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Differences between gingivitis and periodontitis associated microbial flora in the beagle dog

Relationship of plaque parameters to histological parameters of periodontal disease

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The predominant dental plaque flora was compared between two groups of adult beagle dogs, (1) ten with gingivitis and (2) ten with advanced periodontitis. Plaque from a maxillary third premolar was cultured under strict anaerobic conditions. Specimens comprising the marginal periodontal tissues were taken at the plaque sampling site and analyzed for some histological parameters of periodontal disease. The periodontitis dogs scored significantly higher for crevice depth, length of ulcerated crevice epithelium, area of inflamed connective tissue, and loss of attachment. Supragingival periodontitis plaque had significantly higher anaerobic to aerobic ratio, proportions and CFU of esculin negative streptococci, but lower proportions of *Actinomyces viscosus*. Subgingival periodontitis plaque had significantly higher anaerobic to aerobic ratio, microscopic counts of spirochetes, total viable CFU, proportions and CFU of esculin negative streptococci and *Fusobacterium nucleatum*, as well as CFU of *Bacteroides asaccharolyticus*, Gram negative bacilli and coccobacilli, but significantly lower proportions of *A. viscosus* and unspeciated actinomycetes. The total viable CFU, proportions and CFU of esculin negative streptococci correlated with all four histological parameters of periodontal disease. The CFU of *B. asaccharolyticus*, bacilli, and coccobacilli correlated with the length of ulcerated crevice epithelium and loss of attachment, but *F. nucleatum* only with the area of inflamed connective tissue and loss of attachment.

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Introduction

In most studies on the etiology and pathogenesis of periodontal disease in the beagle dog, only the quantity of dental plaque has been correlated with the severity of the periodontal lesion. A few investigations have been aimed at determining the composition of the canine microbial flora associated with gingivitis (Krasse & Brill 1960, De Castro & Going 1964, Soames & Davies 1975, Listgarten, Lindhe & Parodi 1979, Syed, Svanberg & Svanberg 1980) and with periodontitis (Wunder, Briner & Calkins 1976, Newman et al. 1977, Williams et al. 1979, Syed, Svanberg & Svanberg 1981), but only Courant et al. (1968) made an attempt to compare the bacterial flora in periodontal health and disease. However, considering the various levels of anaerobiosis described by

Aranki et al. (1969), Courant's study was conducted using less than strict anaerobic conditions, and only a few selected organisms were isolated for identification.

The aim of the present study was two-fold: to compare the cultivable microbial flora associated with gingivitis and periodontitis using strict anaerobic techniques and to determine the relationship, if any, between certain microorganisms and some histological parameters of periodontal disease.

Material and Methods

Twenty female beagle dogs were obtained from the Laboratory Research Enterprises Inc., Kalamazoo, Michigan. The animals are genotypically and phenotypically highly standardized. After six to twelve weeks in the nursery, where the pups are kept in groups of about 100 animals, two or three dogs are placed in woven steel rod cages where they remain for the rest of their stay. They are fed a standard pellet dog food (Bench and Field 26, Martin Feeds, New Paris, Indiana) and water *ad libitum*.

Two age-matched sets of dogs, ranging in age from three to six years, were selected by oral examination from a group of 800 animals, 10 dogs for having minimal gingival inflammation and no detectable loss of connective tissue attachment (Gingivitis group) and 10 dogs with advanced periodontitis.

Table 1

Distribution of Plaque Index (PII) scores and Gingival Index (GI) scores of upper left third premolar (p^3)

Dogs	PII			GI		
	1	2	3	1	2	3
Gingivitis		9	1	1	9	
Periodontitis		1	9		10	
Signif.		$p < 0.002$			NS	

Plaque Collection Procedure

The animals were anesthetized with an intravenous injection of Pentothal (Abbott, North Chicago, Illinois). The Plaque Index score (Silness & Löe 1964) and the Gingival Index score (Löe & Silness 1963) were then assessed for the upper left third premolar (P^3). Supragingival plaque was collected from a two millimeter wide zone adjacent to the gingival margin of the distal root of P^3 using a stainless steel plaque spoon (1.7 × 15 mm) held in a hemostat. The plaque spoon was immediately placed in a 1 dram screw cap vial filled to the rim with prereduced transport fluid (RTF, Syed & Loesche 1972) and taken into an anaerobic glove box (Aranki et al. 1969) containing a gaseous mixture of 85 % nitrogen, 10 % hydrogen, and 5 % carbon dioxide.

The remaining supragingival deposits were then removed with a curette operated in a disto-mesial and slightly coronal direction. Subgingival plaque was collected with a stainless steel wire loop (wire diameter 0.3 mm, outer diameter of loop 1.5 mm) held in a hemostat. The loop was introduced into the crevice at a slight angle to the tooth surface, moved apically until slight resistance was encountered, and then moved back and forth in a mesio-distal direction with light pressure toward the tooth surface to free any adherent plaque. The loop holding the plaque sample was immediately placed in RTF and taken into the anaerobic glove box. This method of sampling provided a standard volume sample as plaque was retained only in the interior of the loop.

Plaque Culturing and Characterization of Organisms

Plaque culturing was done inside the anaerobic glove box, and the procedures have been described in detail in a previous paper (Syed et al. 1980). Each sample was dispersed for 20 seconds with an ultrasonic dis-

rupter (Kontes, Vineland, New Jersey; frequency 23,500 cycles/s, power output 1,200 watt/sq. in.) followed by Vortex mixing for 15 seconds. Serial fivefold dilutions in RTF were prepared, and 50 µl aliquots of the last three dilutions were dispersed with an Eppendorff pipette and spread with sterile L-shaped glass rods on duplicate plates of the following prereduced media: enriched trypticase soy agar (ETSA, Syed et al. 1980), ETSA with 5 % sucrose, and Schaedler agar (Baltimore Biological Laboratories, Cockeysville, Maryland) with menadione and 3 % defibrinated sheep blood. The procedure was completed 30 to 60 minutes after collection of the plaque. The inoculated plates were incubated at 37°C in the glove box for six to eight days. One plate from each sample showing 75 to 150 well-isolated colony forming units (CFU) was kept in the glove box and used for isolation and subculturing of the organisms. The remaining plates were used for determination of total and differential CFU and then discarded. A duplicate set of ETSA plates from each specimen was incubated aerobically to determine the aerobic CFU.

Subculturing of the organisms on the primary ETSA plates was done inside the glove box. Prior to transfer into broth media the colonies were examined for purity using a stereomicroscope equipped with a zoom lens and illuminator. The microscope was also used when picking the colonies. Each colony was transferred into basal esculin broth (Syed & Loesche 1978) and the growth used for esculin hydrolysis, nitrate reduction and indole production tests, Gram stain, and inoculation of an ETSA plate for aerobic incubation. The purity of the subcultures was confirmed by microscopic examination of Gram stained preparations of the broth cultures and stereomicroscopic examination of the growth on agar media used for various tests. Samples of the broth were also passed into suitable media for catalase production, glucose fermentation, and gelatinase tests (Syed 1976). Gas-liquid chromatographic analyses of volatile and non-volatile fatty acid end products (Holdeman & Moore 1975) from representative isolates were done either to confirm the identification or facilitate the characterization of the organisms. The organisms were classified according to the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons 1974), the *Anaerobic Laboratory Manual of Determinative Bacteriology* (Holdeman & Moore 1975), and a taxonomic scheme (Syed & Loesche 1978).

Spirochete counts and anaerobic to aerobic ratio: Following sonication and appropriate dilution of the samples the microscopic counts of spirochetes were determined in a Petroff Hausser chamber with buffered formalin-crystal violet added to the suspending medium (Aranki et al. 1969). Since the number of spirochetes was low in the gingivitis samples, the whole chamber (400 squares) was used.

The anaerobic to aerobic ratio was determined as the ratio of total colonies grown anaerobically to total colonies grown aerobically. Anaerobic counts included strictly anaerobic and facultative organisms. Aerobic counts included aerobic and facultative organisms.

Biopsy Procedure

Specimens comprising parts of the tooth and the adjacent marginal periodontal tissues (Schroeder et al. 1973) were taken at each plaque sampling site, fixed in 5 % glutaraldehyde and 4 % paraformaldehyde buffered at pH 7.3 (Karnovsky 1965), decalcified in formic acid-citrate (Luna 1968), and embedded in Epon (Luft 1961). Two micron thick bucco-lingual sections were taken and stained with methylene blue and basic fuchsin. Tracings of the sections were obtained by microscopic projection (linear

Table 2

Histological parameters of periodontal disease in gingivitis and periodontitis dogs

	Gingivitis $\bar{x} \pm SE$	Periodontitis $\bar{x} \pm SE$	Signif.
Loss of Attachment*	0.03 ± 0.01	2.48 ± 0.44	p = 0.0001
Crevice Depth*	1.13 ± 0.11	2.28 ± 0.37	p = 0.004
Length of Ulcerated Crevice Epithelium*	0.26 ± 0.07	1.63 ± 0.30	p = 0.0001
Area of Inflamed Connective Tissue**	0.20 ± 0.03	1.61 ± 0.35	p = 0.0003

* Measurements given in mm.
** Measurements given in mm^2.

mag. 55 ×) and analyzed for the following four parameters: (1) crevice depth, measured from the free gingival margin to the free surface of the junctional epithelium, (2) length of ulcerated crevice epithelium, identified as crevice areas without any light microscopically distinguishable epithelium, (3) area of inflamed connective tissue, comprising the cell rich/collagen poor tissue portions, and (4) loss of attachment, measured from the cemento-enamel junction to the apical termination of the junctional epithelium.

Statistical Analysis

When comparing gingivitis and periodontitis dogs, the Median test was used for ordinal level measurements (Plaque Index, Gingival Index) and Student's t-test for ratio level measurements (histological measurements, plaque parameters). The relationship between subgingival microorganisms and the histological parameters of periodontal disease was analyzed by the Pearson Product-Moment Correlation test.

Results

Clinical Parameters

The upper left third premolar of the periodontitis dogs had significantly more supragingival plaque than those of the gingivitis dogs with a predominance of Plaque Index scores of 3. The clinical signs of gingival inflammation were similar in both groups with a Gingival Index score of 2 (Table 1).

Table 3

Plaque parameters of supragingival and subgingival plaque*

	Supragingival Plaque			Subgingival Plaque		
	Gingivitis $\bar{x} \pm SE$	*Periodontitis* $\bar{x} \pm SE$	Signif.	*Gingivitis* $\bar{x} \pm SE$	*Periodontitis* $\bar{x} \pm SE$	Signif.
Viable CFU per sample × 10^6	18.0 ± 4.5	32.9 ± 8.8	NS	0.09 ± 0.05	3.2 ± 0.8	p = 0.0003
Anaerobic/Aerobic Ratio**	5.4 ± 1.4	46.9 ± 18.7	p = 0.03	8.1 ± 2.7	76.6 ± 29.1	p = 0.02
Microscopic Counts of Spirochetes × 10^6	3.9 ± 0.8	6.4 ± 1.3	NS	ND	15.0 ± 4.7	p = 0.003

* *One sample was taken from 10 dogs in each group.*

** Anaerobic/aerobic ratio = $\frac{\text{All CFU recovered anaerobically}}{\text{All CFU recovered aerobically}}$

ND = Not detectable

Table 4

Differences in the proportions of the predominant cultivable plaque flora

	Supragingival Plaque			Subgingival Plaque		
	Gingivitis $\bar{x} \pm SE$	Periodontitis $\bar{x} \pm SE$	Signif.	Gingivitis $\bar{x} \pm SE$	Periodontitis $\bar{x} \pm SE$	Signif.
A viscosus	2.9 ± 1.1*	0.08 ± 0.075	p = 0.05	1.6 ± 0.5	0.3 ± 0.2	p = 0.05
Unspeciated actinomycetes				3.5 ± 1.0	0.8 ± 0.4	p = 0.03
Streptococci (esculin neg.)	4.4 ± 1.5	12.3 ± 2.2	p = 0.008	2.2 ± 0.8	10.9 ± 3.9	p = 0.02
F. nucleatum				20.7 ± 6.5	43.0 ± 8.4	p = 0.05

* Percentage of total cultivable bacteria on anaerobically incubated ETSA plates.
** Anaerobic and facultative streptococci which were not speciated.

Histological Parameters

Specimens taken at the plaque sampling site in the gingivitis dogs showed insignificant loss of connective tissue attachment ($\bar{x} = 0.03$ mm), whereas the periodontitis specimens revealed considerable breakdown of the supporting tissues ($\bar{x} = 2.48$ mm, Table 2). The periodontitis specimens also displayed significantly deeper crevices, more extensive ulceration of the crevicular epithelium, and larger areas of inflamed connective tissue.

Plaque Parameters

Supragingival plaques of both groups were similar in total viable CFU and microscopic counts of spirochetes, whereas the anaerobic to aerobic ratio was significantly higher in the periodontitis samples (Table 3). Subgingival samples from the periodontitis dogs had significantly higher total viable CFU, anaerobic to aerobic ratio, and microscopic counts of spirochetes (Table 3).

Bacteriological Findings

The predominant cultivable flora was analyzed for differences in the proportions of microorganisms associated with gingivitis and periodontitis. Only those results which showed a significant difference between the two groups are given in Table 4. The gingivitis dogs had significantly higher proportions of *A. viscosus* in supragingival and subgingival plaque and unspeciated actinomycetes in subgingival plaque. The periodontitis dogs had significantly higher proportions of esculin negative streptococci in supragingival and subgingival plaque and

Table 5

Differences in viable counts $\times 10^4$ of predominant cultivable flora

	Supragingival Plaque			Subgingival Plaque		
	Gingivitis $\bar{x} \pm SE$	Periodontitis $\bar{x} \pm SE$	Signif.	Gingivitis $\bar{x} \pm SE$	Periodontitis $\bar{x} \pm SE$	Signif.
Gram pos. rods (esculin neg.)				1.0 ± 0.7	9.4 ± 2.9	p = 0.005
Streptococci (esculin neg.)	48.0 ± 17.5	447.2 ± 186.9	p = 0.02	0.2 ± 0.18	42.7 ± 17.8	p = 0.01
F. nucleatum				2.3 ± 1.8	122.1 ± 41.3	p = 0.003
B. asaccharolyticus				0.7 ± 0.3	81.9 ± 32.2	p = 0.008
Gram neg. bacilli				0.1 ± 0.03	21.8 ± 7.2	p = 0.002
Gram neg. coccobacilli				1.5 ± 0.7	12.4 ± 4.5	p = 0.01

Table 6

Relationship between microorganisms of subgingival plaque and histological parameters of periodontal disease

	Crevice depth		Length of Ulcerated Crevice *Epithelium*		Area of Inflamed *Connective* Tissue		Loss of Attachment	
	Corr. coeff.	Signif. p value	Corr. coeff.	Signif. p value	Corr. coeff.	Signif. p value	Corr. coeff.	Signif. p value
Total viable CFU	0.63	0.004	0.82	0.0001	0.62	0.004	0.82	0.0001
Viable counts of:								
Gram positive organisms								
Esculin negative rods	0.55	0.01	0.55	0.02	0.66	0.002	0.63	0.004
Unspeciated actinomycetes			0.62	0.005				
Esculin negative streptococci	0.64	0.003	0.79	0.0001	0.52	0.02	0.75	0.0002
Gram negative organisms								
F. nucleatum					0.60	0.006	0.60	0.007
B. asaccharolyticus	0.60	0.007	0.85	0.0001			0.75	0.0002
Bacilli			0.70	0.0009			0.56	0.01
Coccobacilli			0.81	0.0001			0.56	0.01
Proportions (%) of:								
Esculin negative streptococci	0.65	0.003	0.67	0.002	0.57	0.01	0.67	0.002

significantly higher proportions of *F. nucleatum* in subgingival plaque.

The two groups were compared for differences in the absolute levels of viable CFU of the various organisms (Table 5). Esculin negative streptococci were the only organisms that were found in significantly higher numbers in both supragingival and subgingival plaque in the periodontitis dogs. This is a heterogeneous group of organisms and includes both anaerobic and facultatively anaerobic streptococci. They were not *Streptococcus mutans* or *Streptococcus sanguis*. We were not able to speciate these organisms by the criteria that were used in this study. Therefore, these organisms were treated as a group rather than a single species. Subgingival samples from the periodontitis dogs also had significantly higher counts of esculin negative Gram positive rods, *F. nucleatum*, *B. asaccharolyticus*, and Gram negative bacilli and coccobacilli.

The bacteriological parameters of the subgingival samples were analyzed for correlations, if any, with the histological parameters of periodontal disease (Table 6). Loss of attachment and the length of ulcerated crevice epithelium showed a high correlation with the total viable CFU as well as the CFU of esculin negative streptococci and *B. asaccharolyticus*, and a moderate correlation with the CFU of esculin negative Gram positive rods and Gram negative bacilli. Also, loss of attachment displayed a moderate relation with the CFU of *F. nucleatum* and Gram negative coccobacilli. The length of ulcerated crevice epithelium revealed a moderate relation with the CFU of unspeciated actinomycetes and a high relation with the CFU of Gram negative coccobacilli. Crevice depth and the area of inflamed connective tissue were correlated moderately with the total viable CFU and the CFU of esculin negative Gram positive rods and streptococci. In addition, a moderate relation was seen between crevice depth and the CFU of *B. asaccharolyticus* as well as between the area of inflamed connective tissue

and the CFU of *F. nucleatum*. In terms of proportions only the esculin negative Gram positive streptococci correlated significantly with the severity of the lesion and showed a moderate relation with all four histological parameters of periodontal disease.

Discussion

In the present study on the dental microbial flora, beagle dogs with advanced periodontitis were compared with those that had gingivitis but negligible loss of connective tissue attachment. The two groups of dogs had similar GI scores, but the periodontitis dogs scored significantly higher for histological parameters of periodontal disease such as crevice depth, length of ulcerated crevice epithelium, area of inflamed connective tissue, and loss of attachment. The results showed that periodontal pockets harbored significantly more Gram negative anaerobes and spirochetes than did gingivitis sites. *F. nucleatum* accounted for a major portion of the periodontal pocket flora which also contained significantly higher proportions of esculin negative streptococci.

It has been reported that Gram negative anaerobic organisms account for a major portion of the subgingival flora of the beagle dog (Newman et al. 1977, Williams et al. 1979, Syed et al. 1980, 1981) and that the mean proportions of *B. melaninogenicus (B. asaccharolyticus)* are higher in advanced than incipient periodontal lesions (Newman et al. 1977). Our results indicate that the proportions of *B. asaccharolyticus* in samples from periodontal pockets are high (20.3 ± 5.4 %; $\bar{x} \pm$ SE), but not significantly different from the gingivitis samples (19.0 ± 8.4 %; $\bar{x} \pm$ SE). However, the levels of subgingival *B. asaccharolyticus* exhibited strong correlations with the length of ulcerated crevice epithelium and loss of attachment, and the subgingival periodontitis samples had significantly higher CFU of this organism than did the subgingival gingivitis samples. These findings imply a relationship between *B. melaninogenicus (B. asaccharolyticus)* and the severity of periodontal disease in the beagle dog. *B. asaccharolyticus* and closely related strains also have been described as important pathogens in actively destructive periodontal disease in the *Macaca arctoides* monkey (Slots & Hausmann 1979). This is in agreement with human studies which implicate this organism as a periodontal pathogen (Slots 1977, Loesche et al. 1981) that is isolated from sites with the most inflammation and suppuration (Tanner et al. 1979). However, *B. asaccharolyticus* isolated from the oral flora of beagles (Syed 1980) and *Macaca arctoides* (Slots & Hausmann 1979) show catalase activity whereas the human oral strains are catalase negative and have higher contents of guanine – plus – cytosine of the deoxyribonucleic acids (Coykendall, Kaczmarek & Slots 1980).

Newman et al. (1977) found similar and fairly low proportions of *F. nucleatum* in subgingival samples from incipient (~ 2 %) and advanced (~ 4 %) periodontal lesions in the beagle dog. In comparison with these figures, the mean proportions of *F. nucleatum* in our study were about ten times higher in both the gingivitis and periodontitis samples. Our findings are compatible with what has been described for the human subgingival flora in that *F. nucleatum* may account for a considerable portion of the cultivable flora recovered from relatively healthy sites (Tanner et al. 1979). However, the general pattern seems to be that subgingival *F. nucleatum* are low in clinically healthy sites (Slots 1977, Newman et al. 1978), increase with increasing GI scores (van Palenstein Helderman 1975) and, together with *B. melaninogenicus (B. asaccharolyticus)*, account for the major portion of the viable CFU in periodontal pockets (Slots 1977). This is analogous to our find-

ings in that, although high in the subgingival gingivitis samples, the proportions of *F. nucleatum* were significantly higher in the subgingival periodontitis samples and together with *B. asaccharolyticus* accounted for an average of about 60 % of the cultivable subgingival periodontitis flora.

Our gingivitis samples harbored streptococci at levels similar to what has been reported for the beagle dog (Wunder et al. 1976, Williams et al. 1979). The proportions of esculin negative streptococci were significantly higher in the periodontal pockets but lower than what has been described for periodontal pockets in humans (Gibbons et al. 1963, 1964, Dwyer & Socransky 1968). Their less than strict anaerobic techniques most likely discriminated against the fastidious Gram negative anaerobes resulting in an overestimation of the Gram positive organisms e.g. streptococci. When the human subgingival flora of periodontal pockets was cultured under strict anaerobiosis, Gram positive cocci (Williams, Pantalone & Sherris 1976) and facultatively anaerobic cocci (Slots 1977) accounted for about 15 % and 6 % respectively, which is similar to our findings in periodontal pockets in the beagle dog.

Actinomyces species were among the predominant cultivable organisms of the supragingival and subgingival gingivitis flora in the beagle dog (Syed et al. 1980) but were present in low proportions in periodontitis dogs, particularly in the subgingival samples (Syed et al. 1981). High proportions of actinomycetes have been reported for the human gingivitis flora (Loesche & Syed 1978, Slots et al. 1978), but these organisms were found to be less numerous in destructive disease sites (Tanner et al. 1979). This is in keeping with the results of the present study in that *Actinomyces* species were very low in the periodontal pockets.

Although Courant et al. (1968) could not detect any spirochetes in dogs with periodontitis, these organisms were seen in most sites with gingivitis (Swensen & Muhler 1947, Krasse & Brill 1960) and found to predominate in the apical part of the gingival crevice (Soames & Davies 1975). In our study, the spirochete counts were similar in supragingival gingivitis and periodontitis plaque, but although found in significant numbers in the subgingival periodontitis flora, spirochetes were not detected in the subgingival gingivitis samples. These findings suggest a relationship between spirochetes and the severity of periodontal disease in the beagle dog, which also has been reported for humans (Socransky et al. 1963, Lindhe, Liljenberg & Listgarten 1980). However, our data reveal no correlation of spirochetes to crevice depth, length of ulcerated crevice epithelium, area of inflamed connective tissue or loss of attachment. Spirochetes require some essential nutrients that can be provided by other organisms of the oral flora and might be contained in the crevicular fluid (Loesche 1976). Thus, it is conceivable that the establishment of a complex flora associated with periodontal disease will favor the growth of spirochetes. The seepage of exudate from the periodontal lesion might help explain why the surface portion of subgingival plaque has a layer of spirochetes (Listgarten 1976) and why the apical part of subgingival plaque may consist entirely of spirochetes (Soames & Davies 1975).

A fall in the oxidation-reduction potential occurs with increasing age of the plaque (Kenney & Ash 1969) which will favor the growth of facultative and strict anaerobic organisms, and bleeding will provide for growth factors that are essential for some organisms (Loesche 1968). Such considerations should call for caution when drawing any conclusions regarding the cause and effect relationship between the bacteriological and histological parameters of periodontal disease. It is true that in the present

study the total viable CFU were significantly higher in the periodontal pockets and showed a strong correlation with the length of ulcerated crevice epithelium and loss of attachment. However, not only the proportions of *F. nucleatum* and esculin negative streptococci but also the CFU of these two organisms as well as the CFU of other Gram negative organisms such as *B. asaccharolyticus,* bacilli and coccobacilli were significantly higher in the subgingival periodontitis samples and also showed a significant correlation with the length of ulcerated crevice epithelium and loss of attachment. These results indicate that specific organisms are correlated with some histological parameters of periodontal disease in the beagle dog and suggest that, in this analysis, not only the proportions but also the CFU of the organisms should be taken into consideration. It is tempting to hypothesize that when the numbers of certain microorganisms reach a critical level their pathogenic potential is increased beyond the capacity of the host defense system resulting in breakdown of the tissues. If this is true it might help explain why, although in significantly lower numbers, periodontitis associated bacteria also are found in gingivitis sites.

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