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Subgingival Microflora Associated With Puberty

Jayne E. Delaney

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Master of Dental Science

SUBGINGIVAL MICROFLORA ASSOCIATED WITH PUBERTY

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1984
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Jayne E. Delaney
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Introduction

Periodontitis is a chronic, inflammatory disease which primarily affects adults. It is bacterially mediated and is thought to begin as a chronic gingivitis, which is reversible, and to spread into an irreversible periodontitis. Although children almost universally exhibit gingivitis, periodontitis is rarely detectable until after the age of 30 and even the low percentage of cases classified as localized juvenile periodontitis do not appear to develop until after puberty. This has been explained traditionally on the basis of the time of exposure to the plaque bacteria; however, recent studies indicate major differences between children and adults in terms of the clinical gingival response to plaque and the histopathologic features of gingivitis. Early studies have also indicated that at least one organism which is associated with adult periodontitis is not detectable in children. These bacteriology studies suggested that the subgingival microbiota changes around the time of puberty, but they did not use techniques which would allow optimal recovery of anaerobic bacteria and they were performed before it was possible to differentiate some important species of Bacteroides. Recent studies, using current microbiological techniques, report few bacteriological changes with pubertal development.

The present study examined the subgingival bacterial flora in children around the age of puberty. Since puberty is actually a range of time in which many complex changes take place in the host, one must evaluate several different parameters to accurately assess developmental age. Therefore, the predominant cultivable microbiota
was correlated to stages of skeletal, dental, and sexual maturation, as well as clinical indices of gingival health, to determine if ecological changes in the composition of the subgingival microbiota occur in association to development.
Review of the literature

The relationship of age to bacterial disease susceptibility

Many common infections have a more common occurrence in either children or adults, but not both. For example, sexually transmitted diseases are more common in adults, due to an increased exposure to the etiologic organisms. On the other hand, the extremely high incidence of otitis media in young children may be based more on anatomy and immunology than on exposure. The Eustachian tube is more easily obstructed in the young child, as there is less cartilage support. Also, the shorter Eustachian tube is horizontally placed, rather than at a 45 degree angle: mucous from the nasopharynx during upper respiratory tract infections easily contaminates the middle ear. Moreover, aerobic and anaerobic organisms isolated from middle ear infections were commonly part of the normal oropharyngeal microflora (Brook, 1979; Brook and Schwartz, 1981). Susceptibility to otitis media, as well as to other bacterial and viral infections, may also be due to the immunological immaturity of young children. Infections such as diphtheria, pertussis, measles, and mumps are most severe in infancy and become progressively less severe as the child enters adolescence and adulthood. This is due to acquired immunity.

Tinea capitis, commonly known as ringworm infection of the scalp, is a disease of children, and is very rare after puberty. Two species of fungus are commonly associated with the infection, Microsporum audouinii and Microsporum canis, although many species of fungus can cause ringworm. The hairs of the scalp are invaded by the fungus,
altering their structure and causing them to break. Scaling of the scalp skin occurs as keratin is affected. Interesting from the standpoint of developmental interaction with infection, ringworm infections resolve spontaneously at puberty. This may be explained by the local physiologic changes of the skin which occur with puberty, namely, the marked increase in sebaceous secretions. Fatty acids found in these secretions are fungistatic. Conversely, this same physiologic change of skin at puberty is positively related to the occurrence of acne after puberty. Acne vulgaris, the most common disease of the skin, affects more than 80% of the population, usually during the second and third decades of life (Report of Study Group, 1979). Acne tends to be more severe among boys than girls, since androgen production increases to a higher level in males. Androgens directly stimulate the sebaceous glands; they increase in size and increase in production of their holocrine product, sebum. The sebum provides the nutrients for proliferation of the anaerobic diphtheroid Propionibacterium acnes, which produces a lipase that hydrolyzes the triglycerides of sebum to free fatty acids. Free fatty acids are then responsible for the inflammation in acne lesions. Also, unsaturated fatty acids found on the skin stimulate growth of P. acnes in vitro, creating a vicious cycle. The role of hormones in the pathogenesis of acne can be demonstrated in altered hormone conditions. Oral contraceptive pills influence acne. There is often an improvement in acne when a high estrogen pill is taken, while the androgenic, anti-estrogen activity of some of the synthetic progesterone pills, combined with low estrogen levels, exacerbates acne. In addition, premenstrual flares of acnes may occur from puberty to menopause.
Susceptibility of females to vaginal tract infections also changes with age and hormonal development. Prepubertal girls and post-menopausal women rarely develop Candida vaginitis. The highest incidence of the condition is in diabetic women, pregnant women and in women using oral contraceptive pills for one year or more. From puberty until menopause, under the influence of estrogen, glycogen increases in the vaginal mucosa. Prior to recent studies utilizing anaerobic techniques for cultivation of the vaginal flora, the normal vaginal flora was thought to consist primarily of an aerobic lactobacillus, which after puberty was able to colonize the vagina, metabolize the glycogen, and produce lactic acid. Lactic acid thus created a vaginal pH of about 5.0, thought adequate to prevent colonization of most other bacteria. We now know that the vaginal flora of women and children is quite complex, consisting primarily of anaerobic organisms (Bartlett et al., 1977; Hammerschlag et al., 1978). The elevated glycogen levels in the vaginal epithelium, formerly thought to selectively promote growth of Lactobacillus species, can favor the growth of Candida. Mead (1982) proposed that Candida vaginitis is primarily an infection of the vaginal secretions rather than an inflammation of the vaginal wall, since the condition occurs only in a setting where vaginal secretions are either normally or exogenously increased. Thus vaginal secretions can be likened to the gingival crevicular fluid in bathing bacterial components and influencing composition of the flora.

**Relationship of age to periodontal disease**

Gingivitis is defined as an inflammation of the gingiva, which is
the soft tissue surrounding the tooth extending to the mucogingival junction. Gingivitis is the most prevalent form of periodontal disease found in children; it can be considered to be ubiquitous in the age range of 6 to 17. Parfitt (1957) found the prevalence of gingivitis to increase as a function of age: below 5% at age 3, 50% at age 6, with a peak of 90% at 11 years of age. Between the ages of 11 and 17, the level fell slightly, but remained between 80% and 90%. The prevalence and severity of gingivitis reaches a peak at different ages for boys and girls: the peak for girls occurs at age 11 and for boys, at age 13 (Parfitt, 1957; Sutcliffe, 1972). Thereafter, the prevalence falls slightly, and the severity of the inflammation falls sharply. These differences may reflect difference based on sex, or may reflect a difference in oral cleanliness.

In spite of the almost universal occurrence of gingivitis in children, periodontitis, as defined by loss of attachment, is uncommon in children under the age of 16. Epidemiologic studies which relied solely on probing depths reported a relatively high incidence of children with one or more pockets >3mm of depth. Downer (1970) found that 24% of 11-14 year old English school children had deepened periodontal pockets; Sheiham (1969) examined a similar population, but tried to disregard the "swelling" of the gingiva due to gingivitis; he reported deepened pockets in 11% of the children. Lennon and Davies (1974) examined 15 year-old English school children for loss of periodontal attachment, and reported a frequency of 41% of the children with >1 mm loss of attachment. These investigators, in their attempt to report the prevalence of incipient periodontitis, were most likely overestimating the frequency of occurrence of true periodontal
pockets in children due to the high levels of gingivitis found in these school children. Hull et al. (1975) and Davies et al. (1978) used radiographs to study alveolar bone loss in 11 to 14 year old children. The criteria used in determining periodontal bone loss included: 1) crestal bone >3mm from the cemento-enamel junction; 2) crestal irregularity (notching); or 3) widened periodontal ligament space. These studies reported periodontal bone loss in 44% to 51.5% of the children. However, based on our current understanding of the normal radiographic appearance of bone, and on the variable appearance of bone surrounding erupting and newly-erupted teeth, only the first criterion is valid in determining the prevalence of periodontitis in school age children. Re-analyzing data reported by Hull and coworkers (1975), only 5/602 children had crestal bone >3 mm from the cemento-enamel junction, giving an incidence of periodontal bone loss of 0.8% in 14 year-old English schoolchildren (Blankenstein et al., 1978). This finding is consistent with a similar study done by Blankenstein et al. (1978) of 1645 English and Danish children from 13 to 15 years of age. This group found that radiographic evidence of periodontal bone loss occurred in <1% of the children.

In contrast to the low incidence of true periodontal attachment loss in children below the age of 16, adults (i.e., over age 18) do have significantly more periodontal bone loss associated with periodontitis. Three out of four American adults with a natural dentition have some form of periodontal disease and one out of four are in danger of losing their teeth due to periodontitis (National Center for Health Statistics, 1971).

The character of periodontal disease also appears to differ in
adults and children. Matsson (1978) found a marked difference in the development of gingivitis in preschool children and young adults, using a 21 day experimental gingivitis protocol. Development of gingivitis in six children from 4 to 5 years of age was compared to that in six dental students from 23 to 29 years of age. Because of the difference in the normal gingival margin between primary and permanent dentitions, Matsson used objective methods to score gingivitis, such as registering the amount of gingival exudate, the amount of crevicular leukocytes, and the number of bleeding units after probing with a standardized pressure. These methods were intended to reflect vascular as well as cellular changes associated with the gingival inflammation. As the amount of plaque levels increased in both groups, the amount of gingival exudate and the tendency to gingival bleeding increased to high values in the adults, while only a small rise was seen in the children. Comparing gingival units with a similar plaque development, there was a real difference in the tendency to develop gingivitis between preschool children and adults. Similarly, Mackler and Crawford (1973) found that six of eight preschool children that they studied failed to develop gingivitis during 26 days of an experimental gingivitis protocol.

Löe and coworkers (1978) studied the rate of periodontal bone destruction from adolescence to 40 years of age in a group of Norwegians and in a group of Sri Lankans. Loss of attachment progressed with age in both groups, and was more severe and rapid in the Sri Lankans than in the Norwegians. The 15 year-old Sri Lankans also had a far greater incidence and severity of periodontitis than that found in Norwegian, English, or Danish children: 30% exhibited
localized lesions measuring between 2 and 9 mm and more than 1% had one or more root surfaces with 10 mm loss of attachment or more. Morphological differences exist between the periodontium of children and adults. Zappler (1948) described the child's periodontium as follows: "The gingiva is more reddish, lacks stippling, is flabbier, has rounded or rolled margins, and has a greater sulcular depth. The cementum is thinner and less dense. The periodontal membrane is wider, the fiber bundles are less dense per unit area, and there is increased hydration, and a greater blood and lymph supply. The alveolar bone has a thinner lamina dura, fewer trabeculae, larger marrow spaces, decreased degree of calcification, a greater blood and lymph supply, and flatter alveolar crests associated with the primary teeth." Ruben and coworkers (1971) noted that the marginal gingiva of the child does not have the dense, well-oriented and organized collagen fiber systems seen in adult gingiva, but rather consists of numerous more delicate collagen and reticulum fibers, deficient in the "bundle" arrangement evident with adulthood. Incompletely erupted permanent teeth had a long epithelial adherence to the enamel surface, and the gingival wall, from the base of the epithelial attachment to the gingival crest, was relatively flaccid. In another study, the differences in lymphocyte and plasma cell densities in biopsies of inflamed gingiva from adults and young children were examined (Longhurst et al., 1977). Their data showed that the adult tissue had a considerably greater density of plasma cells than the child tissue while the lymphocyte density in the two age groups was similar. The results indicated that periodontal disease in the deciduous dentition differed from the disease in the adult in that the disease did not
progress to the plasma cell-dominated lesion of established gingivitis found in adults. However, in a more recent study of gingival biopsies from children during the deciduous dentition, Longhurst's group quantitated the inflammatory cell infiltrates in gingival connective tissue using electron microscopy, and found that the childhood lesions did have much in common with the early lesion of adult gingivitis, as there was an apical distribution of transformed B-lymphocytes identified mainly as plasma cells (Longhurst et al., 1980). Lymphocytic cells contributed two-thirds of the cells of the connective tissue infiltrate compared with a little over three quarters found by Schroeder et al. (1973) in infiltrates from gingiva of permanent teeth in adolescents.

In summary, children differ from adults in incidence of periodontitis, in their clinical response to plaque accumulation, in their periodontal anatomy, and in their immunological response to subgingival plaque.

**Etiology of periodontal diseases**

Bacteria play a major role in the etiology of gingivitis and periodontitis. Initial observations (Lovdal et al., 1958) indicated a relationship between dental calculus and periodontal disease. Calculus was thought to play a role as an irritant, with other local and host factors also playing a part. Waurhaug (1956) challenged the postulation that periodontal disease was caused by rough dental calculus which resulted in inflammation of the connective tissue in unclean mouths. He examined the healing of the periodontal tissues of experimental animals after producing a rough subgingival surface with a diamond point, and compared the histologic appearance of brushed
versus unbrushed sides of the mouth. He concluded that there was greater inflammation of the connective tissue on the unbrushed sides, indicating that it was not the rough surface that irritates the tissue, but the bacteria or their toxins which were found in plaque that had been retained on the rough tooth surface. Epidemiological studies, such as the Norwegian study of male employees of a manufacturing company (Lovdal et al., 1958), demonstrated that with increasing age, the men had greater amounts of calculus, less effective toothbrushing, and a greater incidence of periodontal destruction, thus supporting the relationship of dental plaque and age to the etiology of periodontal disease. Rosebury (1953) concluded that other etiological factors occurred in periodontal disease, including local trauma, food stagnation and impaction, nutritional deficiencies, endocrine factors, emotional factors, toxic factors, and predisposing infections. He proposed that there was a dynamic architectural balance of the periodontium, and that disease results if the equilibrium shifts due to the factors listed above.

Clinical Studies Relating Periodontal Disease with a Microbial Etiology:

As a result of initial observations that plaque plays an important role in the etiology of periodontal disease, Ash et al. (1964) began the first of many investigations to study the effect of plaque on gingival health. These early studies involved clinical observations on the effects of oral hygiene and accumulation of plaque and calculus on the development of periodontal disease. Ash found, for example, that even in subjects who had a professional prophylaxis, both plaque and gingivitis scores increased with time during a 60-day
observation period, in spite of subjects following their usual oral hygiene regimen. There was a high positive correlation between the degree of plaque accumulation and the degree of gingivitis in these adults. Löe and his coworkers (Theilade et al., 1966; Löe et al., 1967) extended the work of Ash et al. and developed experimental protocols which examined the sequence of gingival changes after oral hygiene measures were discontinued in young adult subjects who had previously obtained Plaque Indices and Gingival Indices approaching zero. Subjects developed clinical gingivitis within 9 to 21 days in the absence of oral hygiene; this correlated with plaque accumulation. However, when oral hygiene was re-instituted, gingival health was restored within one to two days of the removal of plaque. In another study involving young adults, Löe et al. (1967) went one step further to demonstrate a relationship between plaque and gingivitis; he showed that when subjects rinsed with antibiotics during their period of no oral hygiene, they experienced decreased plaque accumulation. In addition, when compared to controls, they also had less evidence of gingivitis, as evidenced by a decreased amount of gingival exudate, and a decreased leukocyte emigration rate. Comparable clinical results were achieved with topically-applied tetracycline, a broad spectrum antibiotic; with vancomycin, which selectively suppresses Gram-positive bacteria; and with polymycin B, which suppresses Gram-negative organisms. Thus, gingivitis exhibited a correlation with the amount of plaque rather than to specific bacterial segments of the plaque. Silness and Löe (1964) provided additional evidence that there was a positive correlation between plaque and the etiology of periodontal disease. In their study of the the levels of gingivitis,
plaque, and calculus in pregnant and post-partum subjects, they
concluded that the accumulation of plaque paralleled the gingival
changes during pregnancy, while calculus did not. Suomi et al (1971)
tested the hypothesis that after initial prophylaxis, the development
and progression of gingival inflammation and destructive periodontal
disease are retarded in an oral environment with high oral hygiene
levels. Results in this study of matched subjects indicated that
persons instructed in good oral hygiene and given frequent prophylaxes
have cleaner teeth, less gingival inflammation, a slower rate of
apical migration of the epithelial attachment than the controls.
Finally, laboratory studies using Beagle dogs have also given results
indicating that good cleaning of the teeth will result in less loss of
attachment than in uncleaned areas. (Saxe et al, 1967; Lindhe et al,
1975.)

Microbiological Studies Relating Periodontal Disease with a Microbial
Etiology:

Early microbiological studies of the pathogenicity of dental
plaque supported the conclusion that it was the mass of plaque which
determined the amount of disease produced. Plaque collected from
human subjects caused infection in guinea pigs when inoculated
subcutaneously, whether the plaque was taken from subjects with
periodontal disease or from controls who were periodontally healthy
(Rosebury et al, 1950). Microscopically, the plaque samples showed a
similar distribution of bacteria from diseased and healthy mouths,
which appeared about identical. However, there was an apparent
quantitative difference between the two groups. Similarly, Socransky
et al. (1963) found that subgingival plaque from both clinically
healthy and periodontally involved subjects showed no differences in microscopic appearance. They employed both microscopic counts and viable counts, and found the same numbers of morphologic types, with the exception of spirochetes. However, since spirochetes comprised only 1% of the flora in their samples, the authors discounted the role of spirochetes in the etiology of periodontal disease. In these early microbiological studies (Rosebury et al., 1950; Gibbons et al., 1963; Socransky et al., 1963), bacterial samples taken from patients with or without periodontal disease revealed no organisms unique to disease and no real proportional differences in the predominant floras of the two groups.

These studies suggested that periodontal disease, especially gingivitis, results from plaque accumulations. Taken together, the evidence appeared to support the "Nonspecific Plaque Hypothesis", suggested by Loesche (1976), who postulated that all bacterial deposits on the teeth (i.e., plaque) have equal potential to induce disease. Loesche attributed disease to an increase in accumulated plaque deposits which elaborate more toxic products than the host can detoxify without tissue damage. An etiological concept based on the Nonspecific Plaque Hypothesis dictated that disease could be prevented or treated by maintaining plaque mass below the critical threshold (Loesche, 1976). The numerous studies (Suomi et al., 1971; Lindhe et al., 1975) which showed a correlation between a reduced Plaque Index and an absence of disease supported this hypothesis.

However, refined microbiological techniques and new ways of looking at disease development, revealed some evidence that specific bacteria are involved in certain forms of periodontal disease. Two
studies, Loesche et al., (1971) and Loesche and Nafe (1973) stand out: these studies demonstrated that short-term intensive topical kanamycin treatment for 5 days every fifth week for 40 weeks could produce a reduction in gingivitis which persisted for up to 12 weeks after cessation of all treatment, including plaque control. Streptococci levels were reduced from 30% of the cultivable microflora before treatment to 3% 4 weeks after the topical kanamycin treatment, although differences in plaque weight were no longer apparent. This suggested that following antibiotic treatment, recolonization of the dental surfaces involved organisms which had a reduced potential for disease. It has also been noted that the clinical symptoms of an acute form of periodontal disease, acute necrotizing ulcerative gingivitis, can be alleviated by the use of systemic penicillin (Schuesler et al., 1945) or metronidazole (Shinn, 1962), supporting further microbiologically directed disease. The effectiveness of metronidazole is interesting from the standpoint that the drug is active against anaerobic Gram-negative bacteria but not aerobic bacteria.

The plaque flora appears to be spacially stratified. Major gains in our understanding of the relationship between bacteria and periodontal disease and plaque formation came from the ingenious studies of Newman et al. (1974) and Listgarten (1976). These investigators employed electron microscopy, darkfield microscopy, and cultural techniques. Listgarten (1976) demonstrated with electron microscopy of extracted teeth with gingival health, gingivitis, periodontitis, and juvenile periodontitis, the stratification of the microbiota associated with tooth surfaces, both in a corono-apical and
a plaque surface-to-tooth direction, with spirochetes and loosely adherent motile bacteria apparently dominating at the front (apical aspect) of the lesion. Furthermore, Listgarten et al. (1975), in a remarkable experiment showed that when clean epoxy resin crowns were implanted in human mouths, dental plaque consisting of bacteria developed with time in the same spatial relationship. Not only does the plaque vary with location on the tooth, but also with site in the mouth, depending on the disease status of the individual teeth (Listgarten and Hellden, 1978; Slots, 1976.)

Subgingival microbial flora

In recent years, several studies (Slots, 1976; Slots, 1977; Slots, 1978; Newman and Socransky, 1977) have overcome the technical difficulties in anaerobic microbiology to demonstrate apparent proportional differences in the flora associated with different clinical forms of periodontal disease. These studies represent the first successful efforts to define a disease-associated flora, but they are limited in number and the pathology at the sample site generally has been poorly defined.

Gingival health:

The healthy gingival sulcus is dominated by Gram-positive facultatively anaerobic coccii and rods which account for approximately 75% of the cultivable flora. In studies on 7 plaque samples, the majority of the isolates were *Actinomyces* species and *Streptococcus sanguis*; approximately 13% of the flora was comprised of Gram-negative anaerobic rods which were equally divided into *Bacteroides* and *Fusobacterium* species (Slots, 1977). The characteristic flora of the
Transition from gingival health to gingivitis:

Loesche and Syed (1978) studied the developing bacterial flora in essentially "plaque-free" subjects who then refrained from all oral hygiene practices for three weeks. At time 0, *S. sanguis* and *Streptococcus mitis* comprised 62% and *Actinomyces* species accounted for 12% of the cultivable flora. After 1 week of no oral hygiene, streptococci dropped to 43% of the flora and *Actinomyces* species increased to 32% of the isolates. By week 3, *Actinomyces* species comprised 51% of the cultivable flora. *Actinomyces israelii* showed a significant correlation with plaque bulk but not gingivitis, whereas *Actinomyces viscosus* showed a significant correlation with gingivitis but not plaque bulk. Gram-negative species other than *Veillonella* accounted for 5% of the isolates by week 3.

Gingivitis

The bacterial flora associated with chronic gingivitis appears to represent a transition between health and periodontitis. Although clear patterns are not obvious and data have been highly variable, there are significant trends in the microbiological shifts which have been observed. For example, Listgarten's (1976) electron microscopic surveys of intact plaque associated with gingivitis revealed a variety of bacterial morphologies, including dense masses of coccoid and filamentous forms dominated the adherent plaque which was often covered by a loose layer of flagellated bacteria and spirochetes. Slots et al. (1978) has reported a study of 9 gingivitis-associated
bacterial samples. Streptococci accounted for 26.8% of the flora, and Gram-positive anaerobic rods represented 25% of the flora but no single species averaged more than 9% of the flora. *B. melaninogenicus* isolates, which comprised 7.7% of the cultivable flora, were predominantly of the *intermedius* subspecies. The data were very variable, with Gram-negative rods ranging from 0 to 48.9% of the flora.

**Periodontitis**

Prior to 1970, cultural studies of periodontitis involved the evaluation of pooled plaque samples from many sites in the oral cavity of patients with disease. Not only do different sites in the same mouth vary bacteriologically, but supragingival plaque differs significantly from subgingival plaque (Listgarten, 1976). The earlier practice of combining supragingival and subgingival plaque (i.e., pooling plaque) from several teeth may have obscured real differences in the subgingival flora of sites with active disease. Recently, complete bacteriological studies of single site samples from patients with chronic periodontitis have been reported. Williams et al. (1976) looked at 8 samples from 4 patients with periodontitis. These samples were collected by means of a curette from a "mid-crevicular" site and probably included most of the subgingival plaque in the pocket. The bacterial isolates were dominated by Gram-positive rods, the majority of which were *Actinomyces*. Although 30% of the flora were Gram-negative anaerobic rods, the isolation of *Bacteroides* species was variable. Williams et al. (1976) noted that bacteriologically, except for higher proportions of *Actinomyces* species, diseased sites were similar to control sites from the same
patients. Slots (1977) also examined 8 samples but surgically exposed the periodontal pocket and sampled only the leading front of the disease lesion, which presented a very different bacteriological profile. *B. melaninogenicus*, predominantly subspecies *asaccharolyticus* (*B. gingivalis*), represented 31.7% of the flora, and *Actinomyces* species accounted for about 16% of the isolates. Although *B. melaninogenicus* predominated in 5 of the 8 samples, this organism was less than 10% of the flora in the other 3 samples.

**Transition from gingivitis to periodontitis:**

It is almost impossible to study carefully the bacterial changes associated with the progression of gingivitis to periodontitis in humans. Most forms of periodontitis are thought to be slowly progressing chronic diseases; therefore, identification of active lesions requires longitudinal examination. The bacterial transitions from gingivitis to periodontitis have recently been demonstrated in an animal model system (Kornman et al., 1981). The cultivable subgingival microflora in the cynomolgus monkey was monitored during the ligature-induced progression of naturally occurring gingivitis to periodontitis. Between 4 to 7 weeks following ligature placement, increased pocket depth and radiographic evidence of bone loss was associated with an increase in *Bacteroides gingivalis* from 1.8% in gingivitis to 34% in periodontitis.

**Juvenile periodontitis:**

A very different bacterial flora has been described for juvenile periodontitis, a rapidly progressive form of periodontal disease which produces extensive bone loss about molar and incisor teeth in patients
under 20 years of age. Electron microscopic studies (Listgarten, 1976) of teeth from four juvenile periodontitis patients revealed a general lack of adherent bacterial plaque. The flora appeared sparse and dominated by Gram-negative filaments. Cultural studies (Slots, 1976; Newman & Socransky, 1977) noted that 50-63% of the cultivable flora was composed of Gram-negative anaerobes. When diseased sites were compared to non-diseased sites in the same mouth in these young individuals, Gram-negative anaerobic rods comprised 59% and 27% respectively in one study (Slots, 1976) and 49% and 9% respectively in another (Newman & Socransky, 1977). While a substantial number of these organisms could not be classified by current taxonomic criteria, a significant number of the isolates from periodontal pockets in juvenile periodontitis patients were Capnocytophaga species (Newman & Socransky, 1977). More recently, Tanner et al. (1979) and Slots et al. (1980) have identified a small Gram-negative organism, Actinobacillus actinomycetemcomitans, which appears to play an important role in juvenile periodontitis. Slots and coworkers (1982) used a selective medium to demonstrate that A. actinomycetemcomitans could be found in almost every juvenile periodontitis lesion sampled, and only infrequently in subjects with periodontal health, chronic adults periodontitis, or in patients with insulin-dependent diabetes mellitus. Moreover, after eradication of the organism with systemic tetracycline therapy, sites which remained free of reinfection with the organism after 5 months showed improvement in clinical attachment, and sites with detectable A. actinomycetemcomitans continued clinical loss of attachment.
Differences between chronic adult periodontitis and juvenile periodontitis

The different bacteriological pictures observed in the same clinical condition; i.e., chronic periodontitis, may be due to the differences in sampling described above or to the fact that clinical disease is crudely diagnosed by the mere presence of a deepened periodontal pocket, which in no way distinguishes active from inactive disease. It does appear, however, that advanced periodontitis is characterized by a nonsaccharolytic, nonmotile Gram-negative flora, whereas saccharolytic motile Gram-negative organisms are associated with juvenile periodontitis.

Summary of the microbial etiology of periodontal disease

Gingival health and gingivitis appear to be associated with a subgingival flora consisting primarily of Gram-positive facultative anaerobes, while the microflora of periodontitis is predominated by Gram-negative anaerobes. The complexity of periodontal diseases have greatly limited attempts to identify specific periodontopathic organisms. The demonstration by Kornman et al. (1981) of dramatically increased numbers of a specific periodontopathic organism found during active periodontitis in the monkey model comprises most of the "proof" we have to date for specific organisms' etiologic involvement in adult periodontitis. Unlike the clearer implication of Actinobacillus actinomycetemcomitans as the organism of primary importance in juvenile periodontitis, future studies of chronic adult periodontitis may demonstrate one or many periodontopathic organisms which interact or increase from very low levels in gingival health or gingivitis to only slightly higher levels with disease activity resulting in loss of
attachment. Thus, it appears at this time that the bacteria associated with periodontal disease are part of the normal, or indigenous, flora, and do not represent an "infection" per se, but rather an alteration in the development of the normal flora.

Development of the normal flora

The child is sterile in utero. The newborn acquires bacteria after the amniotic membrane ruptures, and from the mother's birth canal during a vaginal delivery. Neonates also acquire bacteria on all body surfaces from the local environment. The development of an intestinal flora during the neonatal period occurs very early. Rotimi and Duerden (1981) found that only staphylococci, streptococci, and enterococci appeared in the fecal flora on the first day of life, but by the second day, representatives of all the bacterial groups appeared. Some bacteria demonstrated a transient colonization of the gastrointestinal tract: the isolation rate of staphylococci rose to 74% on the second and third days, then fell to 48% by the sixth day. No anaerobes were isolated from any of the babies on the first day of life, but on the second day, bifidobacteria were present in the fecal specimens of 74% of these breast fed infants, who also received supplemental feedings with a commercial cow's milk preparation. By the third day, the isolation rate of bacteroides, primarily B. fragilis, was approaching the adult level. The fecal flora acquired during the first week of life was predominantly anaerobic and was similar in composition to the normal adult fecal flora. Differences in infant feeding do lead to differences in initial colonization of the infant's intestinal tract. Yoshioka et al (1983) recently compared the fecal flora of breast-fed and bottle-fed infants. In
both groups of infants, the intestine was first colonized with enterobacteria. On day 6, bifidobacteria were the predominant organisms in the stool of the breast-fed infants, exceeding enterobacteria by a ratio of 1,000:1, whereas enterobacteria were the predominant organisms in the formula-fed infants, exceeding bifidobacteria by approximately 10:1. At one month of age, bifidobacteria were the most prevalent organisms in both groups but were fewer in number in the bottle-fed infants. The development of the oral microflora during the neonatal period is more complex. Eight hours after birth, the mouth is colonized by viridans streptococci and *Streptococcus salivarius* (Rotimi and Duerden, 1981). Thereafter, great variability in the bacterial composition occurs for the next few days (Socransky et al, 1971). Organisms such as *Neisseria* spp., *H. influenzae*, enterococci, and enterobacteria were minor or transient members of the oral flora during the first week of life. This is in contrast to the high intestinal colonization rate of the anaerobic organisms in these same infants, suggesting that the organisms passed through without infecting the mouths of the neonates. Also, in the absence of the teeth and gingival crevice in these predentate infants, organisms of importance in oral diseases, such as *Streptococcus mutans* and spirochetes, are not retained in spite of apparent continual exposure to these organisms.

As seen by the development of the intestinal and oral microflora in infants, the sterile unborn child is exposed to maternal vaginal and fecal flora, and to environmental microorganisms. A transient colonization by these organisms occurs, followed by the development of a flora unique to the environment, i.e., the relatively anaerobic
microflora of the intestinal tract, and the predominantly aerobic microbiota of the infant's oral cavity. Then as the host changes with the development of the child (discontinuation of breast-feeding; eruption of teeth), new ecologies are set up reflecting the altered environment.

**Development of the subgingival flora**

The gingival crevice develops with the eruption of a tooth, and provides a unique ecological niche within the oral cavity. Very little is known about the development of the complex microbiological ecosystem of the gingival crevice, and how subsequent changes in the host influence the microflora.

As noted earlier, the newborn infant is exposed to a vast number of anaerobic and facultatively anaerobic bacteria during and immediately after birth (Socransky et al., 1971). The oral cavity is colonized mainly by alpha-hemolytic streptococci and *Streptococcus salivarius*, with minor or transient appearance of anaerobic bacteria (Rotimi and Duerden, 1981). *Nocardia, Actinomyces*, and *Veillonella* are found with increasing frequency in infants during the first year of life (McCarthy et al., 1965). Ellen (1976), specifically examining the oral cavities of predentate infants and in infants with teeth, found *A. naeslundii* in 40% of the predentate infants and 82% of the infants with teeth. *A. viscosus* was not detected before the eruption of teeth, and only infrequently in infants with teeth.

Further, DeAraujo and Macdonald (1964) observed that the cultivable subgingival microflora of five preschool children (age 3 to 7) consisted of 29.7% Gram-positive facultative rods; 20.4% Gram-positive facultative cocci, with 16.8% being *Streptococcus mitis*;
16.3% Gram-negative anaerobic cocci (Veillonella alcalescens); 16.3% Gram-positive anaerobic rods. Also present although less often among the 211 microbial strains studied from the five children were: Gram-negative facultative rods, Gram-negative anaerobic rods, Neisseria sp., and Peptostreptococcus sp. Spirochetes were also detected by darkfield examination of subgingival plaque in 10 of the 15 children. Interestingly, they were able to cultivate black-pigmented Bacteroides from the subgingival plaque of only 4 of the 15 preschool children. Bailit et al. (1964) used scrapings from the gingival sulcus of 320 children from 5 to 15 years of age to look for the presence of Bacteroides melaninogenicus (now called black-pigmented Bacteroides). In the 5 to 7 year age group, 40% of the cultures were positive for Bacteroides, and by age 13, nearly 100% of the cultures were positive. The largest increase in cultures positive for black-pigmented Bacteroides occurred from age 7 to 9. In contrast, Kelstrup (1966), found much lower amounts of black-pigmented Bacteroides in 149 children from 5 to 16 years of age. Using a cotton swab screening technique, collecting marginal plaque from many teeth, he was able to culture the organism from the gingiva in about 20% of the children from age 5 to 12, in 46% of the 14-year-olds, and in 70% of the 16-year-olds. Subsequently, 44 of the children who had detectable black-pigmented Bacteroides with the cotton swab plaque collection were studied further. Subgingival plaque was sampled from at least 5 sites in each child, anaerobically cultured, and the presence or absence of detectable black-pigmented Bacteroides in the subgingival plaque of individual sulci was determined. In children from age 4 to 8, representing the deciduous and early mixed dentitions, black-pigmented
Bacteroides could not be cultured from the subgingival plaque of any of the 106 teeth sampled. In the adolescent sample, from 13 to 15 years of age, only 11 of 86 sulci had cultivable black-pigmented Bacteroides. In another study, black-pigmented Bacteroides was observed in all eight preschool children who were following an experimental gingivitis protocol (Mackler and Crawford, 1973). Spirochetes were observed after sixteen days with no oral hygiene. The bacterial findings of these studies of subgingival plaque in children and adolescents can only suggest that changes in plaque composition are occurring around the time of puberty, i.e., from 11 to 14 years of age. Unfortunately, the microbiologic techniques available at the time of these latter studies did not allow optimal recovery of anaerobic bacteria, as well as the conclusions being drawn relying solely on chronologic age to determine the developmental status of the child. Rapid changes in skeletal, dental, and sexual maturation during puberty, as well as the wide variation in the timing and rate of development of each child, were not considered, and clearly dictate that more precise measures of pubertal development be used. Further, these early studies lacked the criteria necessary for the characterization of the gingival health of the sampled sites, as well as the systemic health of the individual. Two recent studies have attempted to account for these earlier shortcomings. Yanover and Ellen (1983) reported preliminary results of a longitudinal study of 26 "normal" children, who were developmentally characterized according to chronologic age and onset of menstruation. Gingivitis, crevicular fluid flow, and probing depth were measured. Subgingival plaque from one site was examined with darkfield microscopy and anaerobically cultured for black-pigmented Bacteroides. Microscopic counts averaged
88% cocci, and no spirochetes were detected. Black-pigmented Bacteroides, in each case identified as B. intermedius, were detected in only 8 of the 26 subjects, and detection was unrelated to age or maturation. Presence or levels of other bacterial species was not investigated in this study. More recently, Gusberti et al. (1983) reported their cross-sectional observations of 77 insulin-dependent diabetic children. These investigators plan to follow these children through sexual maturation, as assessed with Tanner staging. In addition, they assessed gingivitis and probing depth, as well as tests for the child's metabolic control of the diabetes; plaque (supragingival + subgingival) from a first molar and a lower incisor was sampled for microscopic counts and anaerobic cultivation on selective and nonselective media. These investigators found that the proportions of black-pigmented Bacteroides was not related to chronologic age nor to sexual maturation, although an age-related effect on the plaque proportions of Capnocytophaga ssp. and Actinomyces naeslundii was noted. The nature of the metabolic disease affecting these children, and their moderate-to-severe gingivitis (also, localized juvenile periodontitis in one subject) make it difficult to compare results of this study with other studies of the subgingival microflora associated with puberty.

Thus, there are essentially no definitive studies in the literature which relate age, sexual maturation, and somatic development to changes in the subgingival microflora which may be associated with the transition of gingivitis to periodontitis. Therefore, the present study was undertaken to establish similarities and differences in the subgingival microflora between varying ages around the time of puberty.
Specific Objectives

1) To determine the microbial composition of subgingival plaque from circumpubertal females, using microscopic and cultural techniques.

2) To determine the dental, skeletal, chronologic, menarchal, and sexual development of each subject.

3) To determine if cross-sectional correlations exist between developmental parameters around the time of puberty and the microbial composition of subgingival plaque.
Hypothesis

The composition of the subgingival microflora changes during puberty. These changes may contribute to the apparent increase in the susceptibility to periodontitis after puberty.
Materials and Methods

Clinical Procedures

Selection of Subjects and Consent

Twenty-two female subjects were selected from the list of patients who had been accepted by the Department of Orthodontics for treatment by orthodontic residents at the University of Connecticut Health Center. Females were chosen as subjects since onset of menses provided an additional parameter which, along with breast development and pubic hair development, reflect hormonal changes during puberty. Parents of the subjects were contacted by letter and by phone concerning the possible participation of their daughter in the study. Only subjects with no systemic illness or handicap were chosen; in addition, subjects could not have taken systemic antibiotics for a period of two months prior to their participation in the study. Since subjects were undergoing the pre-treatment data base collection prior to placement of orthodontic bands and brackets, recent radiographs were available for determination of skeletal age and dental age. Both the subjects and their parent were required to sign a consent form prior to participation in the study. This group of subjects represented a population of healthy pre-, circum-, and post-pubertal females.

Clinical Appointment Procedures

After the parent and subject had read and signed the consent form, the following data were collected:

1. Medical history questionnaire completed by subject and parent,
including menarchal status, history of any systemic illnesses or frequent infections of mucosal surfaces, and family history of early tooth loss or systemic illnesses.

2. Intraoral photographs were taken.

3. Sampling of subgingival dental plaque from the mesiobuccal aspects of both mandibular first molars, following careful removal of supragingival plaque. Subgingival plaque from each site was removed from the sulcus using a sterile Gracey curette, placed into a sterile vial of transport fluid, and transferred to the anaerobic chamber.

4. Periodontal evaluation of sites sampled, using:

   Plaque Index (Löe and Silness, 1963): The Plaque Index uses a 0 to 3 scoring scale. A score of 0 indicates no plaque adjacent to the gingiva. A score of 1 indicates plaque adhering to the free gingival margin and adjacent tooth surface. Plaque is detected only when a probe is run across the tooth surface. A score of 2 indicates a moderate accumulation of soft deposits within the gingival pocket, on the gingival margins and/or adjacent tooth surfaces, and can be seen without staining or use of a probe. A score of 3 indicates an abundance of soft debris within the gingival pocket or on the gingival margin and adjacent tooth surface. This scoring system was chosen because it is weighted toward plaque adjacent to or in contact with the gingiva, and has been used principally in studying the relationship between early gingivitis and early plaque.

   Papillary Bleeding Index: The measurement of gingivitis used in this study reflected the tendency of the gingival tissue to bleed. The diagnostic criteria for the Papillary Bleeding Index developed by Saxer and Muhlemann (1975) are: a score of 0 if no bleeding occurs
after probing; a score of 1 if there is bleeding some seconds after probing; a score of 2 if the gingival tissue bleeds immediately after probing; and a score of 3 if bleeding on probing spreads towards the marginal papilla.

**Probing Depth:** Probing depth was measured in millimeters from the gingival margin, using a Michigan 'O' periodontal probe.

5. **Assessment of Developmental Age:** If all children reached a given developmental level at the same chronologic age, determining the level of maturation would be simple. However, children with the same chronologic ages show a great deal of variation in their development. There are differences both in the age and in the rate at which maturation occurs, as well as sex differences. Because of this lack of correlation between developmental age and chronologic age, investigators have utilized additional parameters to assess the level of maturation in children. Tanner (1962) uses four systems to assign a developmental age to a growing child. These are:

1. skeletal age
2. dental age
3. morphological age (size, height) or shape age
4. secondary sex character age

**Skeletal Age:**

Skeletal maturity serves as a good index of biologic maturation and correlates well with other maturation parameters. It is based on the progression of secondary ossification of epiphyseal centers as seen radiographically. The hand and wrist are convenient to use for examination because of the large number of epiphyses and the small amount of exposure to radiation required. The Skeletal Maturation
Assessment system (Fishman, 1982) was used to score skeletal age in subjects in this study. The system uses four stages of bone maturation found at six anatomical sites located on the thumb, third finger, fifth finger and radius. The sequence of the four ossification stages progresses through epiphyseal widening on selected phalanges, the ossification of the adductor sesamoid of the thumb, the 'capping' of selected epiphyses over their diaphyses, and the fusion of selected epiphyses and diaphyses. Eleven discrete adolescent skeletal maturational indicators, covering the entire period of adolescent development, are found on the six sites. This method of skeletal age assessment was chosen because it offered an organized and relatively simple approach to assessing the level of skeletal maturation, and because its objective methods could be easily repeated by other investigators.

**Dental Age**

Dental age is another indicator of maturational age. The definition of dental age varies, depending whether the investigator is using clinical or radiographic examination of the dentition. The two main categories of dental age are:

1) Eruption age, which is the progressive emergences of the tooth from its alveolus into functional occlusion (Krogman, 1968).

2) Calcification age, which is the stage-sequence of tooth development from the first appearance of the cusps to apical root closure, as seen radiographically.

Fanning (1962) studied the developmental levels of the roots with respect to the chronological age. The method of determining dental
age with radiographic assessment of tooth development was found to be less variable than eruption tables. Moorees et al. (1963) devised a scoring system for permanent teeth according to crown and root formation as seen on standard dental radiographic films. This system was used in the present study to determine dental age. A mean dental age was derived by scoring each developing mandibular cuspid, first and second bicuspids, and first, second, and third molars. Each tooth examined had thirteen possible stages of crown, root, and apex formation (with seven additional scoring stages for the second root of the molars); a mean dental age could be determined based on comparison with the standards from Moorees' study population.

**Secondary Sex Character Age**

Secondary sex character age is useful only after puberty has started. To designate how far a given child has developed, a rating system has been devised by Tanner (1962) to rate the genital development and pubic hair development in boys, and for girls, breast development and pubic hair development is rated. All ratings are done on a one to five scale, and are based on photographic standards devised by Tanner.

Tanner's stages of breast development for girls are:

- **Stage 1:** Pre-adolescent: elevation of papilla only.
- **Stage 2:** Breast bud stage: elevation of breast and papilla as small mound. Enlargement of areolar diameter.
- **Stage 3:** Further enlargement and elevation of breast and areola, with no separation of their contours.
- **Stage 4:** Projection of areola and papilla to form a secondary mound above the level of the breast.
Stage 5: Mature stage: projection of papilla only, due to recession of the areola to the general contour of the breast. (Tanner, 1962)

Tanner's stages of pubic hair development for boys and girls are:

Stage 1: Pre-adolescent: the vellus over the pubes is not further developed than over the abdominal wall; i.e., no pubic hair.

Stage 2: Sparse growth of long, slightly pigmented downy hair, straight or only slightly curled, appearing chiefly at the base of the penis or along the labia.

Stage 3: Considerably darker, coarser, and more curled. The hair spreads sparsely over the junction of the pubes.

Stage 4: Hair now resembles adult in type, but the area covered is still considerably smaller than in the adult. No spread to the medial surface of the thighs.

Stage 5: Adult in quantity and type with distribution of the horizontal pattern. (Tanner, 1962)

The appearance of the breast bud is, as a rule, the first sign of puberty in the female (Falkner, 1978). The progression through breast and pubic hair stages is not simultaneous; however, to obtain a composite sex character rating, the stages can be averaged. Physical examination to determine the Tanner breast and pubic hair stages of subjects in this study was done by Dr. Susan Ratzan, a pediatric endocrinologist from the Department of Pediatrics, University of Connecticut Health Center.

Menarche is also used as a parameter in assessing the developmental status of girls. It occurs concurrently with the
appearance of axillary hair and when the breasts are between Tanner's stage 4 and 5. The number of months since the onset of menses was determined by questioning the subject and her parent.

Microbiological procedures

Sample collection:

Subgingival bacterial plaque samples were collected from the mesiobuccal aspect of both mandibular first molars. The site was isolated, dried, and supragingival plaque was removed using a sterile curette. A new, sterile Gracey curette was used to sample the subgingival plaque. The tip was placed into a sterile vial containing glass beads and 0.5 ml pre-reduced transport fluid (RTF) (Syed and Loesche, 1973) without ethylenediamine-tetraacetate, and agitated so that adherent plaque was deposited into the vial. Two sample vials for each subject were transported directly to a Coy anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Michigan), where all dispersion, diluting, and plating procedures were performed. The anaerobic chamber was maintained under an atmosphere of 5% carbon-dioxide and 10% hydrogen balanced with nitrogen (Aranki et al., 1969).

Laboratory processing

Sample vials were vortexed for 30 seconds to disperse the plaque sample, and 20 µl of the sample was removed for microscopic counts. Dilutions of 1:6 and 1:40 were made using additional RTF, and the diluted plaque samples were plated onto selective and nonselective media using an automatic plating and diluting device (Spiral Systems, Bethesda, Maryland). The Spiral Plater allowed rapid and reproducible plating while giving a two log dilution of the sample on a single
The two dilutions of each sample were plated onto:

a. *Mitis salivarius agar*, for quantitative determination of streptococci colony counts;

b. *Actinobacillus medium* (Slots et al., 1980), an agar medium with a Trypticase soy agar base containing bacitracin and horse serum to allow the selective isolation of *Actinobacillus actinomycetemcomitans*; and,

c. *Enriched Trypticase Soy Agar* (ETSA) (Syed et al., 1980), a nonselective blood medium enriched with menadione and Vitamin K to aid growth of anaerobic organisms.

Streptococcal plates were incubated at 37°C in 5% CO₂ plus air for 48 hours, and Slot's plates were incubated in the anaerobic chamber for 4 days and examined under a binocular dissecting microscope for colony morphology and decomposition of 3% H₂O₂. Presumptive *Actinobacillus actinomycetemcomitans* colonies were subcultured for confirmation. Duplicate ETSA plates of each dilution were plated; one set was incubated at 37°C in 5% CO₂ plus air for 5 days to determine colony count in facultatively anaerobic conditions and the other set was incubated in the anaerobic glove box at 37°C for 5 to 7 days. Appropriately diluted anaerobically incubated plates were selected for subculturing.

**Subculturing and biochemical tests**

A portion of the surface of plates with well dispersed colonies were scribed by means of a stainless steel template. The inscribed area includes two sectors termed "sector III" and "sector IV". Within
sector III, $8.33 \times 10^{-4}$ ml of the sample dilution was plated in a spiral pattern. Sector IV included $3.28 \times 10^{-3}$ ml of the sample. Calculation of the colony forming units per ml of the sample utilized these calibrated sector areas. Duplicate sectors with 30-50 colonies were counted with the aid of a binocular dissecting microscope. The total number of colonies within one sector was quantitated; then colony morphology types were characterized using color, shape and circumference, and the frequency of each colony type was determined. Representative colonies of each morphology type were picked from the sector and each one subcultured by streaking onto ETSA plates. After 4 days of growth in the anaerobic chamber, each subculture was checked for purity by means of a dissecting microscope.

Pure isolates were then picked into Basal Anaerobic Broth (BAB) with 1% glucose (Syed and Loesche, 1978), Basal Esculin Nitrate Broth (BEN) (Loesche et al, 1972), and into a sterile milk + DMSO solution for freezing using liquid nitrogen. The frozen strains were stored for further biochemical tests. In addition, all strains of black pigmented Bacteroides, easily detected due to their brown pigmentation and colony morphology, were picked into broth for inoculation of the Minitek biochemical series (BBL Minitek Biochemical Differentiation Discs, Cockeysville, MD) and into sterile water for the An-Ident System (API, Plainville, NY), which uses both miniaturized conventional and chromogenic substrate tests for the identification of anaerobes.

After incubation in the anaerobic chamber, each BAB culture was used for preparation of a Gram stain, for streaking aerobic blood plates for incubation in a candle jar, and to determine the level of
acid production in this glucose broth.

Each BEN culture was also allowed to grow to turbidity in the anaerobic chamber, and was used for biochemical tests of esculin hydrolysis, nitrate reduction, nitrite reduction, indole production, and the presence of catalase (Holdeman and Moore, 1977).

Specific biochemical tests utilizing the Minitek system were: esculin hydrolysis, and acid production following utilization of cellobiose, dextrose, and lactose. Inoculated wells containing the appropriate disk were allowed to incubate in the anaerobic chamber for 48 hours before reactions were read.

Twenty-one rapid amino-peptidase and carbohydrate utilization tests were performed in only 3 to 4 hours, using the An-Ident system of chromogenic substrates. Inoculated strips were aerobically incubated at 37°C, and reactions were read after the addition of Cinnamaldehyde Reagent, Kovac's Reagent, or 3% H₂O₂.

**Identification**

Results were entered into a Heath Kit 90 computer for storage and identification, and organisms were classified to the species level where possible, using schemes derived from the Virginia Polytechnic Institute Anaerobe Laboratory Manual (Holdeman and Moore, 1977). The black pigmented *Bacteroides* were classified according to the scheme proposed by Johnson and Holdeman (1983).

**Microscopic Counts**

The 20 µl aliquot of the vortexed sample in RTF was used for determining the total bacterial count and spirochete count. Twenty µl of a known concentration of 1 µm diameter beads in formalin was added
to the sample and vortexed for 5 seconds. The mixed sample (plaque + latex beads) was dispensed in 10 µl portions on a glass microscope slide. Cover slips were placed and were sealed with varnish. Total bacterial cell counts and spirochete counts were determined as follows:

a. **Phase contrast microscope** - In each of 10 to 20 fields, the number of beads and bacteria other than spirochetes were counted, until at least 200 bacteria had been counted. The ratio of bacteria/bead was calculated.

b. **Darkfield microscope** - Beads and spirochetes were counted in as many fields as were required to equal or exceed the number of beads counted with the phase contrast microscope. The ratio of spirochete /bead was calculated.

The number and proportions of total bacteria and spirochetes in the original plaque sample were calculated:

\[
\text{Total bacteria/ml sample} = \frac{\text{bacteria}}{\text{bead}} + \frac{\text{spirochetes}}{\text{bead}} \times \frac{\text{beads/ml}}{}
\]

\[
\text{Spirochetes/ml sample} = \frac{\text{spirochetes}}{\text{bead}} \times \frac{\text{beads/ml}}{}
\]

\[
\text{bacteria} + \text{spirochetes} \quad \text{bead} + \text{bead}
\]
The procedure of duplicate counting on both phase contrast and darkfield microscopes was based on the superiority of the darkfield microscope for the resolution of small spirochetes, and the problems of differentiating bacteria from debris with the darkfield microscope.

**Statistical analysis**

All clinical and developmental parameters were tested for correlation with the microbial findings, using the Spearman Rank Correlation Coefficient. In addition, correlations between stages of sexual maturation (breast development, pubic hair development, and menarchal status) and levels of specific bacteria were tested using the Kruskal-Wallis One-way Analysis of Variance by Ranks, and the Mann-Whitney U-test.
Results

Developmental description of the subjects

Twenty-two healthy females participated in this investigation. Chronologically, they ranged in age from 7.3 to 16.9 years, with a mean of 12.4 ± 2.0 years. Dental age, based on calcification of forming teeth, ranged from 5.3 to 13.5 years, with a mean dental age for the group being 10.9 ± 2.0 years. All eleven stages of the Skeletal Maturation Assessment were seen on evaluation of hand-wrist films in the study population, representing skeletal changes seen well before puberty, as well as during and after puberty. On physical examination of breast and pubic hair development, the 22 subjects ranged from clearly prepubertal (Tanner Stage 1) to fully mature (Tanner Stage 5), with an approximately equal distribution in each maturational stage. Since subjects' breast and pubic hair development were often not at equal stages (i.e., breast development at Stage 3, with pubic hair development at Stage 2) statistical analyses were performed separately for breast and pubic hair development. Ten of the 22 subjects had experienced onset of menses, from 3 to 58 months prior to participating in the study. For statistical analysis of the effect of menarchal status on the subgingival microbiota, the subjects were divided into 4 menarchal groups:

1) Premenarchal by report, and, by Tanner staging, prepubertal (Stage 1) or with only early pubertal development (Stage 2).

2) Premenarchal by report, but with Tanner Stage 3 or 4 development, indicating probable menarche within about one year.
3. One to twelve months postmenarchal by report, with Tanner Stage 3 or 4 development.

4. More than one year postmenarchal by report, with Tanner Stage 4 or 5 development.

See Figure 2 for demonstration of the breast development, pubic hair development, and menarchal status of subjects.

Clinical description of the subjects

Results of the clinical evaluation of gingival health of the 44 molar sites sampled subgingivally were:

**Plaque Index**: Almost all sites exhibited plaque: 40 of the 44 sampled site had Plaque Index score of 1 or 2, with a mean Plaque Index for the group of $1.36 \pm 0.64$ (Figure 3).

**Papillary Bleeding Index**: Most sites exhibited mild gingivitis, with a Bleeding Index of 1 in 28 sites, and a Bleeding Index of 2 in 14 sites. Only 2 sites had no gingivitis as measured by the amount of bleeding on probing. The mean Papillary Bleeding Index for the population studied was $1.27 \pm 0.54$ (Figure 3).

**Probing depth**: Probing depth ranged from 1 mm to 4 mm, with a mean probing depth of $2.75 \pm 0.61$ mm. The majority of the sites (28 of 44) had a probing depth of 3 mm. None of the sites sampled had loss of periodontal attachment (Figure 3).

Microbiological description of the subjects

From the 44 subgingival plaque samples collected in this investigation, 481 bacterial strains were examined and characterized from the predominant cultivable microflora. Results of the characterization of these bacterial strains are:
Gram-negative rods: The mean percentage of Gram-negative rods in the predominant cultivable microflora of sites sampled was $48.2 \pm 24.5\%$, with a range of 2.5 to 100\%.

Gram-positive rods: Ranging from 0 to 86.5\%, Gram-positive rods represented a mean of $31.9 \pm 23.3\%$ of the predominant cultivable microflora for the total study population. Gram-positive rods were detected in 88.6\% of the sites, and for those sites, Gram-positive rods represented a mean of $36.0 \pm 21.6\%$ of the predominant cultivable microflora.

Gram-negative cocci: The percent Gram-negative cocci ranged from nondetectable to 37.3, with a mean of $4.1 \pm 9.8\%$ for all sites sampled. Detected in 20.5\% of sampled sites, Gram-negative cocci represented $21.2 \pm 18.9\%$ for those sites with positive detection.

Gram-positive cocci: With a mean of $7.1 \pm 11.0\%$, the Gram-positive cocci group ranged from nondetectable to 34.2\% of the predominant cultivable microflora of the 44 subgingival sites sampled. Gram-positive cocci were present in 50\% of sites sampled, and represented $14.2 \pm 11.8\%$ of the predominant cultivable microflora for those sites.

Anaerobes vs. facultative anaerobes: A mean of $44.0 \pm 24.6\%$ of the predominant cultivable microflora required strict anaerobiosis, with a range of 1.2 to 92.6\%.

Selected Gram-negative bacterial species

Black-pigmented Bacteroides: Black-pigmented Bacteroides ranged from nondetectable to representing 54.0\% of the predominant cultivable microflora, with a mean of $4.9 \pm 10.0\%$. All BPBs isolated were saccharolytic, with the majority (39 of 46 strains characterized) identified as Bacteroides intermedius. The remaining 7 strains were
identified as *B. melaninogenicus*. Black-pigmented *Bacteroides* were detected in 64% of the subjects and in 45.5% of sampled sites. For those sites with a positive detection, black-pigmented *Bacteroides* represented \(10.8 \pm 12.5\)% of the predominant cultivable microflora.

**Surface Translocating Bacteria:** STBs ranged from nondetectable to 35.7% of the cultivable microflora, with a mean of \(5.7 \pm 7.1\)% of the predominant cultivable microflora for all sites. In the 65.9% of sites with surface translocating bacteria, \(8.6 \pm 7.1\)% of the cultivable microflora was comprised of surface translocating bacteria. *Capnocytophaga* sp. represented a mean of \(0.9 \pm 3.6\)% of the predominant cultivable microflora for all sites, and ranged from nondetectable to \(20.0\)%.

Present in 9.1% of sites sampled, *Capnocytophaga* comprised \(10.1 \pm 6.9\)% of sites positive for the organism. Other species detected included *Wolinella* and *Eikenella*.

**Fusobacterium nucleatum:** *F. nucleatum* ranged from nondetectable to 17.2%, with a mean of \(2.2 \pm 4.4\)% of the cultivable microflora for all sites sampled. Detected in 27.3% of sampled sites, *F. nucleatum* represented \(8.0 \pm 4.7\)% of the predominant cultivable microflora in sites positive for this bacterial species.

**Selected Gram-positive bacterial species**

*Actinomyces:* The total *Actinomyces* sp. in the predominant cultivable microflora ranged from nondetectable to 64.9%, with a mean of \(9.5 \pm 14.8\)% for all sites sampled. Fifty percent of the sites had detectable *Actinomyces*, and in positive sites, *Actinomyces* comprised \(19.0 \pm 16.1\)% of the predominant cultivable microflora. *A. naeslundii* predominated, with a mean of \(8.7 \pm 13.9\)% and a range of 0 to 64.9% of the cultivable microflora. For sites with detection, *A. naeslundii*
made up 19.0 ± 15.1% of the cultivable microflora. *A. viscosis* was detected in 4 of the 44 plaque samples, and ranged from 0 to 20.0% of the flora. *A. odontolyticus* was seen only rarely, and represented a mean of 0.12% of the predominant cultivable microflora.

**Selective medium for Actinobacillus actinomycetemcomitans**

Using a selective medium, and confirmation with subculturing and characterization, 7 of the 44 subgingival plaque samples, in 6 of the 22 subjects (27.3% of the subjects), had demonstrable *A. actinomycetemcomitans*.

**Selective medium for Streptococci**

The ratio of total colony forming units (CFUs) on *Mitis Salivarius* agar to total CFUs on anaerobically incubated Enriched Trypticase Soy Agar for each plaque sample provided the percent streptococcal CFUs, which ranged from 0 to 24.5% (mean = 3.4 ± 5.0%).

**Microscopic counts**

The total microbial count ranged from 4.6 x 10^6 to 1.2 x 10^8, with a mean of 4.4 x 10^7 ± 3.0 x 10^7. Spirochetes represented a mean of 5.7 ± 9.3% of the microscopic count for all sites sampled, and ranged from nondetectable to 41.2% of the microscopic count. Of the 56.8% of sites with detectable spirochetes, spirochetes comprised 10.0 ± 10.4% of the microscopic count.

**Correlation between developmental parameters**

Tested for correlation with the Spearman rank correlation coefficient, all growth parameters (chronologic age, dental age, skeletal maturation, breast development, and pubic hair development)
were significantly related to each other, with $p<0.0005$. Rho values ranged from .73 to .88, suggesting a strong interrelatedness of the parameters.

**Chronologic age** showed the highest degree of correlation with skeletal maturation ($\rho = .88$), as is seen in figure 4. A rho value of .81 was obtained for correlation with dental age (figure 5), and the lowest rho values of all developmental relationships tested were obtained with correlation of chronologic age and the indicators of sexual maturity, breast and pubic hair development ($\rho = .73$ and .78, respectively; figures 6 and 7).

**Dental age** was more highly correlated with skeletal maturation ($\rho = .84$; figure 8) and breast development ($\rho = .84$; figure 9), than with chronologic age ($\rho = .81$; figure 5) or pubic hair stage ($\rho = .78$; figure 10).

**Skeletal maturation** showed the highest degree of correlation with chronologic age ($\rho = .88$; figure 4), with pubic hair stage ($\rho = .87$; figure 11), dental age ($\rho = .84$; figure 8), and breast development ($\rho = .84$; figure 12) were also highly correlated.

**Correlation between developmental parameters and the predominant cultivable microflora of sites sampled**

Using the Spearman rank correlation coefficient, no significant correlations were observed between the developmental parameters of chronologic age, dental age, skeletal maturation, Tanner breast stage, and Tanner pubic hair stage, and the proportion of the predominant cultivable flora comprised by Gram-negative rods (figures 13-17), Gram-positive rods (figures 18-22), Gram-negative cocci (figures 23-27), or Gram-positive cocci (figures 28-32). Also demonstrating no
statistical significance with the Spearman rank correlation coefficient was the analysis of percent anaerobes in the cultivable microflora and all developmental parameters (figures 33-37).

**Correlations between developmental parameters and selected Gram-negative bacterial species**

**Black-pigmented Bacteroides**: No statistically significant correlations were observed between the levels of black-pigmented Bacteroides and chronologic age, dental age, skeletal maturation, breast development, or pubic hair development when examined with the Spearman rank correlation coefficient (figures 38-42).

**Spirochetes**: Also of no statistical significance were the spirochete proportions of the microscopic counts, when similarly compared to all developmental parameters (figures 43-47).

**Surface Translocating Bacteria**: Although no statistical significance was shown with the Spearman rank correlation coefficient for the levels of surface translocating bacteria and chronologic age (figure 48), dental age (figure 49), or skeletal maturation (figure 50), there were significant negative correlations between the percent STBs in the predominant cultivable microflora and Tanner breast stage ($p<0.01$) and also with Tanner pubic hair stage ($p<0.005$). Figure 51 and figure 52 demonstrate the decreasing levels of STBs with sexual maturation (Tanner breast stage and pubic hair stage, respectively).

**Fusobacterium nucleatum**: No significant correlations were observed between levels of F. nucleatum and developmental parameters, (figures 53-57).

**Actinobacillus actinomycetemcomitans**: No significant correlations were found between detection of the organism and any developmental
parameters. *A. actinomycetemcomitans* was found in subjects with sexual development characterized by Tanner stages 1, 2, 3, and 4.

**Correlations between developmental parameters and selected Gram-positive bacterial species**

*Actinomyces*: The total *Actinomyces* sp. in the predominant cultivable microflora was negatively related to each of the developmental parameters when analyzed with the Spearman rank correlation coefficient. Significant at p<0.005 were chronologic age (figure 58), dental age (figure 59), skeletal maturation (figure 60) and Tanner pubic hair stage (figure 62), while the correlation of Tanner breast stage and % *Actinomyces* was significant at p<0.01 (figure 61). Accounting for this decrease in levels of *Actinomyces* with increasing development are the similar negative correlations of *Actinomyces naeslundii* and development. *A. naeslundii* was negatively related to chronologic age, dental age, skeletal maturation, and pubic hair stage at p<0.005, and with Tanner breast stage at p<0.025 (figures 63-67).

**Analysis of levels of selected bacterial species during sexual maturational stages**

The separation of stages of sexual maturation by Tanner staging of breast and pubic hair development, and by menarchal status lent itself to further analysis with the Kruskal-Wallis one-way analysis of variance by ranks and the Mann-Whitney U-test, to determine if differences could be revealed in the levels of bacterial species during specific pubertal stages.

*Black-pigmented Bacteroides*: No significant differences in
percent black-pigmented Bacteroides in the predominant cultivable microflora of sampled sites were found for subjects for the Tanner breast or pubic hair stages 1 to 5, using the Kruskal-Wallis one-way analysis of variance by ranks. However, significant differences were found for menarchal status (p<0.01). Further analysis with the Mann-Whitney U-test showed differences in the % BPBs in subjects who were prepubertal or premenarchal with only the earliest stages of pubertal development (early premenarchal) versus premenarchal subjects with significant sexual development (late premenarchal), and between this latter group and early post-menarchal subjects (menarche within 12 months), at p<0.01 (figure 68).

**Surface Translocating Bacteria:** The Kruskal-Wallis one-way analysis of variance revealed significant differences in % STB for the Tanner pubic hair stages (p<0.01). The Mann-Whitney U-test revealed differences in % STB between Tanner pubic hair stage 1 and 3 (p<0.01); stage 1 and 5 (p<0.004); stage 2 and 3 (p<0.01) and stage 2 and 5 (p<0.01), as demonstrated in figure 52. No differences were observed for % STB between menarchal groups.

**Actinomyces:** The Kruskal-Wallis one-way analysis of variance by ranks revealed no differences in total Actinomyces or *A. naeslundii* levels with menarchal status. However, differences in total Actinomyces were noted for Tanner breast stages (p<0.01), and differences in *A. naeslundii* were seen for the Tanner breast stages (p<0.001), as well as for Tanner pubic hair stages (p<0.01). Use of the Mann-Whitney U-test demonstrated statistically significant differences in total % Actinomyces between Tanner breast stages 1 and 2 (p<0.000); 1 and 4 (p<0.001); and 1 and 5 (p<0.01). Differences in
% *A. naeslundii* were found between Tanner breast stages 1 and 2 (p<0.000); stages 1 and 4 (p<0.01); stages 1 and 5 (p<0.01); and between Tanner pubic hair stages 1 and 3 (p<0.01); stages 1 and 4 (p<0.001); and stages 1 and 5 (p<0.01).
Discussion

Developmental correlations

The high positive correlations found between developmental parameters in subjects participating in this investigation are in general agreement with the literature. Nicolson and Hanley (1953) found high coefficients of correlation for girls between the ages at which: menarche occurs; the breast reaches stages 2, 3, and 4; the pubic hair reaches stages 2, 3, and 4; Todd skeletal age standards of 9-3/4, 12-3/4, and 16-1/4; and 80%, 90%, and 99% of mature stature is reached. Although all growth parameters were significantly correlated at p<0.0005, the finding of the highest rho value for skeletal versus chronologic age is supported by Green (1961), who found that skeletal age was relatively highly correlated with chronologic age, height, and weight, compared to dental age. In Green's study, the dental age was more correlated with chronologic age than with skeletal age. Historically, dental age has not been found to correlate well with overall developmental age. Nanda (1960) found a low correlation of the age at which all permanent teeth have erupted and the age at which the growth spurt occurred, and a slightly higher correlation of eruption of permanent teeth and menarche. However, differences in methodology between these studies and the present investigation account for the relatively high correlations found between dental age and the developmental parameters of skeletal maturation, Tanner breast stage, chronologic age, and Tanner pubic hair stage. These former investigators relied on clinical examination of tooth eruption, rather than on the calcification age used in this study, which is considered
to be less variable than eruption age (Fanning, 1961).

Of particular interest due to the nature of previous studies of bacterial plaque changes during the pubertal period was our finding of the lowest correlation between chronologic age and Tanner pubic hair stage or breast stage, suggesting that chronologic age is the poorest indicator of sexual maturation, as compared to skeletal maturation or radiographic dental age. Menarche is clearly related to sexual maturational changes, as shown by the pattern of post-menarchal subjects in the Tanner stage 4 and 5 groups (Figure 2). A report of onset of menses, however, divides puberty into only premenarchal and postmenarchal stages, while the addition of physical examination of sexual development provides further information regarding the pubertal development of subjects in studies of this nature.

Clinical assessment of gingival health

The finding of moderate amounts of plaque, mild gingivitis, and a mean probing of 2.75 mm reflects a population with generally healthy gingival tissues. Unlike the peak in frequency of gingivitis at about age 13 for females reported by Sutcliffe (1972), no increase in "puberty gingivitis" was seen in the present study.

The gingival health generally was not assessed in early microbiological studies of periodontally-associated plaque; thus, differences in results based on periodontal health cannot be made. The two recently reported investigations involving subgingival plaque in pubertal populations utilized patient populations which differ from each other and with this investigation: Yanover and Ellen (1983) examined subgingival plaque from sites with shallower gingival crevices (mean sulcus depth = 1.82 mm), a mean Gingival Index of 1.00,
and almost no visible plaque. In contrast, the Gusberti et al. (1983) diabetic population had high levels of gingivitis, as shown with papillary bleeding scores which increased with age and with the level of metabolic control of diabetes in the prepubertal subjects. In addition, loss of attachment was noted in at least one subject (probing depths and plaque levels were not reported).

**Predominant cultivable microflora**

**Gram-positive cocci:** The mean of 7% Gram-positive cocci reflected a lower level than the 40% found in gingival health (Slots, 1977) but a higher level than the 3% in gingivitis (Slots et al., 1978). Our result was comparable to that found by Gusberti et al. (1983), but was very different than Yanover and Ellen (1983), who reported 88% cocci on microscopic examination. This latter result might be explained by contamination of the subgingival plaque sample by supragingival plaque or by the possibility that short Gram-negative rods were scored as cocci.

**Gram-positive rods:** Although highly variable in the present investigation, the mean of 32 ± 23% Gram-positive rods cultivated is similar to the 45% reported by Slots (1977) for gingival health, although his subjects with gingivitis had only 26% Gram-positive rods (Slots et al., 1978). As with Gram-negative rods, the absence of significant changes in levels of Gram-positive rods with increasing development is in agreement with Gusberti et al. (1983).

**Gram-negative cocci:** Found as only 4% of the cultivable microflora in this study, Gram-negative cocci levels were very similar to the reported values of 2% in gingival health (Slots, 1977) and 4%
in gingivitis (Slots et al., 1978). Again, neither this investigation nor that of Gusberti et al. (1983) found significant changes with pubertal development.

**Gram-negative rods:** The finding of a mean percentage of 48.25% Gram-negative rods in the predominant cultivable subgingival microflora in the present investigation is considerably higher than that reported by Slots (1977) for gingival health (about 13%) but similar to that reported for gingivitis in adults, with 40% Gram-negative rods (Slots et al., 1978). In agreement with Gusberti et al. was our finding of no statistically significant changes in the % Gram-negative rods with increasing chronologic, dental, skeletal, or sexual development. Based on the high levels of these microorganisms already present in the circumpubertal females, colonization must occur earlier in development. Yanover and Ellen did not examine the predominant cultivable microflora in their study.

**Anaerobes vs. facultative anaerobes:**

The mean 44% of the predominant cultivable microflora which was strictly anaerobic in this study compares to Slots et al. (1978) finding 43% anaerobes the subgingival microflora in gingivitis, and differs from the 24% found in gingival health (Slots, 1977). Additionally, our result was in the range of that observed by Gusberti et al. (1983), who found over 50% anaerobes in the subgingival microflora of diabetic children. Since no significant increase in % anaerobes was found in either the present study or that of Gusberti et al. (1983), the establishment of substantial populations of strictly anaerobic bacteria in the gingival crevice may occur much earlier.
Selected bacterial species

**Spirochetes:** Representing about 6% of the microscopic count in this study, the proportion of spirochetes was somewhat higher than that reported for the same age range by Gusberti et al. (1983), who found spirochetes as 1-4% of the microscopic count. Yanover and Ellen (1983), on the other hand, found no spirochetes in the subgingival plaque of adolescents, although Yanover generally found very few organisms per microscopic field, and may have been dealing with plaque dilutions such that low levels of spirochetes were missed (personal communication). No investigation, including the present study, has shown changes in spirochete levels with pubertal development. In adults, however, levels of spirochetes in the microscopic count are known to change with gingival health. Listgarten and Hellden (1978) observed spirochetes in 25% of relatively healthy sites (mean = 1.8±3.2%) and in 100% of periodontally involved sites (mean = 37.7±9.6% of the microscopic count).

**Actinobacillus actinomycetemcomitans:** Detected in low numbers in 27% of the subjects in the present study, *A. actinomycetemcomitans* was shown to be detectable in the gingival crevice of 20% of 14-20 year olds with no or only mild gingival inflammation and in 36% of adults with little gingival inflammation (Slots et al., 1980). Current thought is that this organism can be considered part of the normal subgingival microflora of adolescents, although high levels of *A. actinomycetemcomitans* in an adolescent is suggestive of juvenile periodontitis.

**Actinomyces:** The predominance of *A. naeslundii* over *A. viscosus*
and *A. odontolyticus* in the present study is in agreement with results reported by Slots et al. (1978) for subgingival plaque taken from sites with gingivitis, where data shows a 3:1 predominance of *A. naeslundii* over *A. viscosus*. The finding is in conflict, however, with Ellen (1976), who reported up to 24 times more catalase-positive *Actinomyces*-like isolates (*A. viscosus*) than catalase-negative isolates (*A. naeslundii*) in dental plaque from children, in a study using a selective medium for *Actinomyces*. On the other hand, Gusberti et al. (1983), also using a selective medium, reported approximately equal proportions of *A. naeslundii* and *A. viscosus* in subgingival plaque from diabetic children. Our finding of decreasing proportions of *A. naeslundii* in the subgingival plaque with increasing development is in conflict with findings reported by Gusberti et al. (1983), who reported elevated levels only in plaque samples from Tanner stage 3 subjects. It is interesting to note that although their Tanner stage 3 subjects did exhibit significantly higher levels of *A. naeslundii* than did the other developmental groups, the ratio of *A. naeslundii* to *A. viscosus* remained about equal.

**Black-pigmented Bacteroides:** The mean of 5% black-pigmented Bacteroides in the predominant cultivable flora of this population of circumpubertal females is in agreement with Gusberti et al. (1980), for diabetics in a similar age group. Our findings are also similar to those found in adults with gingival health (2%) and gingivitis (8%) (Slots, 1977; Slots et al., 1978). Our detection of black-pigmented Bacteroides in 64% of the subjects is more than double that reported by Yanover and Ellen (1983), who detected black-pigmented Bacteroides in only 31% of their circumpubertal subjects; it was, however, lower
than that reported by Bailet et al. (1964), who was able to detect the organism in gingival scrapings of nearly all children by the age of 13. It is interesting to compare the importance of gingival health in the incidence of detection of black-pigmented Bacteroides subspecies in subgingival plaque samples: Zambon et al. (1981) reported a 20% detection of B. intermedius in subgingival plaque from subjects with gingivitis (no B. gingivalis or B. melaninogenicus were detected), while 70% of the subjects with periodontitis had B. intermedius, 10% had B. melaninogenicus, and 10% had B. gingivalis. In another study, Spiegel et al. (1979) found a higher incidence of certain black-pigmented Bacteroides in subjects with gingival health or gingivitis: one of six subjects with gingivitis had detectable B. gingivalis, and about 50% of subjects with gingival health or gingivitis had detectable B. melaninogenicus. None of the subjects in our study had detectable B. gingivalis, while 45.5% of the subjects had detectable B. intermedius and 22.7% had detectable B. melaninogenicus.

The influence of increasing age and pubertal development on the detection and levels of black-pigmented Bacteroides has been of particular interest for the last 20 years. The seemingly direct relationship between increasing chronologic age and increasing incidence of black-pigmented Bacteroides in the gingival crevice (Bailet et al., 1964; Kelstrup, 1966) has not been demonstrated in this investigation, or in recent reports in the literature (Gusberti et al., 1983; Yanover and Ellen, 1983). Our finding of a significant relationship between levels of black-pigmented Bacteroides and menarchal status is of interest in that the late premenarchal group of
subjects, who had higher levels of black-pigmented \textit{Bacteroides} than either the early premenarchal group (prepubertal) or the early postmenarchal group (menses within one year), were undergoing physiologic changes which might influence the subgingival microflora. The question of which pubertal physiologic changes might be responsible for alterations in the subgingival microflora is open to speculation. Certainly host defense changes do occur with maturation, but literature dealing with immunologic cell changes in the gingiva and gingival crevice have thus far only compared children in the primary dentition or early mixed dentitions to adults. Pubertal host defense changes in periodontal tissues have not been documented.

Less subtle are the increasing levels of circulating hormones seen during puberty, which are responsible for development of the reproductive organs, as well as for breast and pubic hair development. Although studies have not specifically related changes in steroid hormone levels at puberty with periodontal disease or the subgingival microflora, laboratory experiments and observations of the gingival and bacterial responses in altered-hormone states (i.e., pregnancy and with oral contraceptive) suggest an important relationship between steroid hormone levels and changes in the subgingival microflora. Kornman and Loesche (1980) demonstrated increasing levels of \textit{Bacteroides intermedius} in the second trimester of pregnancy. They also demonstrated an interaction of steroids with \textit{B. intermedius} and \textit{B. melaninogenicus}, as progesterone and estradiol could substitute for vitamin K, an essential growth factor for these organisms (Kornman and Loesche, 1982.) Thus, similar changes in the subgingival microflora may be occurring at puberty, as progesterone and estrogen levels are known to increase dramatically (Gruber and Lucas, 1976).
Conclusions

1. Indices of development used in this investigation appeared useful in documenting the developmental age of subjects. The parameters of menarchal status, breast development, and pubic hair development were of particular importance in an examination of microbiological changes in subgingival plaque with sexual maturation, while dental age and skeletal maturation were more reflective of somatic development.

2. In comparing the predominant cultivable microflora of the subgingival plaque in this group of children to that of adults as described in the literature, the microflora appears to be comparable to that found between gingival health and gingivitis. Most groups of microorganisms seen in the gingival crevice in adults were also detected in the children, i.e., spirochetes and black-pigmented Bacteroides.

3. There did appear to be significant changes in the predominant cultivable microflora of the gingival crevice associated with menarche. Levels of Actinomyces naeslundii decreased with increasing maturation, while levels of black-pigmented Bacteroides were higher just prior to menarche than in sexually more immature or mature females.
Figure 1: Flow chart of experimental protocol.
EXPERIMENTAL PROTOCOL

Females (Age 7 - 16)

- Dental Age
- Skeletal Maturation Assessment
- Menarchal Onset
- Sexual Maturation (Tanner Staging)

Clinical Examination
- Plaque Index
- Bleeding Index
- Probing Depth

Microbiological Examination
- Microscopic Counts
- Predominant Cultivable Microflora
Figure 2: Relationship of breast development, pubic hair development, and menarchal status of subjects.

● = premenarchal
■ = postmenarchal
Figure 3: Clinical description of the subjects: Plaque Index, Papillary Bleeding Index, and probing depth.
CLINICAL EVALUATION

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# SITES

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Figure 4: Relationship of chronologic age to skeletal maturation. 
$p<0.0005$, Spearman rank correlation coefficient.
Figure 5: Relationship of chronologic age to dental age.

$p<0.0005$, Spearman rank correlation coefficient.
Breast Stage vs. Chronologic Age

Figure 6: Relationship of Tanner breast stage to chronologic age. 
p<0.0005, Spearman rank correlation coefficient.
Figure 7: Relationship of Tanner pubic hair stage to chronologic age.

p<0.0005, Spearman rank correlation coefficient.
Figure 8: Relationship of dental age to skeletal maturation.
p<0.0005, Spearman rank correlation coefficient.
Figure 9: Relationship of Tanner breast stage to dental age. p<0.0005, Spearman rank correlation coefficient.
Figure 10: Relationship of Tanner pubic hair stage to dental age.

p<0.0005, Spearman rank correlation coefficient.
Pubic Hair Stage vs Skeletal Maturation

Figure 11: Relationship of Tanner pubic hair stage to skeletal maturation.

p<0.0005, Spearman rank correlation coefficient.
Breast Stage vs. Skeletal Maturation

Figure 12: Relationship of Tanner breast stage to skeletal maturation.

p<0.0005, Spearman rank correlation coefficient.
Figure 13: Relationship of percentage Gram-negative rods in the total cultivable microflora to chronologic age. 

p>0.05, Spearman rank correlation coefficient.
Figure 14: Relationship of percentage Gram-negative rods in the total cultivable microflora to dental age.

\( p > 0.05 \), Spearman rank correlation coefficient.
Figure 15: Relationship of percentage Gram-negative rods in the total cultivable microflora to skeletal maturation. p>0.05, Spearman rank correlation coefficient.
Figure 16: Relationship of percentage Gram-negative rods in the total cultivable microflora to Tanner breast stage. 

$p>0.05$, Spearman rank correlation coefficient.
Figure 17: Relationship of percentage Gram-negative rods in the total cultivable microflora to Tanner pubic hair stage. p>0.05, Spearman rank correlation coefficient.
Figure 18: Relationship of percentage Gram-positive rods in the total cultivable microflora to chronologic age.  
p>0.05, Spearman rank correlation coefficient.
Figure 19: Relationship of percentage Gram-positive rods in the total cultivable microflora to dental age.

p > 0.05, Spearman rank correlation coefficient.
Figure 20: Relationship of percentage Gram-positive rods in the total cultivable microflora to skeletal maturation. 

p>0.05, Spearman rank correlation coefficient.
Figure 21: Relationship of percentage Gram-positive rods in the total cultivable microflora to Tanner breast stage. 

p > 0.05, Spearman rank correlation coefficient.
Figure 22: Relationship of percentage Gram-positive rods in the total cultivable microflora to Tanner pubic hair stage. p>0.05, Spearman rank correlation coefficient.
Figure 23: Relationship of percentage Gram-negative cocci in the total cultivable microflora to chronologic age. p > 0.05, Spearman rank correlation coefficient.
Figure 24: Relationship of percentage Gram-negative cocci in the total cultivable microflora to dental age.

p>0.05, Spearman rank correlation coefficient.
Figure 25: Relationship of percentage Gram-negative cocci in the total cultivable microflora to skeletal maturation. p>0.05, Spearman rank correlation coefficient.
Figure 26: Relationship of percentage Gram-negative cocci in the total culturable microflora to Tanner breast stage.
p>0.05, Spearman rank correlation coefficient.
Figure 27: Relationship of percentage Gram-negative cocci in the total cultivable microflora to Tanner pubic hair stage. $p>0.05$, Spearman rank correlation coefficient.
Figure 28: Relationship of percentage Gram-positive cocci in the total cultivable microflora to chronologic age. p>0.05, Spearman rank correlation coefficient.
Figure 29: Relationship of percentage Gram-positive cocci in the total cultivable microflora to dental age.

p>0.05, Spearman rank correlation coefficient.
Figure 30: Relationship of percentage Gram-positive cocci in the total cultivable microflora to skeletal maturation. p>0.05, Spearman rank correlation coefficient.
Figure 31: Relationship of percentage Gram-positive cocci in the total cultivable microflora to Tanner breast stage. 

$p>0.05$, Spearman rank correlation coefficient.
Figure 32: Relationship of percentage Gram-positive cocci in the total cultivable microflora to Tanner pubic hair stage. $p>0.05$, Spearman rank correlation coefficient.
Figure 33: Relationship of percentage anaerobes in the total cultivable microflora to chronologic age.

p > 0.05, Spearman rank correlation coefficient.
Figure 34: Relationship of percentage anaerobes in the total cultivable microflora to dental age.

p > 0.05, Spearman rank correlation coefficient.
Figure 35: Relationship of percentage anaerobes in the total cultivable microflora to skeletal maturation.

$p > 0.05$, Spearman rank correlation coefficient.
Figure 36: Relationship of percentage anaerobes in the total cultivable microflora to Tanner breast stage. 

p>0.05, Spearman rank correlation coefficient.
Figure 37: Relationship of percentage anaerobes in the total cultivable microflora to Tanner pubic hair stage. 

p>0.05, Spearman rank correlation coefficient.
Figure 38: Relationship of percentage black-pigmented Bacteroides in the total cultivable microflora to chronologic age. 

$p > 0.05$, Spearman rank correlation coefficient.
Figure 39: Relationship of percentage black-pigmented Bacteroides in the total cultivable microflora to dental age.

p > 0.05, Spearman rank correlation coefficient.
Figure 40: Relationship of percentage black-pigmented Bacteroides in the total cultivable microflora to skeletal maturation.

p>0.05, Spearman rank correlation coefficient.
Figure 41: Relationship of percentage black-pigmented Bacteroides in the total cultivable microflora to Tanner breast stage.

$p>0.05$, Spearman rank correlation coefficient.

$p<0.05$, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 42: Relationship of percentage black-pigmented *Bacteroides* in the total cultivable microflora to Tanner pubic hair stage.

p>0.05, Spearman rank correlation coefficient.

p>0.05, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 43: Relationship of percentage spirochetes in the total microscopic count to chronologic age.

p>0.05, Spearman rank correlation coefficient.
Figure 44: Relationship of percentage spirochetes in the total microscopic count to dental age.

p>0.05, Spearman rank correlation coefficient.
Figure 45: Relationship of percentage spirochetes in the total microscopic count to skeletal maturation. p > 0.05, Spearman rank correlation coefficient.
Figure 46: Relationship of percentage spirochetes in the total microscopic count to Tanner breast stage.

$\text{p} > 0.05$, Spearman rank correlation coefficient.

$\text{p} > 0.05$, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 47: Relationship of percentage spirochetes in the total microscopic count to Tanner pubic hair stage. 

p > 0.05, Spearman rank correlation coefficient. 

p > 0.05, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 48: Relationship of percentage surface translocating bacteria in the total cultivable microflora to chronologic age.

p>0.05, Spearman rank correlation coefficient.
Figure 49: Relationship of percentage surface translocating bacteria in the total cultivable microflora to dental age.

$p > 0.05$, Spearman rank correlation coefficient.
Figure 50: Relationship of percentage surface translocating bacteria in the total cultivable microflora to skeletal maturation.

*p > 0.05, Spearman rank correlation coefficient.*
Figure 51: Relationship of percentage surface translocating bacteria in the total cultivable microflora to Tanner breast stage.

p<0.01, Spearman rank correlation coefficient.

p<0.05, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 52: Relationship of percentage surface translocating bacteria in the total cultivable microflora to Tanner pubic hair stage.

p < 0.005, Spearman rank correlation coefficient. 
p < 0.01, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 53: Relationship of percentage *Fusobacterium nucleatum* in the total cultivable microflora to chronologic age. p > 0.05, Spearman rank correlation coefficient.
Figure 54: Relationship of percentage *Fusobacterium nucleatum* in the total cultivable microflora to dental age. 

$p > 0.05$, Spearman rank correlation coefficient.
Figure 55: Relationship of percentage *Fusobacterium nucleatum* in the total cultivable microflora to skeletal maturation.

*p>0.05*, Spearman rank correlation coefficient.
Figure 56: Relationship of percentage *Fusobacterium nucleatum* in the total cultivable microflora to Tanner breast stage.

p>0.05, Spearman rank correlation coefficient.

p>0.05, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 57: Relationship of percentage *Fusobacterium nucleatum* in the total cultivable microflora to Tanner pubic hair stage.

p > 0.05, Spearman rank correlation coefficient.

p > 0.05, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 58: Relationship of percentage Actinomyces in the total cultivable microflora to chronologic age.

$p<0.005$, Spearman rank correlation coefficient.
Figure 59: Relationship of percentage Actinomyces in the total cultivable microflora to dental age.

p<0.005, Spearman rank correlation coefficient.
Figure 60: Relationship of percentage Actinomyces in the total cultivable microflora to skeletal maturation. p<0.005, Spearman rank correlation coefficient.
Figure 61: Relationship of percentage Actinomyces in the total cultivable microflora to Tanner breast stage. 

p<0.01, Spearman rank correlation coefficient. 

p<0.01, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 62: Relationship of percentage Actinomyces in the total cultivable microflora to Tanner pubic hair stage.

p<0.005, Spearman rank correlation coefficient.

p<0.02, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 63: Relationship of percentage Actinomyces naeslundii in the total cultivable microflora to chronologic age. 
p<0.005, Spearman rank correlation coefficient.
Figure 64: Relationship of percentage *Actinomyces naeslundii* in the total cultivable microflora to dental age. 

$p<0.005$, Spearman rank correlation coefficient.
Figure 65: Relationship of percentage *Actinomyces naeslundii* in the total cultivable microflora to skeletal maturation.

$p < 0.005$, Spearman rank correlation coefficient.
Figure 66: Relationship of percentage Actinomyces naeslundii in the total cultivable microflora to Tanner breast stage.

$p<0.025$, Spearman rank correlation coefficient.

$p<0.001$, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 67: Relationship of percentage *Actinomyces naeslundii* in the total cultivable microflora to Tanner pubic hair stage.

p<0.005, Spearman rank correlation coefficient.

p<0.01, Kruskal-Wallis one-way analysis of variance by ranks.
Figure 68: Relationship of percentage black-pigmented *Bacteroides* in the total cultivable microflora to menarchal status.


