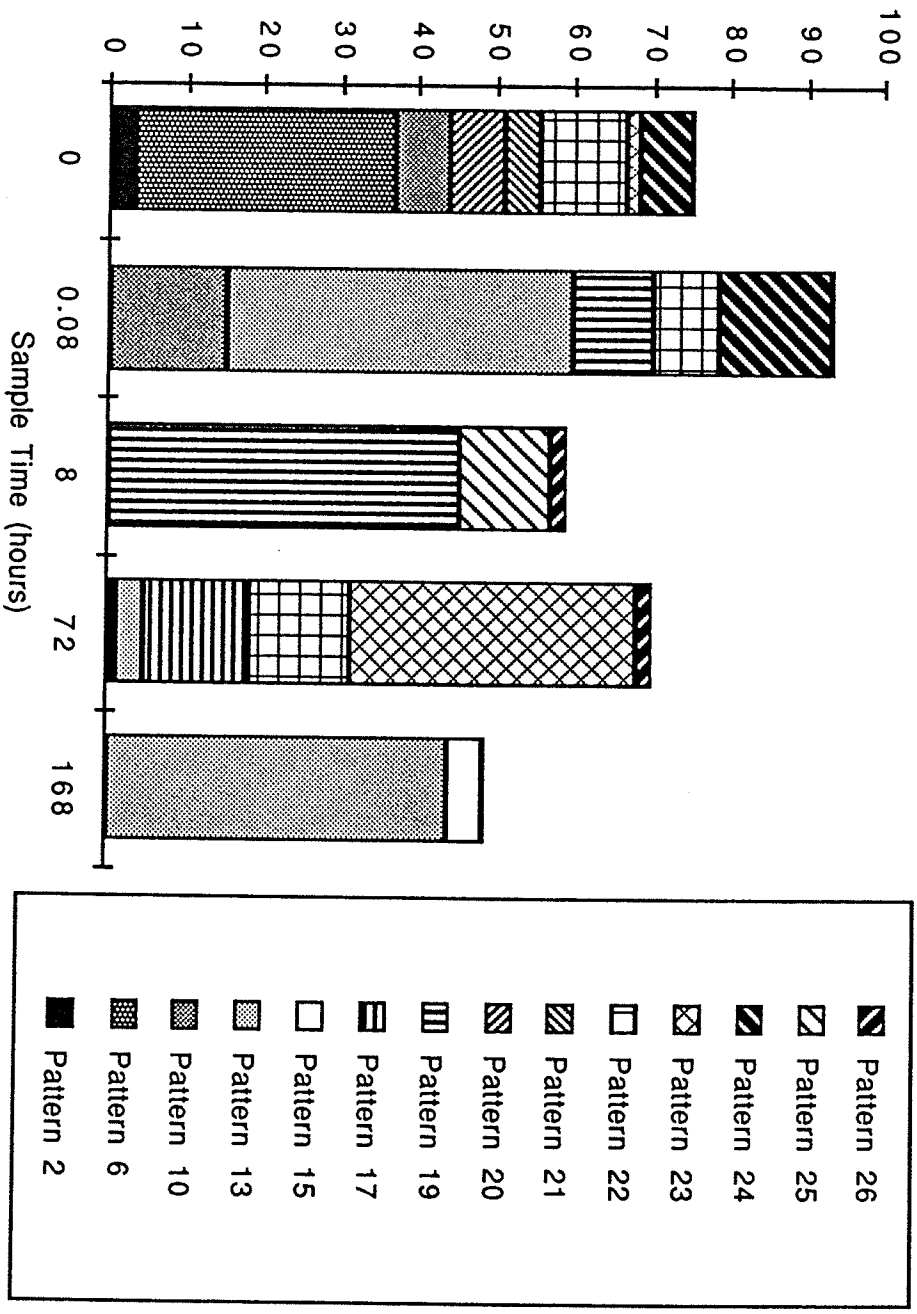
Streptococcal Patterns/Control Sites/Patient-JS



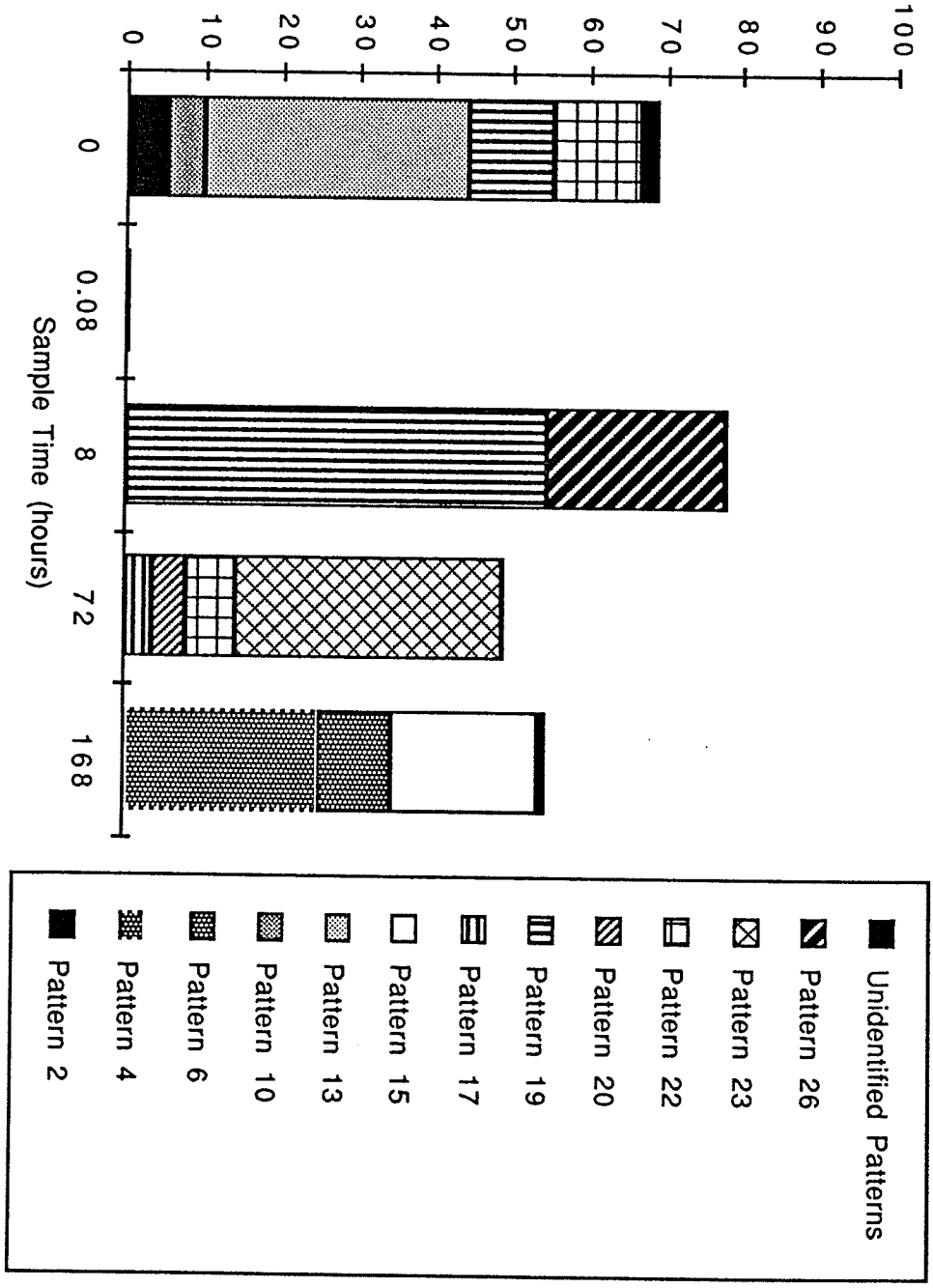
Streptococcal Patterns/Test Sites/Patient-JS



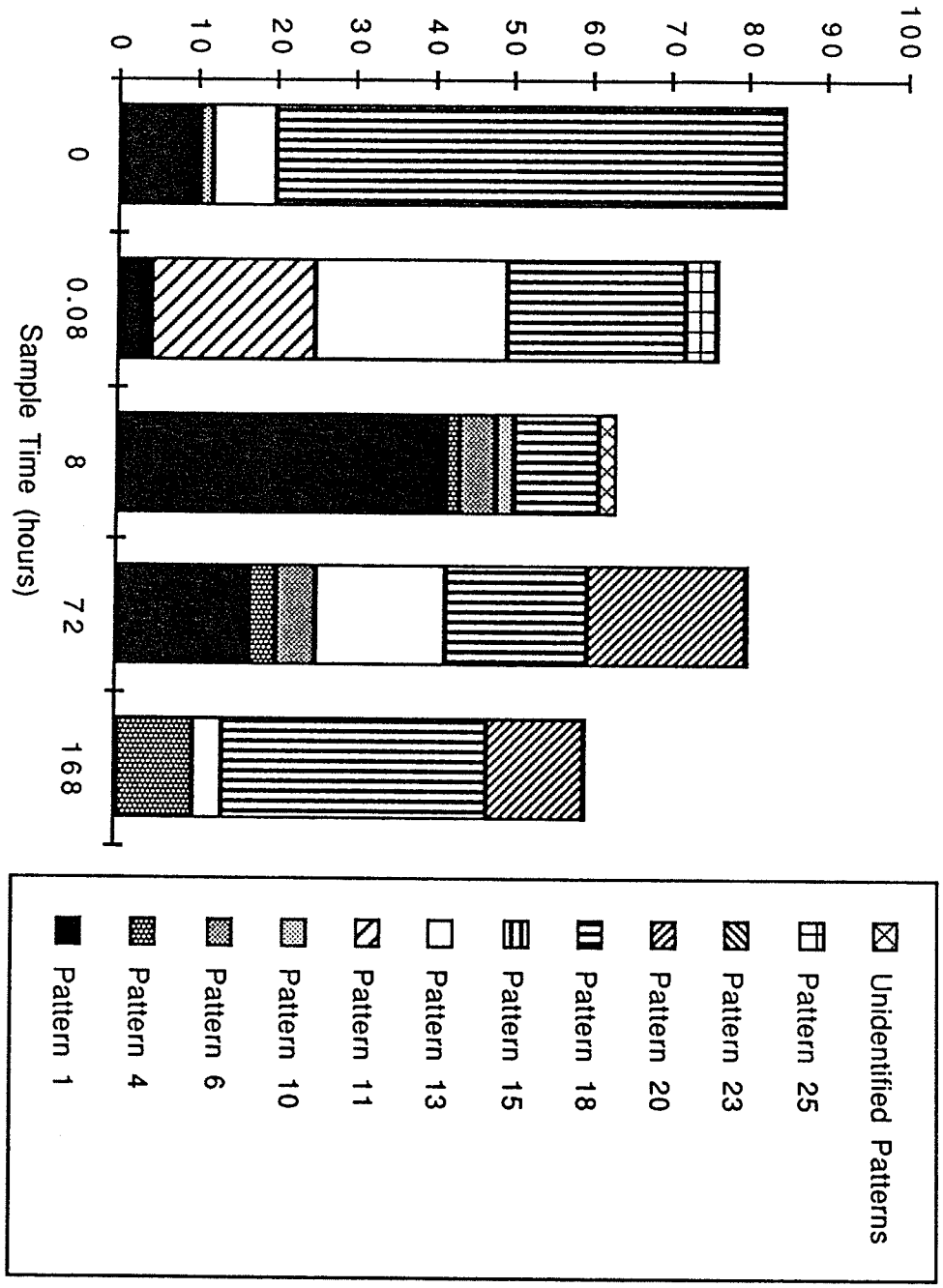
Streptococcal Patterns/Control Sites/Patient-LM



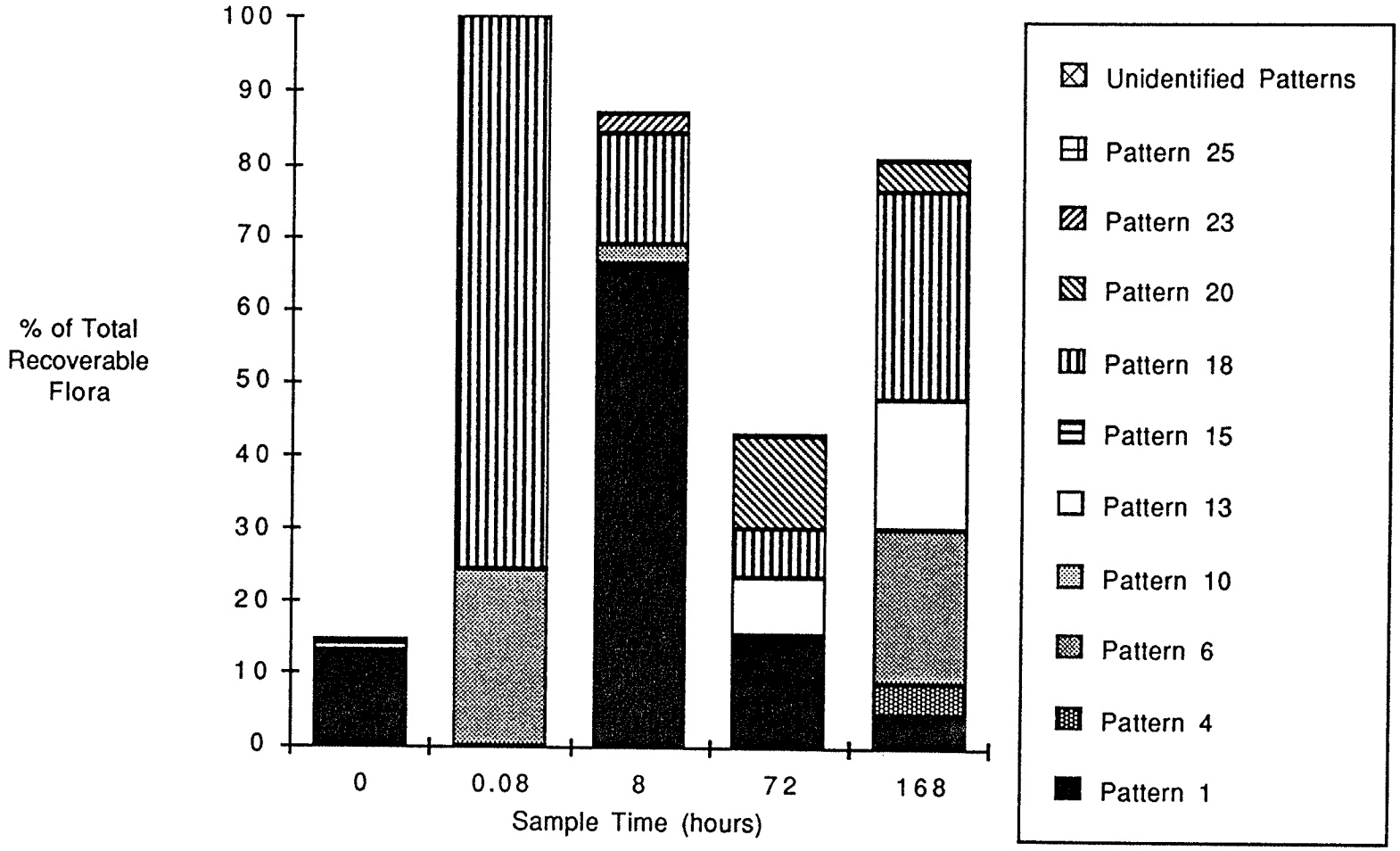
Streptococcal Patterns/Test Sites/Patient-LM



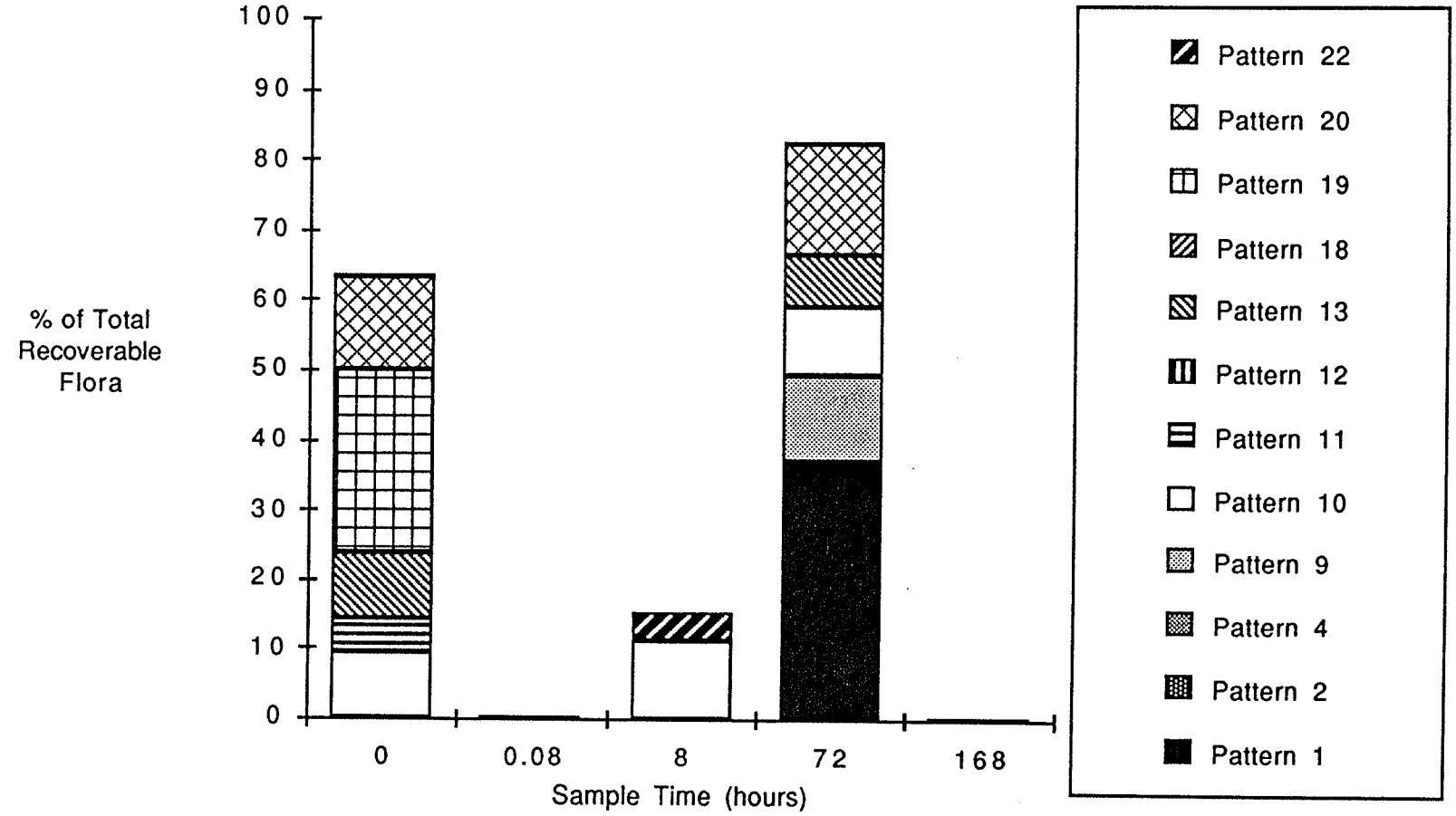
Streptococcal Patterns/Control Sites/Patient-BJ



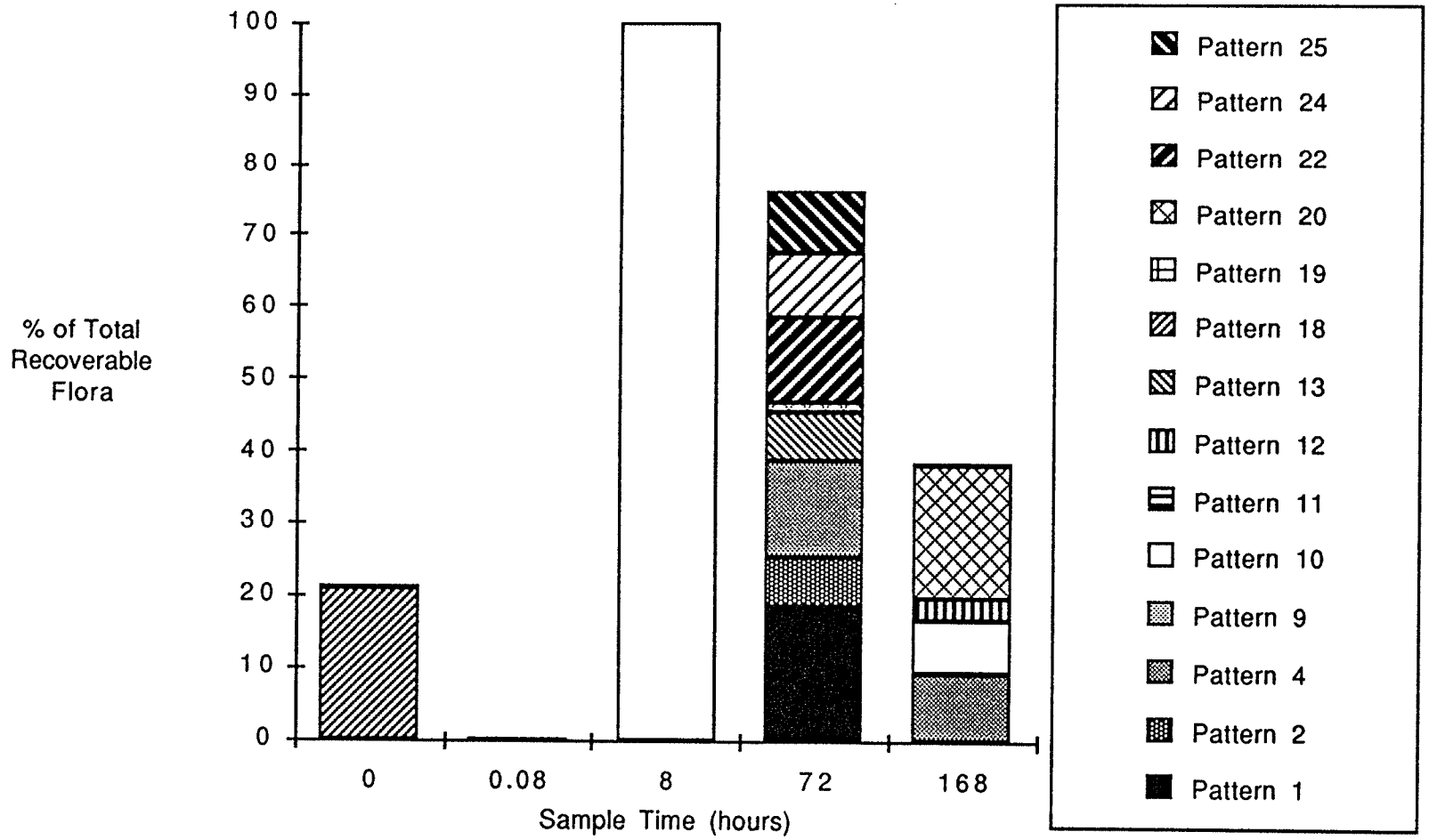
Streptococcal Patterns/Test Sites/Patient-BJ



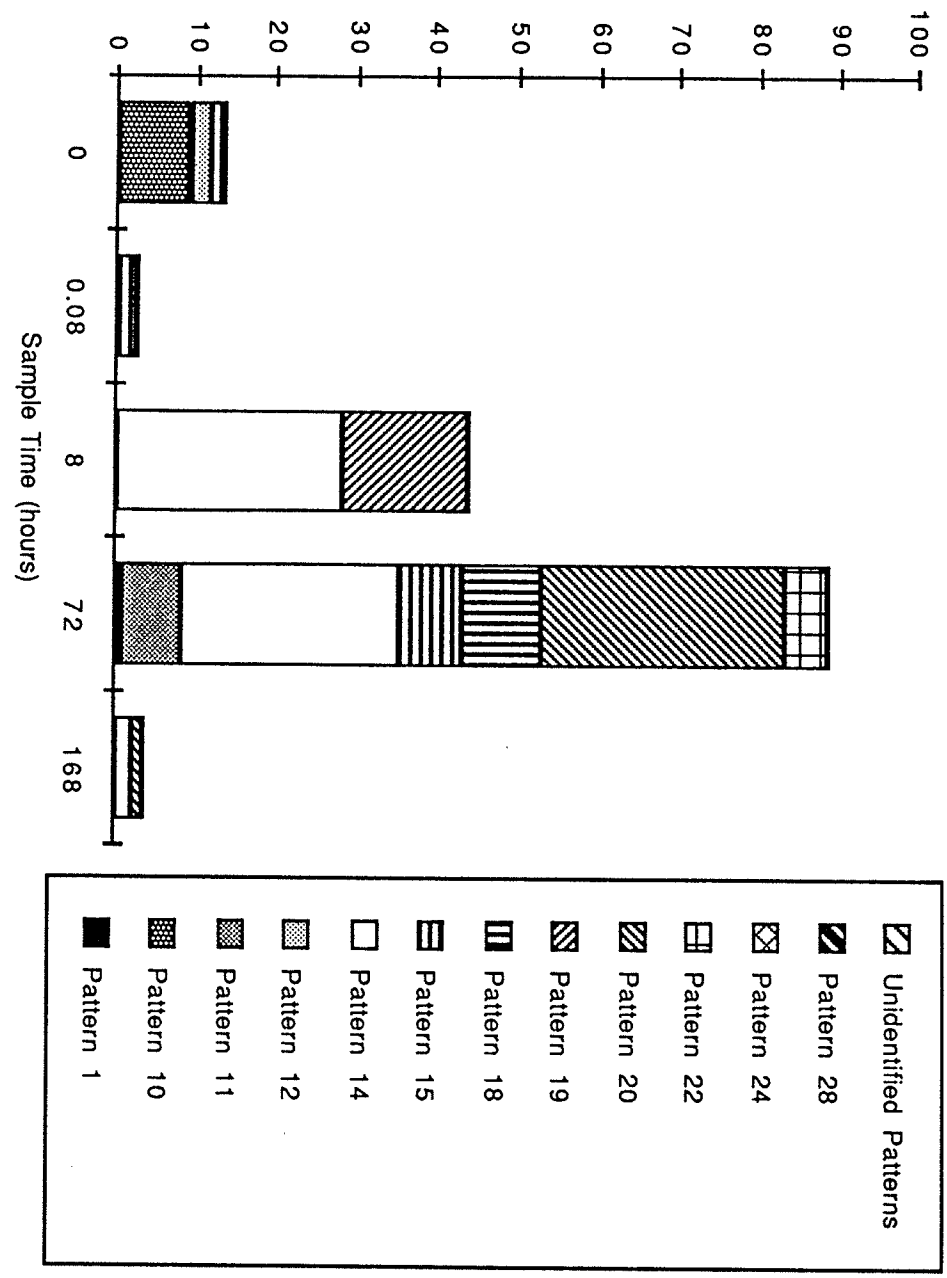
Streptococcal Patterns/Control Sites/Patient-CC



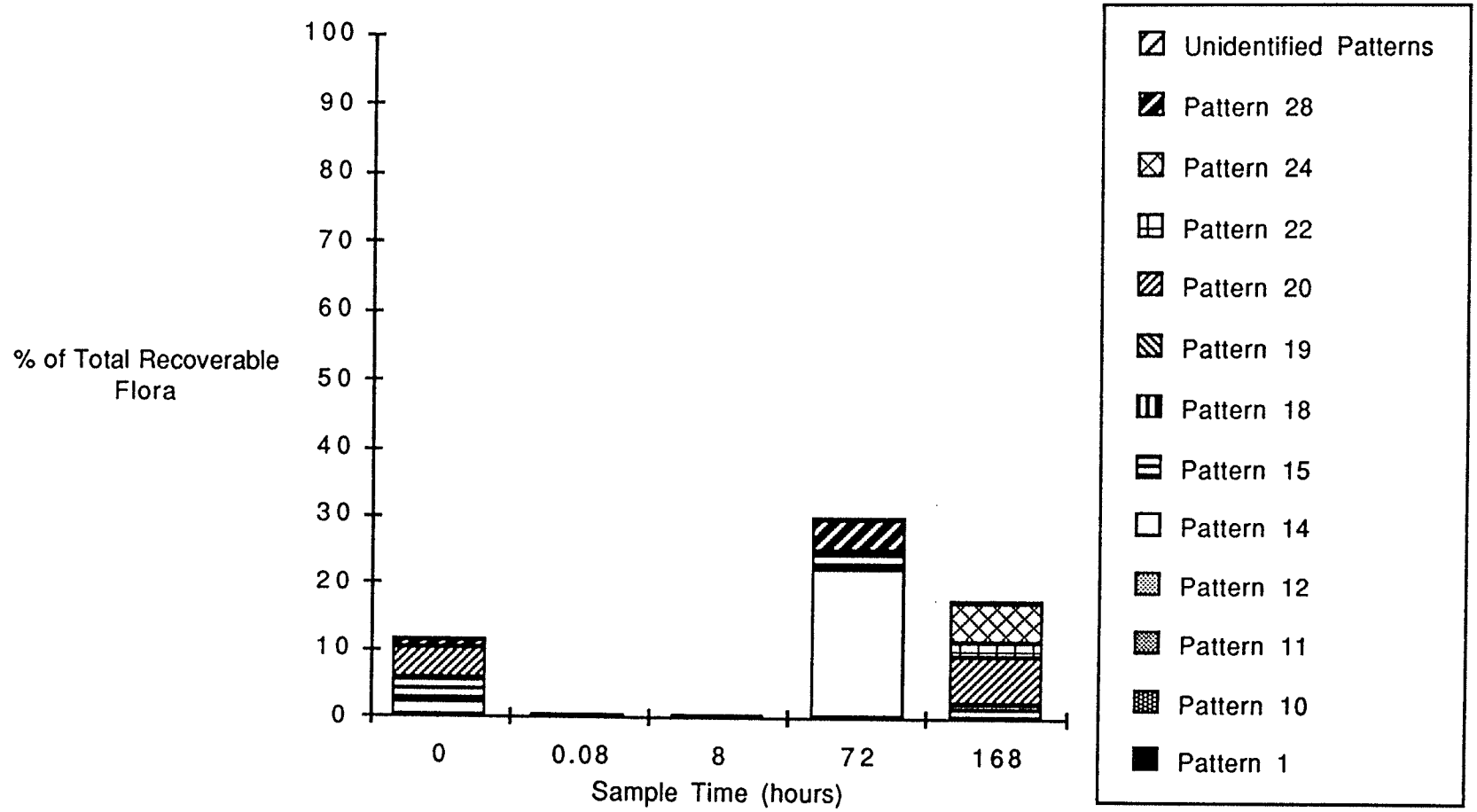
Streptococcal Patterns/Test Sites/Patient-CC



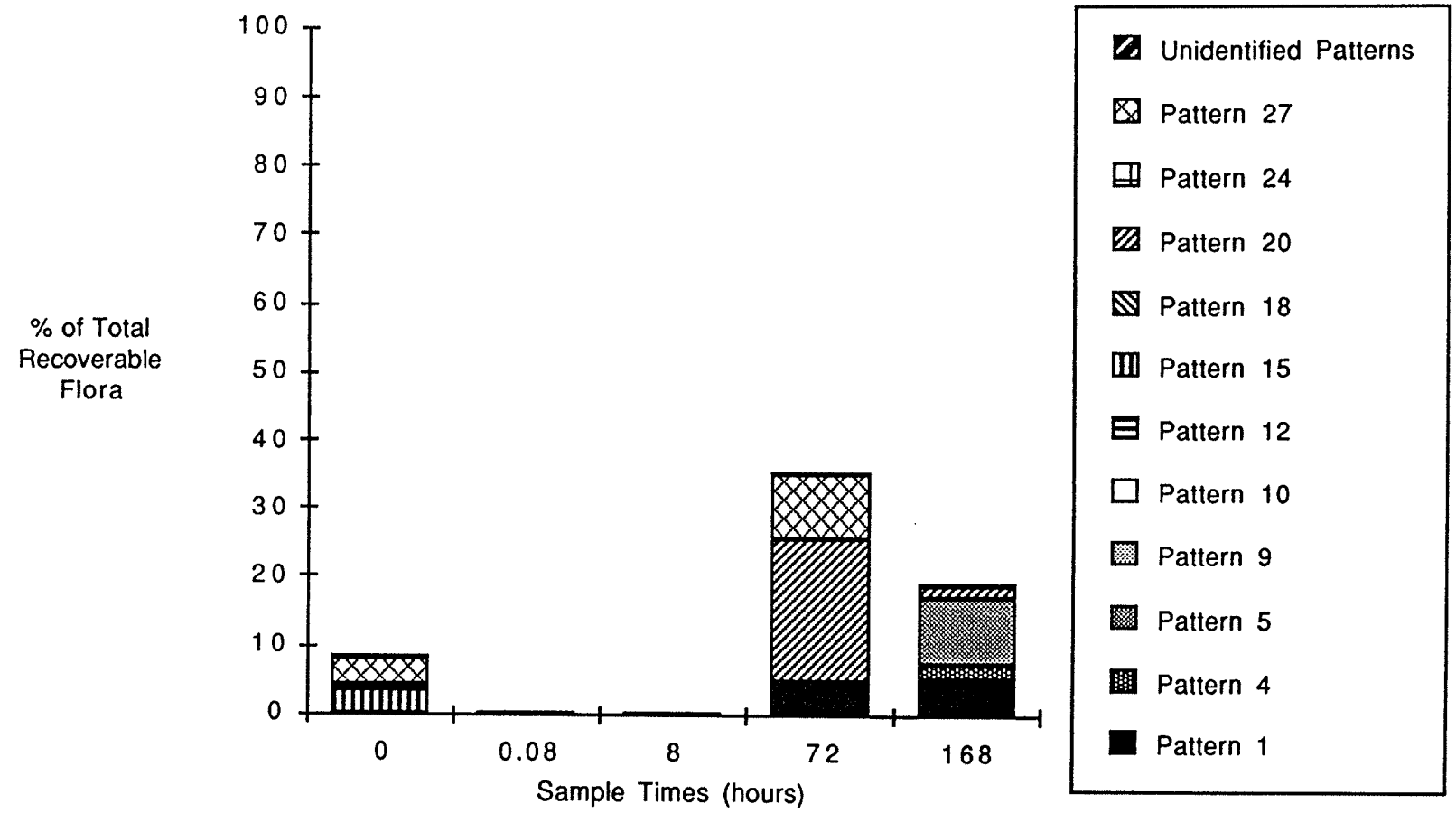
Streptococcal Patterns/Control Sites/Patient-DS



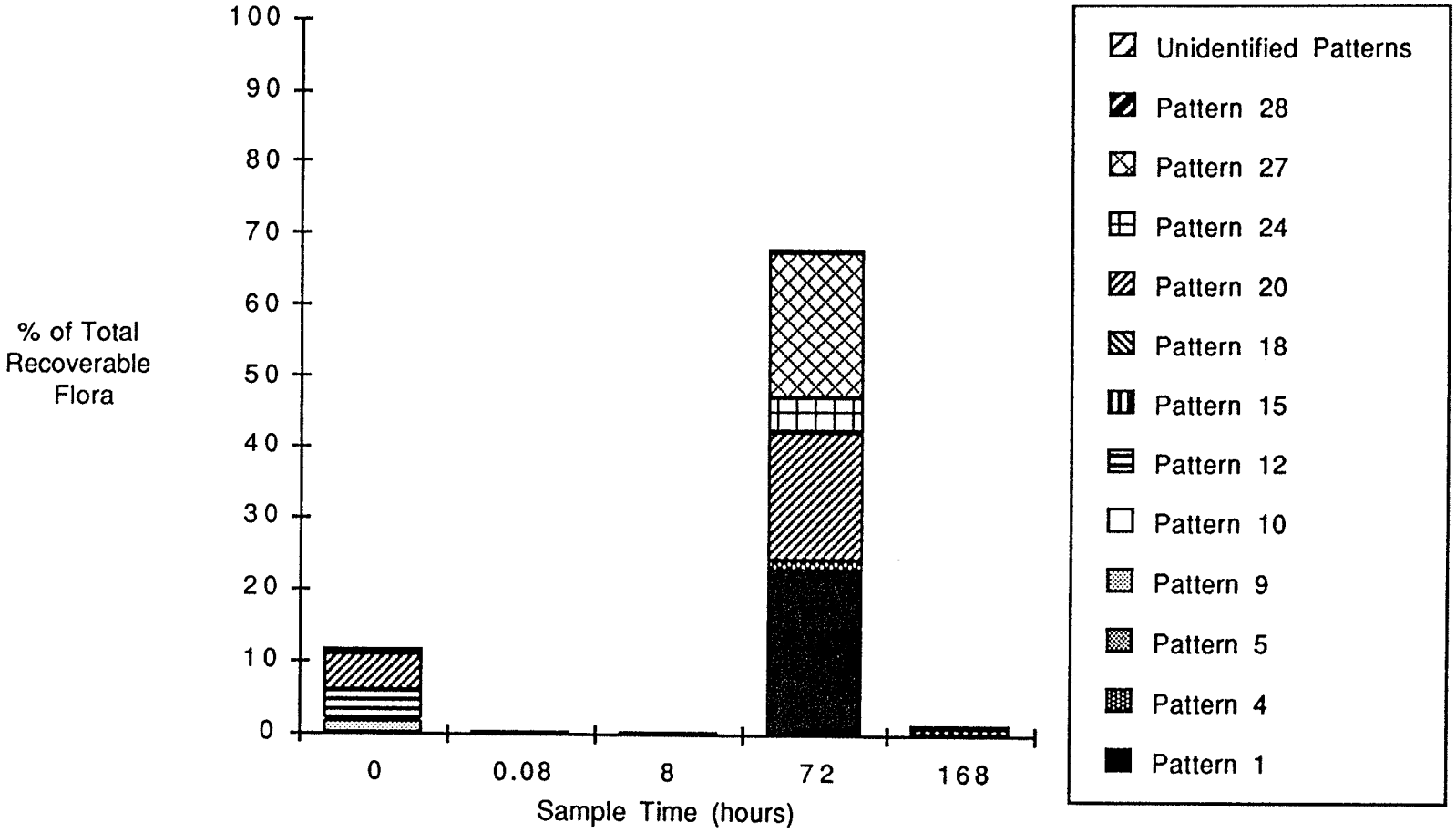
Streptococcal Patterns/Test Sites/Patient-DS



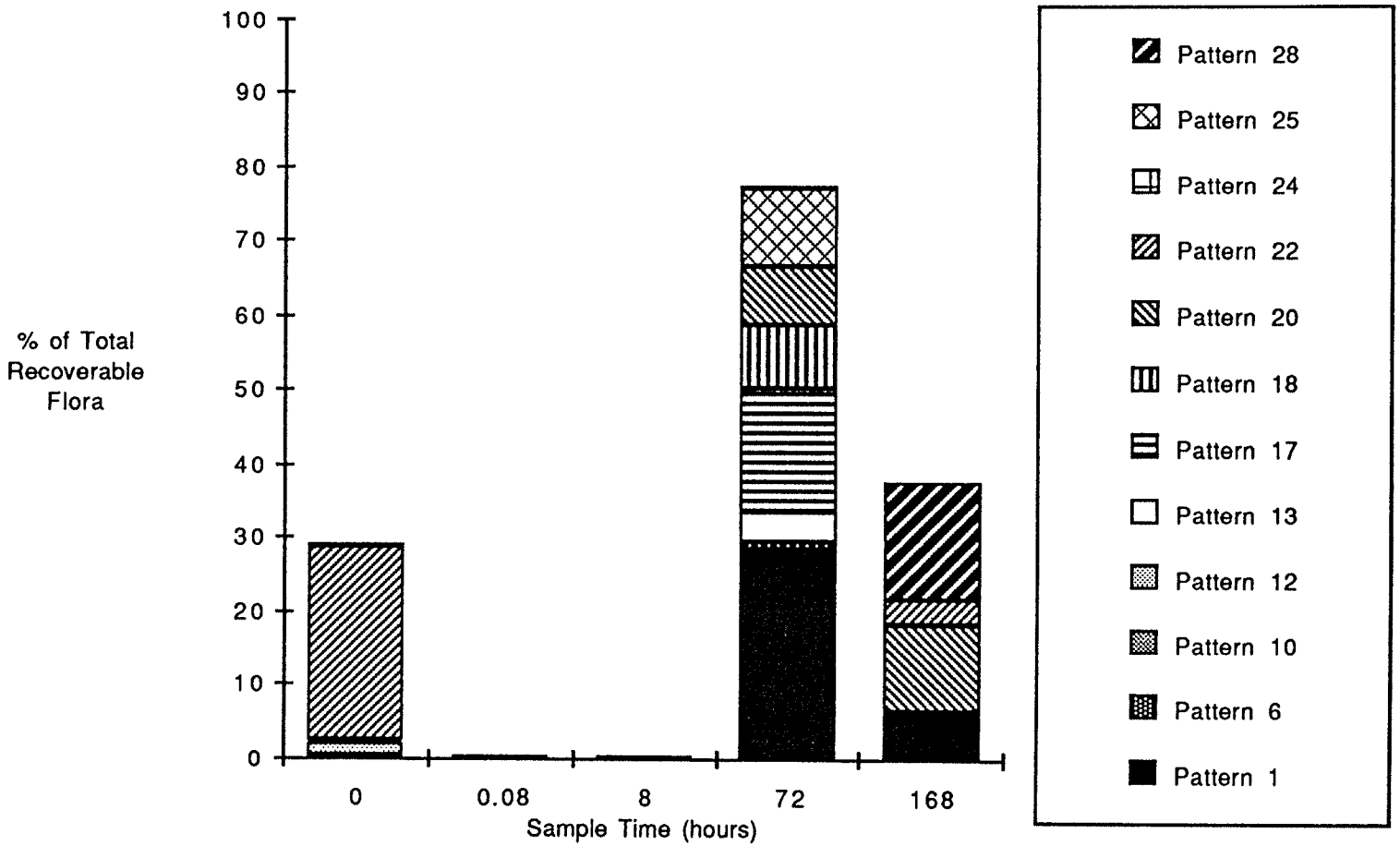
Streptococcal Patterns/Control Sites/Patient-JB



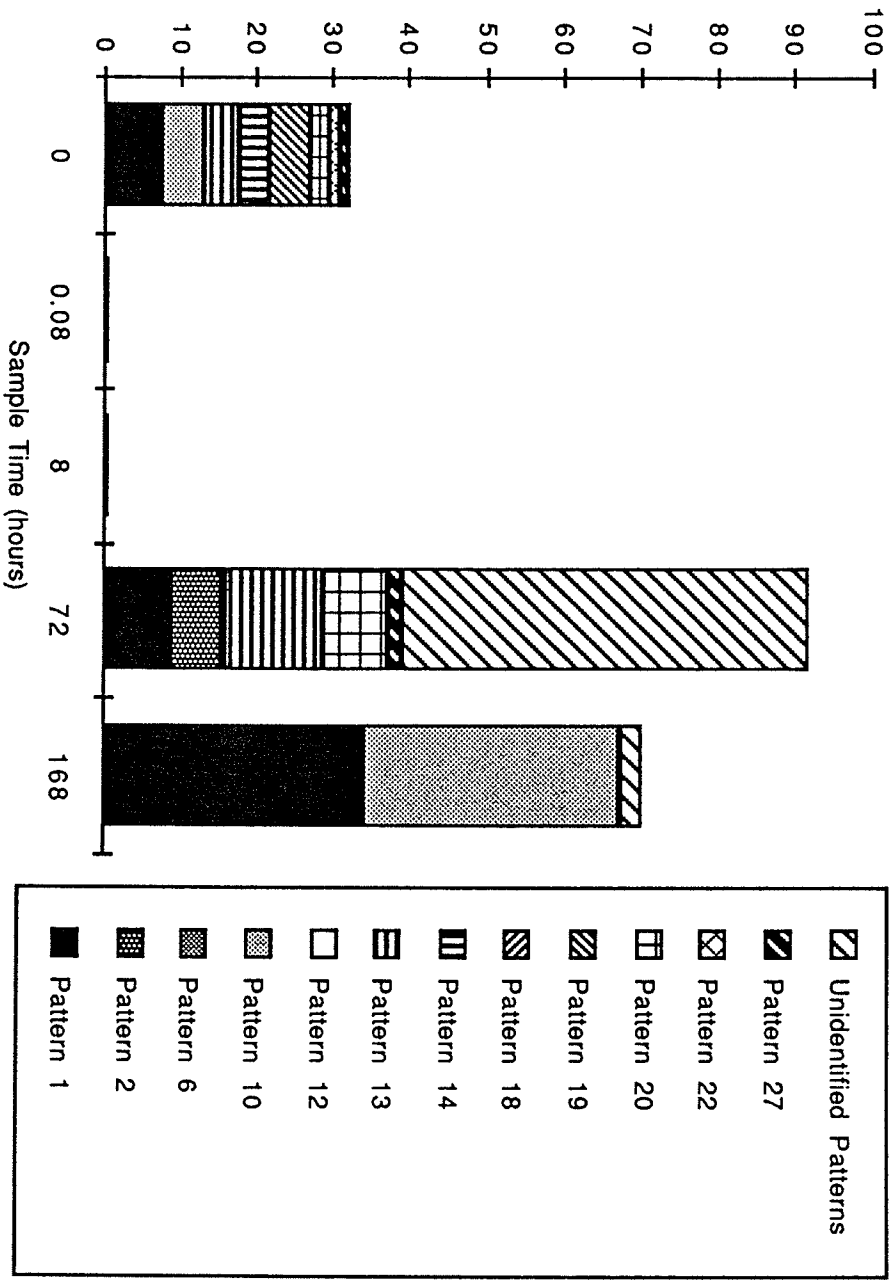
Streptococcal Patterns/Test Sites/Patient-JB



Streptococcal Patterns/Control Sites/Patient-BC



Streptococcal Patterns/Test Sites/Patient-BC



Appendix C

Streptococcal Patterns/species/taxa

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. DS4C/1352 PATTERN 1 DATE June 30/88

ORIGIN SERIES 1 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY H H_2O_2 1

SPECIES mitior Taxon ?

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose	√			
Melibiose	√			
Trehalose		√		
Amygdalin		√		
Inulin		√		

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine		√	
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin		√		
Salicin	√			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	√		
50 ppm	√	-		

API ZYM* + 2, 6, 11, 14
weak + 4, 16, 18

* see Key page 151

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. LM2C/806 PATTERN 1 DATE July 7/88

ORIGIN SERIES 2 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY H H_2O_2 1

SPECIES mitior Taxon ?

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose	√			
Melibiose	√			
Trehalose		√		
Amygdalin		√		
Inulin		√		

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine		√	
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin	√			
Salicin	weak			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	√		
50 ppm	√	-		

API ZYM		
	+	6, 10, 11
	weak +	2, 4, 12, 16, 18

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. BJ1T/936 PATTERN 6 DATE June 30/88

ORIGIN SERIES 1 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY H H_2O_2 0

SPECIES sorbitol fermenter

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol	√			
Raffinose	√			
Melibiose	√			
Trehalose	√			
Amygdalin	√			
Inulin	√			

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine	√		
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin	√			
Salicin	√			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	weak		
50 ppm	√	-		

API ZYM		
	+	6
	weak +	2, 3, 4, 11, 12,

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. DS4C/1357 PATTERN 10 DATE June 30/88

ORIGIN SERIES 1 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY S H_2O_2 0

SPECIES salivarius

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose		√		
Melibiose		√		
Trehalose		√		
Amygdalin		√		
Inulin		√		

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine		√	
Esculin	weak		

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin	√			
Salicin		√		
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	√		
50 ppm	√	√		

API ZYM		
	+	6
	weak +	3, 4, 12, 14, 16

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. JB5C/1250 PATTERN 12 DATE July 9/88

ORIGIN SERIES 3 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY S H_2O_2 0

SPECIES milleri

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose		√		
Melibiose		√		
Trehalose	√			
Amygdalin		√		
Inulin		√		

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine	√		
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin		√		
Salicin	√			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	-		
50 ppm	√	-		

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. LM1T/775B PATTERN 15 DATE July 9/88

ORIGIN SERIES 3 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY H H_2O_2 0

SPECIES sorbitol fermenter

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol	√			
Raffinose		√		
Melibiose	weak			
Trehalose	√			
Amygdalin		√		
Inulin	√			

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine		√	
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin	√			
Salicin	√			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	weak		
50 ppm	-	-		

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. BC1T/1426 PATTERN 19 DATE June 30/88

ORIGIN SERIES 1 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY S H_2O_2 3

SPECIES mitior Taxon ?

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose	√			
Melibiose		√		
Trehalose		√		
Amygdalin		√		
Inulin		√		

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine		√	
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin		√		
Salicin		√		
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	√		
50 ppm	√	-		

API ZYM + 2, 6, 11, 14

weak + 4, 7, 8, 10, 12, 13, 16, 18

STREPTOCOCCAL CHARACTERISTICS

STRAIN NO. JB4C/1213 PATTERN 20 DATE June 30/88

ORIGIN SERIES 1 GRAM STAIN + CATALASE -

HAEMOLYSIS

TYC COLONY H H_2O_2 1

SPECIES sanguis Taxon 5

SERIES A

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Mannitol		√		
Sorbitol		√		
Raffinose		√		
Melibiose	√			
Trehalose	√			
Amygdalin	√			
Inulin	√			

HYDROLYSIS TESTS	positive	negative	COMMENTS
Arginine	√		
Esculin		√	

SERIES B

CARBOHYDRATE FERMENTATION	positive	negative	pH	COMMENTS
Arbutin	√			
Salicin	√			
Dulcitol		√		
D-Xylose		√		
α–Methyl-D-Glucose		√		
Inositol		√		

Fluoride	pH			
	6.5		5.5	
	Growth	Acid	Growth	Acid
10 ppm	√	√		
50 ppm	√	√		

API ZYM		
	+	2, 6, 11
	weak +	3, 4, 5, 12, 18

Key to API ZYM/Enzyme activity tests

Number	Enzyme
1	-(control)
2	alkaline phosphatase
3	esterase
4	esterase lipase
5	lipase
6	leucine aminopeptidase
7	valine aminopeptidase
8	cystine aminopeptidase
9	trypsin
10	chymotrypsin
11	acid phosphatase
12	phosphohydrolase
13	α-galactosidase
14	ß-galactosidase
15	ß-glucuronidase
16	α-glucosidase
17	ß-glucosidase
18	N-acetyl-ß-glucosaminidase
19	α-mannosidase
20	α-fucosidase