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Dental biofilms: difficult therapeutic targets

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Periodontal diseases are infections caused by microorganisms that colonize the tooth surface at or below the gingival margin. While these infections have many properties in common with other infectious diseases, they exhibit unique properties conferred by their site of colonization and the nature of the environment in which they reside. Table 1 presents an overly simplified summary of four crude categories of bacterial infectious diseases. When we think of infections, we most commonly think of the acute infectious diseases. These diseases are typically caused by exogenous agents, have a rapid onset post-colonization and run their course within days to weeks. The source of the organisms is often known, usually another individual exhibiting similar disease. The key characteristics of this category of infection are that the organism or its products gain entry into the body and the infection is usually rapidly resolved by a "cure", by removal of some body part or by demise of the patient. Treatment of these infections is usually supportive, although antibiotics are often used in more severe cases. Examples of such infections include local abscesses caused by organisms such as *Staphylococcus aureus*, upper respiratory infections caused by organisms such as *Streptococcus pneumoniae* or *Haemophilus influenzae* or lower gastrointestinal tract infections caused by organisms such as *Vibrio cholerae* or *Salmonella typhi*.

Chronic bacterial infections such as tuberculosis or leprosy often have a very slow onset post-colonization. The organisms are considered to be exogenous, but they can have characteristics of endogenous infections in that colonization by *Mycobacterium tuberculosis* can occur for years prior to

Table 1. Characterisics of bacterial infectious diseases

Acute	Chronic	Delayed	Biofilm
Examples Upper respiratory Local abscess Gastrointestinal tract	Tuberculosis Leprosy	Rheumatic fever Syphilis Gastrointestinal ulcers Lyme disease Cardiovascular disease?	Caries Periodontal diseases Others
Onset after colonization Rapid	Slow	Delayed	Delayed
Course Days–weeks	Months–years	Years	Years
Causative agent(s) Exogenous	Exogenous Endogenous	Varies	Endogenous
Source Often known	Sometimes known	Often not known	Usually unknown
Characteristics Entry into body, rapid resolution	Entry into body, failure of host to cope	Inauspicious onset, later new form of disease	Biofilm Outside body
Treatment Supportive Antibiotic	Antibiotic Supportive	Antibiotic ?	Physical Antimicrobial Ecological

disease detection. The source of the organisms is sometimes, but not always, known. Typically it is an individual exhibiting similar signs of disease. The major characteristics of chronic infections are that the organisms gain entry into the body and persist for long periods of time, and the host is unable to cope with and eliminate the infecting species. Treatment of these infections usually includes antibiotics, but supportive therapy is essential.

A third class of bacterial infectious diseases is rather intriguing in that the signs and symptoms of disease that are of greatest concern to the patient are delayed months to years after initial local infection. Diseases in this category would include rheumatic fever, syphilis, Lyme disease, gastrointestinal tract ulcers or, conceivably, cardiovascular disease (12, 13, 126). The onset after initial colonization is often years and the damage induced can last for a lifetime. The source of the organisms for some of these infections is often unknown. The diseases are characterized by an inauspicious onset, often with minimal local signs of infection that later lead to a new form of the disease. The most effective treatment of these diseases would appear to be antibiotic therapy during the initial phase of the infection. Treatment in later phases presents much greater difficulty.

The fourth category of disease is the one of immediate concern to the readers of this volume. These are the diseases that result from the formation of biofilms on tooth surfaces. These infections include arguably the most common infectious diseases affecting the human: dental caries and periodontal diseases. The onset of these diseases is usually delayed for prolonged periods of time after initial colonization by the pathogen(s). The course of these diseases typically runs for years. The causative agents in most instances appear to be members of the indigenous microbiota and, thus, the infections might be thought of as endogenous. The source of the infecting agents for any given individual is usually unknown, although transfer from parents or significant others is thought to play a primary role (151, 152, 155, 168, 197). The major characteristics of these diseases are that they are caused by organisms that reside in biofilms outside the body. Their treatment, as we have learned, is complex in that physical, antimicrobial and ecological approaches are required. Biofilm-initiated diseases are by no means unique to the oral cavity. Potera (153) has argued that 65% of infections that affect the human are caused by organisms growing in biofilms.

The explosion in biofilm research

Biofilms have become a "hot" topic in environmental and infectious disease microbiology as well in the popular press. Biofilms may be found virtually anywhere. They colonize a widely diverse set of moist surfaces, including the oral cavity, the bottom of boats and docks, the inside of pipes and rocks in streams. Infectious disease investigators are interested in biofilms that colonize a wide array of artificial devices that have been implanted in the human, including catheters, hip and voice prostheses and contact lenses. Applied environmental microbiologists are interested in preventing the effects of biofilms on fouling industrial processes or, alternatively, in using biofilms productively: for example, in sewage treatment plants. Biofilms consist of one or more communities of microorganisms, embedded in a glycocalyx, that are attached to a solid surface (37, 38, 40, 47, 48, 95, 105, 110, 111, 137, 163, 179, 205, 215). The reason for the existence of a biofilm is that it allows microorganisms to stick to and to multiply on surfaces. Thus, attached bacteria (sessile) growing in a biofilm display a wide range of characteristics that provide a number of advantages over single-cell (planktonic) bacteria.

The nature of biofilms

Biofilms are fascinating structures. They are the preferred method of growth for many and perhaps most species of bacteria (164). This method of growth provides a number of advantages to colonizing species. A major advantage is the protection the biofilm provides to colonizing species from competing microorganisms from environmental factors such as host defense mechanisms, and from potentially toxic substances in the environment, such as lethal chemicals or antibiotics. Biofilms also can facilitate processing and uptake of nutrients, cross-feeding (one species providing nutrients for another), removal of potentially harmful metabolic products (often by utilization by other bacteria) as well as the development of an appropriate physicochemical environment (such as a properly reduced oxidation reduction potential).

A crude analogy to the development of a biofilm might be the development of a city. Successful human colonization of new environments requires several important factors including a stable nutrient supply, an environment conducive to proliferation and an environment with limited potential hazards.

Cities (like biofilms) develop by an initial "attachment" of humans to a dwelling site followed by multiplication of the existing inhabitants and addition of new inhabitants. Cities and biofilms typically spread laterally and then in a vertical direction, often forming columnar habitation sites. Cities and biofilms offer their inhabitants many benefits. These include shared resources and interrelated activities. Inhabitants of cities or biofilms are capable of "metabolic processes" and synthetic capabilities that could not be performed by individuals in an unattached (planktonic) or nomadic state. An important benefit provided by a city or biofilm is protection both from other potential colonizers of the same species, from exogenous species and from sudden harmful changes in the environment. Individuals in the "climax community" of a flourishing city or biofilm can facilitate joint activities and live in a far more stable environment than individuals living in isolation. Cities, like biofilms, require a means to bring in nutrients and raw materials and to remove waste products. In cities, these are usually roads, water or sewage pipes; in biofilms they may be water channels such as those described below. Cities have maximum practical sizes based on physical constraints and nutrient or waste limits; so do biofilms. Cities that are mildly perturbed, such as by a snowstorm or a local fire, usually reform a climax community that is similar to that which was present in the first place, as do biofilms. However, major perturbations in the environment such as prolonged drought or a radioactive cloud can lay waste to a city. Major perturbations in the environment such as a toxic chemical can severely affect the composition or existence of a biofilm. Communication between individuals in a city is essential to allow inhabitants to interact optimally. This is usually performed by vocal, written or pictorial means. Communication between bacterial cells within a biofilm is also necessary for optimum community development and is performed by the production of signaling molecules such as those found in "quorum sensing" or perhaps by the exchange of genetic information. The long-term survival of the human species as well as a species in a biofilm becomes more likely if that species (or the human) colonizes multiple sites. Thus, detachment of cells from biofilms and establishment in new sites is as important for the survival of biofilm-dwellers as the migration of individuals and establishment of new cities is for human beings. Thus, we may regard mixed species biofilms as primitive precursors to the more complex organizations observed for eukaryotic species.

The study of biofilms is technically rather difficult, in part because of the microscopic nature of the participating individuals and, in part, because of the complexity of the ecological relationships that occur on a microscopic level. New techniques have had to be developed in order to examine biofilms. Most of the studies have been carried out on systems other than dental plaque, but the general principles that arise from other systems are likely to be relevant to dental biofilms. Methods such as laser confocal microscopy have been used to examine bacterial cells deep within biofilms (111). Miniature microelectrodes have been employed that permit the measurement of the local environment (118) in biofilms. Programmed microscope stages have been developed that permit accurate images of biofilms in three dimensions (117). Thus, the structure and physical properties of biofilms are becoming clearer.

Properties of biofilm

Structure

Biofilms are composed of microcolonies of bacterial cells (15–20% by volume) that are non-randomly distributed in a shaped matrix or glycocalyx (75–80% volume). Earlier studies of thick biofilms (>5 mm) that develop in sewage treatment plants indicated the presence of voids or water channels between the microcolonies present in these biofilms. The water channels permit the passage of nutrients and other agents throughout the biofilm acting as a primitive "circulatory" system. Nutrients make contact with the sessile (attached) microcolonies by diffusion from the water channel to the microcolony rather than from the matrix. Other models of biofilms, however, have been suggested, including the heterogeneous mosaic and the dense biofilm models (189). Microcolonies occur in different shapes in biofilms which are governed by shear forces due to the passage of fluid over the biofilm. At low shear force, the colonies are shaped liked towers or mushrooms, while at high shear force, the colonies are elongated and capable of rapid oscillation (183). Individual microcolonies can consist of a single species but more frequently are composed of several different species.

Exopolysaccharides – the backbone of the biofilm

As mentioned above, the bulk of the biofilm consists of the matrix. It is composed predominantly of water and aqueous solutes. The "dry" material is a mixture

of exopolysaccharides, proteins, salts and cell material. Exopolysaccharides, which are produced by the bacteria in the biofilm, are the major components of the biofilm, making up 50–95% of the dry weight (189). They play a major role in maintaining the integrity of the biofilm and confer other beneficial properties. Bacteria can produce several different polysaccharides depending on the physiological state of the bacteria and the presence of specific substrates. All biofilms contain exopolysaccharides, although they can vary quite markedly in both bacterial composition and the composition of the extracellular matrix. Some exopolysaccharides are neutral, such as the mutan from *Streptococcus mutans*, whereas others are highly charged polyanionic macromolecules. Different ionic charge and concentrations of exopolysaccharides will alter the confirmation and cause rapid changes in the three-dimensional gel network of polysaccharides. Similar effects may also be produced by provision of sucrose or other sugars. The exopolysaccharides can be degraded and utilized by bacteria within the biofilm. One distinguishing feature of oral biofilms is that many of the microorganisms can both synthesize and degrade the exopolysaccharides.

Exopolysaccharides can exist in both ordered or disordered forms. At high temperatures and often at very low ionic concentrations, the disordered form predominates, although few biofilms exhibit total absence of an ordered structure (188). Biofilm matrices are complex structures that contain masses of fibers of varying size, structure, composition and rigidity that interact with each other, with cells and with surface matrices. A wide range of possible conformations, flexibility and configurations can be expected among different classes of polysaccharides. The density of the fibrillar masses will affect accessibility of both cells and surfaces to nutrients and other solutes.

The chemical composition and tertiary structure of the exopolysaccharides will determine whether it forms an effective adhesive. It will also affect the hydrophilic or hydrophobic nature of the surface. Exopolysaccharides aid in protecting microbial cells within the biofilm by preventing desiccation and attack by harmful agents. They may also bind essential nutrients such as cations to create a local nutritionally rich environment favoring specific microorganisms. The exopolysaccharide matrix could also act as a buffer and assist in retaining extracellular enzymes (and their substrates), enhancing substrate utilization by bacterial cells. Exopolysaccharides are effective in maintaining biofilm structure through the formation of networked, cross-linked linear macromolecules. In most mixed biofilms, numerous types of polysaccharide are found, complicating the network structure. The quantity of exopolysaccharides in a biofilm does not necessarily reflect the proportion of the bacterial species that produce it. Loss or removal of one type of exopolysaccharide may have a more drastic effect on the biofilm matrix than another even if the removed polymer is not dominant.

Physiological heterogeneity within biofilms

Cells of the same microbial species can exhibit extremely different physiological states in a biofilm even though separated by as little as 10 μm. Xu et al. (219) grew *Pseudomonas aeruginosa* in an aerated continuous flow reactor and measured various physiological properties by dyes and indicators. DNA indicating the presence of bacterial cells was detected throughout the 110-μm-thick biofilm. Protein synthesis could be detected in the outer 30 μm, respiratory activity in the outer 24 μm and RNA determined by acridine orange and *in situ* hybridization in the outer 21 μm. The authors suggest that antibiotics that kill actively growing cells would affect the outer layer of the biofilm, but the remaining cells would not be affected.

The use of microelectrodes has shown that pH can vary quite remarkably over short distances within a biofilm (204). Two-photon excitation microscopy of *in vitro* plaque made up of ten intraoral species showed that, after a sucrose challenge, microcolonies with a pH less than 3.0 could be detected adjacent to microcolonies with pH exceeding 5.0 (204). The number of metal ions can differ sufficiently in different regions of a biofilm, so that a difference in ion concentration can produce measurable potential differences (40). Bacterial cells within biofilms can produce enzymes such as β-lactamase against antibiotics or catalases, superoxide dismutases against oxidizing ions released by phagocytes. These enzymes are released into the matrix, producing an almost impregnable line of defense. Bacterial cells in biofilms can also produce elastases and cellulases, which become concentrated in the local matrix and produce tissue damage. Measurement of oxygen and other gases has demonstrated that certain microcolonies are completely anaerobic even though composed of a single facultative species and grown in ambient air (48). Carbon dioxide and methane can reach very high concentrations in specific micro-

colonies in industrial biofilms. Thus, studies to date indicate that sessile cells growing in mixed biofilms can exist in an almost infinite range of chemical and physical microhabitats within microbial communities (42).

Factors affecting biofilm development and behavior

Hydrodynamics affect both the physical shear stress at a wetted surface and the rate at which nutrients are transported to the surface of the biofilm (181). Both shear and mass transfer can influence biofilm development. Biofilms grown under high shear are generally thinner and denser than those grown under lower shear (203). Hydrodynamics can also affect biofilm structure. Stoodley et al. (182–184) grew non-defined tap water, defined mixed species and pure cultures of *P. aeruginosa* biofilms under laminar flow (low shear) and turbulent (high shear) conditions. The biofilms grown in low shear were made up of roughly circular cell clusters separated by voids, whereas clusters formed in turbulent flow were elongated to form filamentous streamers in the direction of the flow (185). Some streamers oscillated rapidly, and others that appeared to be more firmly attached to the surface oscillated at the top only. The elongated cell clusters had the advantage of streamlining, which might have reduced drag and thus maintained attachment of the streamer. Oscillation of the streamers may also increase mixing and the transport of solutes through the biofilm (172) and increase the surface area-to-volume ratio, minimizing mass transfer limitations.

Biofilms can also be affected by changes in nutrient concentration. Stoodley et al. (183) demonstrated that adding nutrients to a biofilm increased both mass and structure. A ten-fold increase in carbon and nitrogen concentration to a mixed species biofilm grown under turbulent flow changed the structure from one of ripples and streamers to large cell clusters. In addition, the thickness of the biofilm increased five-fold within 24 hours. Return to the original nutrient levels resulted in an immediate loss of biomass and the reappearance of ripples and streamers. These changes were thought to be caused by drag caused by changes in fluid shear as a result of change in the thickness and surface coverage of the biofilm. The significance of nutritional deprivation on biomass and microbial composition will be discussed further in the section describing the treatment of periodontal infections.

Detachment of cells from biofilms

The detachment of cells from biofilms is essential to allow colonization of new habitats by bacteria. Detachment, however, is probably the least well understood biofilm phenomenon (181). It appears from *in vitro* studies that cells detach in different fashions. Some of these include the detachment of single cells in a continuous predictable fashion (erosion), the sporadic detachment of large groups of cells (sloughing) or an intermediate process whereby large pieces of biofilm are shed from the biofilm in a predictable manner. The more predictable intermediate process results in detached clusters consisting of about 10^4 cells. *In vitro*, the detachment rate was shown to be about six clusters per mm^2 of surface per hour. Unlike single cells, the detached cell cluster may be protected from the host defense systems similar to the protection afforded the biofilm from which it was shed (84).

The rate of detachment of bacteria from biofilms in the oral cavity is not clear. It has been argued that the rate of growth in many biofilms grown *in vitro* may be very slow and that detachment may be an uncommon event (206). The cells in such biofilms may be metabolically active and capable of growth once released from the biofilm. Other considerations suggest that the detachment of cells from intraoral biofilms may be an active ongoing process. The mean total viable counts of bacteria in saliva average about 10^8 per ml (15, 72, 162). The total volume of saliva secreted per day is approximately 1500 ml, suggesting that as many as 1.5×10^{11} bacteria are swallowed per day. These bacteria must come from somewhere, either the biofilms growing on the teeth and soft tissues or perhaps from some limited growth in saliva itself (18, 49–51, 194). Thus, the number of organisms in saliva and the critical importance of transmission of bacterial species reinforces the notion that detachment of bacteria from intraoral biofilms is an important event about which too little is known.

Related to the phenomenon of detachment of biofilm cells in groups is the possibility of bodily movement of biofilm structures en masse on solid surfaces. This possibility has been demonstrated in *in vitro* studies of mixed biofilms that showed movement of intact biofilm structures across solid surfaces while remaining attached to them (181). This has implications for the colonization of surfaces by biofilms. The formation of a biofilm is generally thought to be initiated by colonization of planktonic cells that become attached and then multiply. The

data of Stoodley et al. (181) suggest that the colonization of "preformed" biofilm structures can occur as these structures move to adjacent areas. This may provide certain advantages in that formation of the biofilm is not reliant on planktonic cells, which are known to be more susceptible to antimicrobial agents (66).

Quorum sensing

Some of the functions of biofilms depend on the ability of the bacteria and microcolonies within the biofilm to communicate with one another. Quorum sensing in bacteria "involves the regulation of expression of specific genes through the accumulation of signaling compounds that mediate intercellular communication" (157). Quorum sensing is dependent on cell density. With few cells, signaling compounds may be produced at low levels; however, autoinduction leads to increased concentration as cell density increases. Once the signaling compounds reach a threshold level (quorum cell density), gene expression is activated. Cell signaling has been studied extensively in luminescent bacteria and appears to be mediated by an *N*-acyl homoserine lactone encoded by a *lux*1 gene. A similar system has been shown to exist in certain other gram-negative species. The high cell concentrations in biofilms present an ideal situation for quorum sensing, as even small microcolonies (<10 cells) may induce gene expression since the signaling compounds may be concentrated within the microcolony and are not degraded. Quorum sensing may give biofilms their distinct properties. For example, expression of genes for antibiotic resistance at high cell densities may provide protection. Quorum sensing also has the potential to influence community structure by encouraging the growth of beneficial species (to the biofilm) and discouraging the growth of competitors. It is also possible that the physiological properties of bacteria in the community may be altered through quorum sensing. The possible role of quorum sensing in influencing the properties of biofilms was first suggested by Cooper et al. (36).

Signaling is not the only way of transferring information in biofilms. The high density of bacterial cells growing in biofilms facilitates the exchange of genetic information between cells of the same species and across species or even genera. Conjugation (78), transformation (212), plasmid transfer (3, 11, 25, 112, 119) and transposon transfer (165) have all been shown to occur in naturally occurring or mixed-species biofilms prepared *in vitro*. Of particular interest was the demonstration of transfer of a conjugative transposon conferring tetracycline resistance from cells of one genus, *Bacillus subtilis*, to a *Streptococcus* species present in dental plaque grown as a biofilm in a constant-depth film fermenter (165).

Attachment of bacteria

The key characteristic of a biofilm is that the microcolonies within the biofilm attach to a solid surface (76). Thus, adhesion to a surface is the essential first step in the development of a biofilm. In the mouth, bacteria can attach to a wide variety of surfaces, including the oral soft tissues, the pellicle-coated teeth and other bacteria. Many bacterial species possess surface structures such as fimbriae and fibrils that aid in their attachment to different surfaces. Fimbriae (pili) are proteinaceous hair-like appendages 2–8 nm in diameter (56) composed of protein subunits called fimbrillins (fimbrins). Some fimbriae also carry adhesins. Fimbriae have been detected on a number of oral species including *Actinomyces naeslundii* (75), *Porphyromonas gingivalis* (221), and some strains of streptococci such as *Streptococcus salivarius* (74), *Streptococcus parasanguis* (58) and members of the *Streptococcus mitis* group (75). Examination of the fimbriae of oral strains indicate that they are very thin, flexible and 2–3 nm in diameter, thus differing from the larger more rigid fimbriae found on other bacteria such as *Escherichia coli*. Fibrils can also be found on a number of oral bacterial species. They are morphologically different and shorter than fimbriae and may be densely or sparsely distributed on the cell surface (73). Oral species that posses fibrils include *S. salivarius* (74), *S. mitis* group (75), *Prevotella intermedia*, *Prevotella nigrescens* (53) and *S. mutans* (83).

As will be discussed later, *A. naeslundii* is one of the most important colonizing species on tooth surfaces. This species, together with other *Actinomyces*, comprises a major segment of the microbiota attached to the tooth and may be thought of as part of the "scaffolding" structure of dental plaque. For this reason, the intense examination of its means of attachment has been warranted (16, 71, 97, 104, 125). The fimbriae of *A. naeslundii* are the best characterized of the gram-positive oral bacteria. They are about 3–4 nm wide and <1.5 μm long. Two major types of fimbriae have been identified although they cannot be distinguished ultrastructurally from one another. Some strains of *A. naeslundii* carry both, whereas others carry only one (220). Type 1 fimbriae are associated

with adhesion of *A. naeslundii* to salivary acidic proline-rich proteins and to statherin deposited within the salivary pellicle (27). Type 2 fimbriae are associated with attachment of *A. naeslundii* to glycosidic receptors on epithelial cells, polymorphonuclear leukocytes and oral streptococci (16, 97, 104). This lectin-like adhesion to these substrates is inhibited by galactose and *N*-acetyl galactosamine (210).

The best characterized fimbriae of the oral gram-negative bacteria are those of *P. gingivalis*. Three types of fimbriae have been identified that are up to 3 μm long and 5 nm wide, the major class of which is composed of fimbrillin. Fimbriae appear to be the major adhesion-mediating determinants of *P. gingivalis*. The fimbrillin polypeptide binds proline-rich proteins, statherin, lactoferrin, oral epithelial cells, oral streptococci, *A. naeslundii*, fibrinogen and fibronectin (109). The fimbriae of *P. gingivalis* have chemotactic properties and demonstrate cytokine induction, both of which are necessary for *P. gingivalis* to invade epithelial cells (207) and endothelial cells (52). Recent studies have suggested the presence of fimbrial-like structures on *Peptostreptococcus micros* (106), although their role in adhesion, if any, has yet to be elucidated.

Factors other than fimbriae or pili are important in the initial attachment of other bacterial species. Force-generating movement is thought to be an important first step in biofilm formation by gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *Pseudomonas fluorescens* and *V. cholerae* (154). Active motility due to the production of flagella or twitching motility due to type IV pili are thought to increase the number of initial interactions between bacterial cells and solid surfaces and to help to overcome initial repulsive forces between bacteria and the surface. Mutants that do not possess flagella or type IV pili show delayed biofilm formation or do not produce biofilms. This mechanism is not an absolute requirement by all taxa, since gram-positive species such as those that predominate on tooth surfaces are not motile.

Although fimbriae or pili are important for the attachment of certain species to solid surfaces, they are not the only means of initial attachment. Cell surface proteins of *Staphylococcus epidermidis* (80, 81) and *Caulobacter crescentus* (108) are important in the initial attachment of these species to solid surfaces, and a capsular, polysaccharide adhesin of *S. epidermidis* can also mediate the attachment of this species to solid surfaces (138).

Recent studies have suggested that the adhesins involved in initial attachment to solid surfaces are in many instances different from the molecules involved in forming a multi-layered structure (45, 80). While one set of proteins mediates attachment of *S. epidermidis* to solid surfaces (80, 81), a second set of proteins (90) or polysaccharide (133, 167, 223) mediate cell-to-cell attachment in building a 3 dimensional structure. Similarly, *E. coli* adheres to surfaces by means of Type 1 fimbriae (61), but the development of a complex three-dimensional structure appears to be due the production of an exopolysaccharide, colanic acid (45).

Coaggregation of bacteria

The association of bacteria within mixed biofilms is not random. It has been shown that there are specific associations among bacteria in dental biofilms. Socransky et al. (174) examined over 13,000 subgingival plaque samples from 185 adult subjects and used cluster analysis and community ordination techniques to demonstrate the presence of specific microbial groups within dental plaque (Fig. 1). Six closely associated groups of bacterial species were recognized. These included the *Actinomyces*, a yellow complex consisting of members of the genus *Streptococcus*, a green complex consisting of *Capnocytophaga* species, *Actinobacillus actinomycetemcomitans* serotype a, *Eikenella corrodens* and *Campylobacter concisus* and a purple complex consisting of *Veillonella parvula* and *Actinomyces odontolyticus*. These groups of species are early colonizers of the tooth surface, and their growth usually precedes the multiplication of the predominantly gram-negative orange and red complexes (Fig. 1). Fig. 2 indicates that certain complexes are observed together more frequently than others in subgingival plaque. For example, it is extremely unlikely to find red complex species in the absence of members of the orange complex. In contrast, members of the *Actinomyces*, yellow, green and purple complexes are often observed without members of the red complex or even the red and orange complexes.

Similar relationships have been demonstrated in *in vitro* studies examining interactions between different oral bacterial species (100). These studies of oral bacteria have indicated that cell-to-cell recognition is not random but that each strain has a defined set of coaggregation partners. Further, functionally similar adhesins found on bacteria of different genera may recognize the same receptors on other bacterial cells. Most human oral bacteria adhere to other oral bacteria. This cell-to-cell adherence is known as coaggregation.

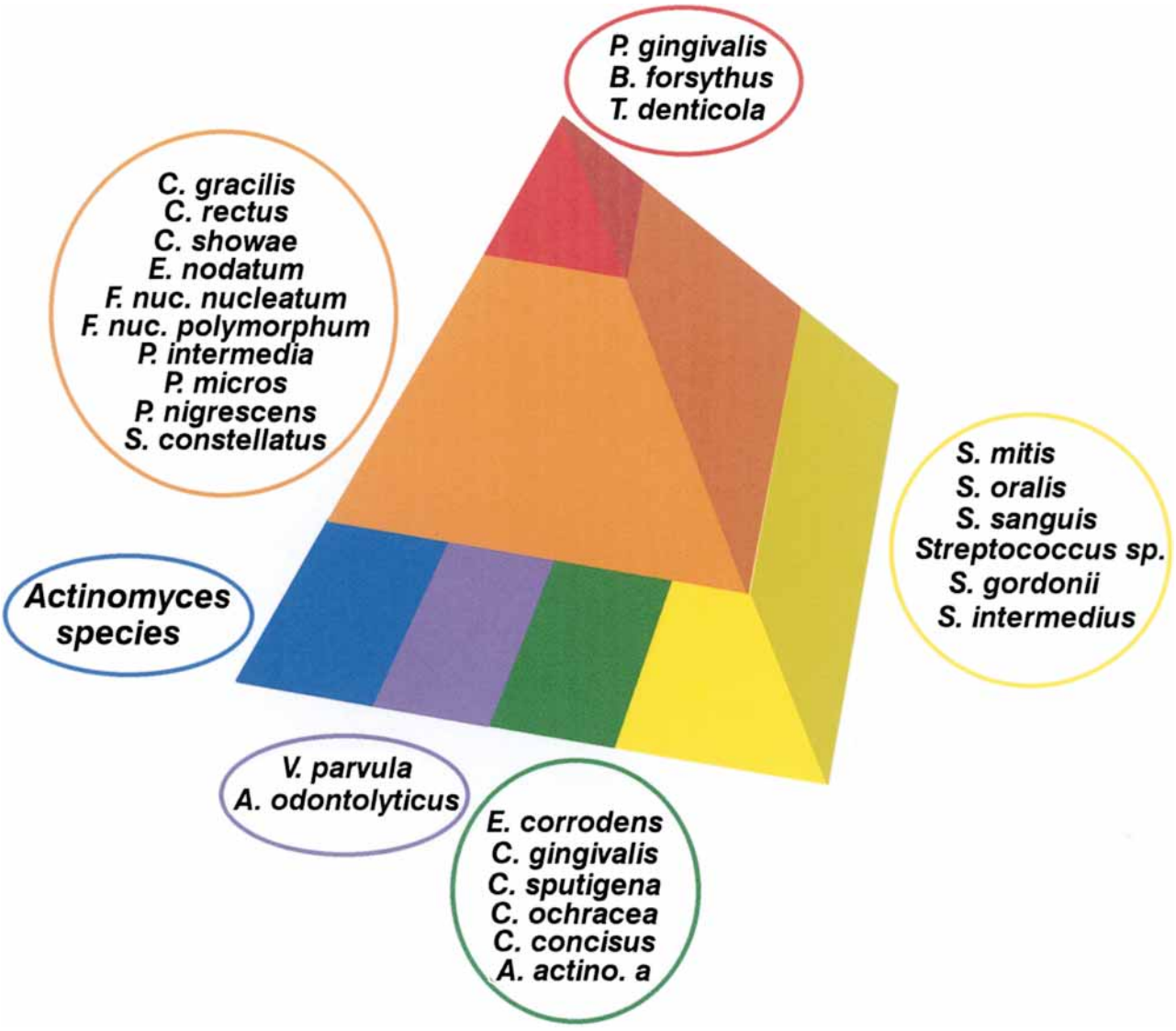


Fig. 1. Diagram of the association among subgingival species (adapted from Socransky et al. (174)). The data were derived from 13,321 subgingival plaque samples taken from the mesial aspect of each tooth in 185 adult subjects. Each sample was individually analyzed for the presence of 40 subgingival species using checkerboard DNA-DNA hybridization. Associations were sought among species using cluster analysis and community ordination techniques. The base of the pyramid is comprised of species thought to colonize the tooth surface and proliferate at an early stage. The orange complex becomes numerically more dominant later and is thought to bridge the early colonizers and the red complex species which become numerically more dominant at late stages in plaque development.

As discussed earlier, the first step in forming a biofilm is attachment of an organism to a solid surface. In supragingival plaque, the acquired pellicle that forms on a clean tooth surface within a few hours is derived from the saliva and gingival crevicular fluid. This "conditioning film" provides several receptors for the binding of oral bacteria such as acidic proline-rich proteins that bind *Streptococcus gordonii* (64, 87) and *A. naeslundii* with type 1 fimbriae (26, 62, 141). Other species that bind to the acquired pellicle include fusobacteria via statherin and *Veillonella* species (136) and *S. mutans* (169) via glucans catalyzed by glucosyltransferases in the presence of sucrose.

Streptococci make up 47–82% of the microbiota colonizing the cleaned tooth surface (100). The majority of viridans streptococci adhere to saliva-coated hydroxyapatite, although certain strains of *S. gordonii* do not (64, 87). *Streptococcus* species, unlike other oral species, show a high degree of coaggregation among strains which may contribute to their dominance as early colonizers (101, 103). This is in

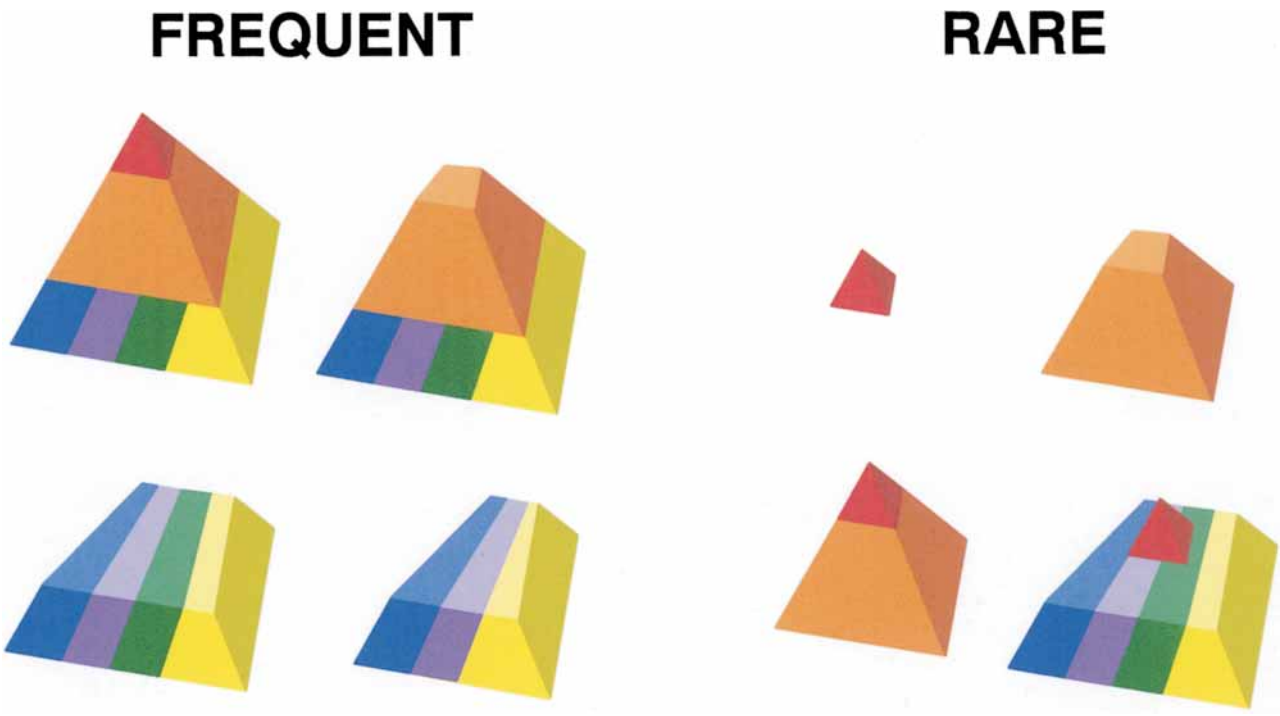


Fig. 2. Diagrams of frequently (left side) and infrequently (right side) detected associations among complexes in subgingival plaque samples

accordance with the description of a "yellow complex" consisting of *Streptococcus* species (174), which appears to be an early colonizing complex. With time, oral biofilms become more complex and are joined by or replaced by other species. For example, species from five different genera including *Haemophilus parainfluenzae* (120), *Prevotella loescheii* (208), *S. gordonii* (28, 101, 209), *A. naeslundii* (99) and *Veillonella atypica* (88) recognize the same carbohydrate receptor on *Streptococcus oralis* (100). Coaggregation between *Actinomyces* and *Veillonella* has also been demonstrated, although the molecules mediating this association are unknown. The coaggregation between *Capnocytophaga ochracea* and *Streptococcus* species depends on receptors different from those responsible for coaggregation among the streptococci. It is interesting that the *in vitro* patterns of coaggregation are similar to the relationships among the *Actinomyces*, the yellow, purple and green complexes found *in vivo*.

The gram-negative species that become numerically more prominent in the dental biofilm at a later stage are dominated by *Fusobacterium nucleatum* (140). This species coaggregates with all oral bacteria tested thus far *in vitro* including strains of *P. gingivalis, Treponema denticola, A. actinomycetemcomitans, P. intermedia, Eubacterium* species, *Selenomonas* species and *Actinomyces* species (2, 102, 103). *F. nucleatum* is also able to bind to statherin, a phosphoprotein found in the acquired pellicle (63). The ability of this species to coaggregate with multiple different species as well as bind to the salivary pellicle may explain its dominance in more complex dental biofilms. The red and orange complexes contain many of these coaggregating species such as *F. nucleatum, P. intermedia, Eubacterium* spp., *P. gingivalis* and *T. denticola*.

The streptococci and *F. nucleatum* provide examples of functional similarity. Seven species from different genera recognize the receptors on *S. oralis*, whereas ten different species recognize the same adhesin on *F. nucleatum*. It is unlikely that these two examples are the only ones to exist in dental biofilms. Thus, the observation in both *in vivo* and *in vitro* studies that coaggregation of many oral bacteria is not random might be explained by the functional similarity of molecules, associated with coaggregation, found on the surface of a wide range of bacteria (100).

Factors affecting attachment of biofilms

Both physical and chemical factors can affect the attachment of biofilms to a surface. Physical prop-

erties, such as the roughness of the surface, can increase surface area and hence increase colonization. Roughness also provides protection from shear forces and increases the difficulty of cleaning (17). Dental plaque formation, for example, starts in cracks, grooves and irregularities of the tooth surface where the initial colonizing bacteria are protected. Further, supragingival plaque formation, after initial colonization has occurred, was shown to occur more rapidly on a roughened surface (158).

The chemical composition of a surface also impacts on bacterial colonization since it may contain beneficial or detrimental components. For example, metals such as brass (an alloy of copper and zinc) have antimicrobial properties due to dezincification and the antimicrobial properties of copper. Polyvinyl chloride on the other hand, contains carbon, hydrogen and chloride, which may encourage bacterial growth (164). Conditioning films, such as dental pellicle on the teeth, may coat the surface and influence colonization. The role of conditioning films on microbial attachment is unclear, but it has been proposed that the strength of the biofilm depends on the cohesiveness of the conditioning film rather than direct bacterial contact with the bare surface (17). The liquid medium surrounding the surface, for example, saliva surrounding the teeth, also influences bacterial attachment and biofilm morphology (183). The flow rate of this medium influences bacterial attachment and biofilm morphology. Biofilms grown under laminar flow conditions (low shear) developed as patchy microcolonies and consisted of roughly circular cell clusters separated by interstitial voids. Biofilms grown under turbulent flow conditions were also patchy but consisted of patches of ripples and elongated streamers that oscillated in the flow (181). The interstitial voids, which act as transport channels, decrease as the biofilm develops, reducing mass transport characteristics and ultimately controlling the growth rate of the biofilm because of reduction in nutrient and possibly oxygen availability (23). As will be described later, this decrease in growth rate may affect antibiotic resistance.

Medical problems due to biofilms

Costerton et al. (43) pointed out that a wide range of human infections are due to biofilms. These include dental caries, periodontal disease, otitis media, musculoskeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, meloidosis and cystic fibrosis pneumonia. Characteristic of these infections is the persistence and chronicity of the infections as well as the difficulty in their eradication. For example, the continued presence of *P. aeruginosa* in the lungs of patients with cystic fibrosis has proven to be an enormous source of discomfort to the patient and a frustrating challenge to the physician. Of particular interest to the dental investigator are the biofilms that occur on implanted devices, since these biofilms share the property with dental biofilms of being formed on a non-shedding surface. Both types of biofilms have the potential to become quite complex in microbial composition and reach rather massive size. Dentists, like physicians, use implants that provide non-shedding surfaces for potential biofilm colonization (98, 114, 139, 191).

There has been an increased use of implantable medical devices made from biomaterials over the last decade and hence an increase in biofilm infections associated with such devices (8). Such infections are particularly difficult to treat despite the fact that many infections of implants are caused by organisms that are not normally considered pathogenic for humans. Three types of implantable devices exist: totally implanted (hip joints, knee joints, pacemakers, heart valves, etc.), partly implanted (such as central venous catheters and tooth implants) or non-implanted (many catheters and voice prostheses), which do not require surgery for implantation or removal. In the first category, infection is greatest at the time of surgical implantation. For the second and third categories, infection is related to the length of time of implantation. *Staphylococcus* species, particularly, *S. epidermidis*, are the most common causative agents of infection of most devices, often causing a late presenting infection due to their "low-grade" pathogenicity. *S. aureus, P. aeruginosa* and other aerobic gram-negative bacilli tend to be associated with more serious infections and earlier acute presentation. Heart valves are often infected late by oral streptococci. *Propionibacterium acnes* has been isolated from >50% of removed hip prostheses (193) while *Candida* species accounted for the majority of infecting agents of voice prostheses (142). Biofilm infections of medical devices, particularly category 1, consist almost always of a single species. Biomaterial-related infections are characterized by chronicity and persistence and lack of susceptibility to antimicrobial agents. Microbial access to the device is usually during surgical implantation, since most of the infecting species are incapable of invasion, although late infections can occur due to bacteremia. *S. aureus* is capable of in-

vasion from a trivial wound. The oral cavity is a source of both *Staphylococcus* and *Streptococcus* infections of implanted heart valves.

Bacteria rarely adhere to the naked surface of the prosthesis but to a conditioning film derived from the host such as blood components on heart valves. The reduced oxygen tension within the biofilm can change the properties of the infecting bacteria. For example, *S. aureus* growing in an environment with low oxygen tension is able to produce α toxin. Reduced oxygen tension also can lead to nutrient limitation and selection of small-colony variants where the cells show pleomorphism and are often mistaken for mixed cultures. Microbial growth is also slowed, and protein and cell wall synthesis is decreased. The low membrane potential leads to an insusceptibility to aminoglycosides, platelet microbicidal proteins and other antibacterial substances which rely on microbial energy to reach their intracellular target (156). Thus, small-colony variants are able to evade the inflammatory response and the immune system and are unaffected by therapeutic concentrations of antimicrobials. It has been suggested that small-colony variants represent the true biofilm phenotype found on implanted medical devices (10).

The search for effective means of treating biomaterial-related infections is very active, given the high cost of such infections both financially and in terms of morbidity and quality of life. Antimicrobial agents are unlikely to be effective at typical dosages for the treatment of biomaterial-related infections. The minimum inhibitory concentration for vancomycin for an organism in the planktonic state might be 1 μg/ml, whereas in biofilm mode it will be 1000 μg/ml. Infected hip replacements have been treated with a combination of ciprofloxacin and rifampicin. Successfully treated cases were diagnosed within a few weeks of surgery, and the antibiotics were administered for 3 to 6 months.

Current research is evaluating the enhancement of antimicrobial activity using microcurrents – "bioelectric effect" (38). Long-term use of antibiotics prophylactically is not suggested for category 2 or 3 devices, although daily oral application of an anti-*Candida* drug in subjects with category 3 devices has been shown to decrease *Candida* infection (113). Further, the use of bioadhesive slow-release lozenges containing miconazole have also been effective (198). The use of surfactants produced by dietary lactobacilli and *Streptococcus thermophilus in vitro* has been shown to reduce *Candida* biofilms (195). Thus, live yogurt may delay *Candida* colonization.

Other studies are evaluating the effects of incorporation of antimicrobial agents into the device materials. Antibiotic powder mixed in bone cement immediately prior to use has been shown to produce a significant decrease in relapse rates when used in patients with already infected hip prostheses (186). The coating of implant materials with antimicrobial agents has produced mixed results because these coatings are eluted from the surfaces and are easily obliterated by the conditioning film (79, 94, 135, 159, 171). Better results have been obtained by impregnating the device material with antimicrobial agents, particularly minocycline and rifampicin. Impregnated catheters were shown to prevent infection for 8 days (46). Cerebrospinal fluid shunts impregnated with antimicrobial agents protecting both the inner and outer surfaces provided protection for 2 months (9).

Mechanisms of increased antibiotic resistance of organisms in biofilms

As will be discussed elsewhere in this volume, antibiotics have been and continue to be used effectively in the treatment of periodontal infections. However, the indiscriminate use of antimicrobial agents and biocides has the potential of leading to the development of resistant bacteria (55, 116, 148). It has also been suggested that resistance to one type of antimicrobial such as a biocide can be transferred to a different type of antimicrobial such as an antibiotic (116). Thus, it is important to understand the factors leading to antimicrobial resistance in biofilms such as dental plaque.

It has been recognized for considerable periods of time that organisms growing in biofilms are more resistant to antibiotics than the same species growing in a planktonic (unattached) state (4, 5, 21, 39–41, 85, 86, 144, 145). Although the mechanisms of resistance to antibiotics of organisms growing in biofilms are not entirely clear (24), certain general principles have been described. Almost without exception, organisms grown in biofilms are more resistant to antibiotics than are the same cells grown in a planktonic state. Estimates of 1000 to 1500 times greater resistance for biofilm-grown cells than planktonically grown cells have been suggested (37), although these estimates have been considered to be too high by some investigators (180). The mechanisms of increased resistance in biofilms differ from species to species, from antibiotic to antibiotic and for biofilms growing in different habitats. One im-

portant mechanism of resistance appears to be the slower rate of growth of bacterial species in biofilms, which makes them less susceptible to many but not all antibiotics (6, 19, 43, 219). It has been shown in many studies that the resistance of bacteria to antibiotics, biocides or preservatives is affected by their nutritional status, growth rate, temperature, pH and prior exposure to subeffective concentrations of antimicrobial agents (20, 22, 211). Variations in any of these parameters can lead to a varied response to antibiotics within a biofilm. The matrix performs a "homeostatic function", such that cells deep in the biofilm experience different conditions such as hydrogen ion concentration or redox potentials than cells at the periphery of the biofilm or cells growing planktonically. The growth rates of these deeper cells will be decreased allowing them to survive better than faster-growing cells at the periphery when exposed to antimicrobial agents. In addition, the slower-growing bacteria often overexpress "nonspecific defense mechanisms" including shock proteins and multi-drug efflux pumps (arcAB) and demonstrate increased exopolymer synthesis (65).

The exopolymer matrix of a biofilm, although not a significant barrier in itself to the diffusion of antibiotics, does have certain properties that can retard diffusion. For example, strongly charged or chemically highly reactive agents can fail to reach the deeper zones of the biofilm because the biofilm acts as an ion-exchange resin removing such molecules from solution (65). In addition, extracellular enzymes such as β-lactamases, formaldehyde lyase and formaldehyde dehydrogenase may become trapped and concentrated in the extracellular matrix, thus inactivating susceptible, typically positively charged, hydrophilic antibiotics. Some antibiotics such as the macrolides, which are positively charged but hydrophobic, are unaffected by this process. Thus, the ability of the matrix to act as a physical barrier depends on the type of antibiotic, the binding of the matrix to that agent and the levels of the agent employed (143). Since reaction between the agent and the matrix will reduce the levels of the agent, a biofilm with greater bulk will deplete the agent more readily. Further, hydrodynamics (47) and the turnover rate of the microcolonies will also affect antibiotic effectiveness (107).

Alteration of genotype and/or phenotype of the cells growing within a biofilm matrix is receiving increased attention. Cells growing within a biofilm express genes that are not observed in the same cells grown in a planktonic state, and they can retain this resistance for some time after being released from the biofilm. For example, Brooun et al. (19) demonstrated that cells of *P. aeruginosa* liberated from biofilms were considerably more resistant to tobramycin than planktonic cells, suggesting that the cells became intrinsically more resistant when growing in a biofilm and retained some of this resistance even outside the biofilm. Using mass transfer calculations and penetration measurements (using attenuate total reflectance Fournier transform infrared spectroscopy), it has been shown that antibiotic resistance may be due not only to the inability of some antimicrobial agents to penetrate the biofilm but also resistance at the cellular level (187). *P. aeruginosa* grown in planktonic and in sessile states differ by a number of genes that could explain the increased resistance of a species in the sessile state (145). Studies that have examined dental pathogens in a planktonic state should be redone using sessile organisms whose bacterial envelopes contain gene products that may be completely different. This recognition has implications for both antibiotic usage and vaccine development.

The presence of a glycocalyx, a slower growth rate and development of a biofilm phenotype cannot provide a total explanation for the phenomenon of antibiotic resistance. These features probably delay elimination of the target bacteria, allowing other selection events to take place (65). Recently, the notion of a subpopulation of cells within a biofilm that are "super-resistant" was proposed. Such cells could explain remarkably elevated levels of resistance to certain antibiotics that have been suggested in the literature. Brooun et al. (19) examined the contribution of multi-drug resistance pumps to antibiotic resistance of organisms grown in biofilms. These "pumps" can extrude chemically unrelated antimicrobial agents from the cell. Since extrusion places the antibiotics outside the outer membrane, the process offers protection against antibiotics that target cell wall synthesis. The investigators examined the dose-response killing of two strains of *P. aeruginosa* in an *in vitro* system. One of the strains was a deletion mutant that did not possess the pump, whereas the other strain was an overproducing mutant. Ofloxacin was 50–100 times more effective in killing the deletion mutant (no "pump") than the overproducing strain at low antibiotic concentrations. However, to the authors' surprise, as the concentration of the antibiotic was increased, the difference in killing of the two strains decreased so that, at levels >10 μg/ml, there was no difference in killing rate. They postulated the presence of a "super-resistant" subpopulation of cells for both strains when

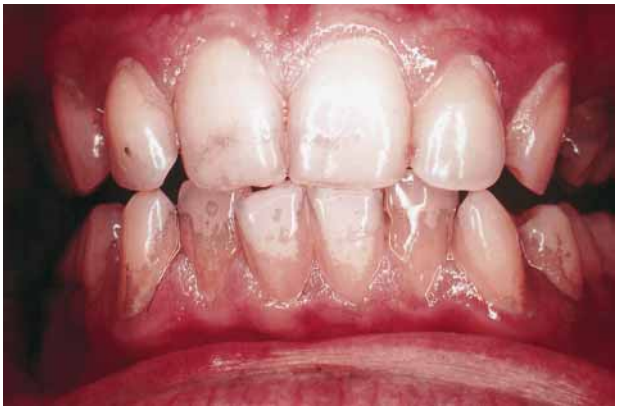


Fig. 3. Clinical photograph of a subject exhibiting tooth stain and supragingival dental plaque

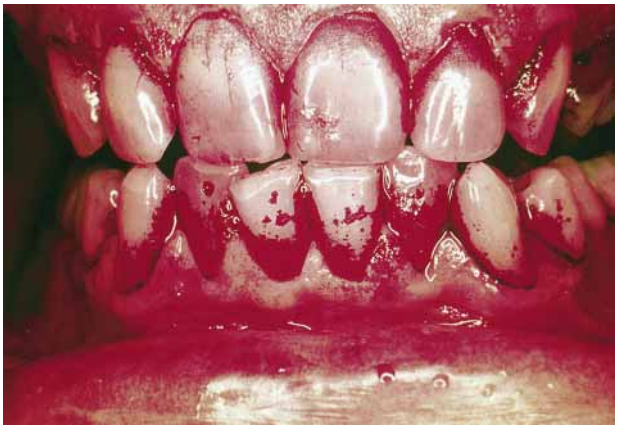


Fig. 4. Clinical photograph of the subject in Fig. 3 after staining with disclosing solution

grown as biofilms. This subpopulation represented about 10^5 of the 10^8 cells in the original biofilms. No "super-resistant" subpopulation was detected when the same strains were grown in a planktonic state. In the planktonic state, deletion mutants were killed much more rapidly than the overproducing mutants throughout the tested antibiotic concentration range. Ciprofloxacin showed similar patterns of killing for deletion and overproducing mutants in the planktonic state. However, there was no difference in the killing rates of the two mutants when grown as biofilms. A "plateau" of killing was detected for both strains when grown in biofilms, suggesting "super-resistant" cells.

Clearly, the detection of increased antibiotic resistance of cells within biofilms must lead us to re-examine our clinical and research procedures. If cells in biofilms differ in antibiotic resistance from cells grown in a planktonic state, how do we interpret *in vitro* antibiotic sensitivity data provided to us by medical and dental microbiology laboratories? When antimicrobial drugs are developed, should the testing be carried out in biofilm-grown or planktonic cells? What role, if any, should antibiotics play in the treatment of periodontal infections?

The oral biofilms that lead to periodontal diseases

The rather extensive section on biofilm biology presented above provides a background to help understand the ecology of the incredibly complex community of organisms that colonize the tooth surface and lead to periodontal diseases. Fig. 3 presents a clinical photograph of a subject with less than optimal home care. Evident in this photograph is stain on the tooth surfaces that may have resulted from smoking, coffee or tea drinking. Of greater concern is the occurrence of a thin film of bacterial plaque on many of the tooth surfaces along with the quite obvious plaque formation in regions such as the mesial buccal surfaces of the upper left and lower right canines. These biofilm (plaque) regions are highlighted in Fig. 4, which shows the same dentition after staining with a disclosing solution. The thin films such as those on the lower incisors might consist of biofilm communities that are 50 to 100 cells in thickness. Thicker plaques such as those on the upper left and lower right canines might consist of biofilms that are 300 or more cell layers in thickness. The number of organisms that reside on the mesial surface of the upper left or lower right canine probably exceeds 300 million. This number is remarkable in that it exceeds the entire population of the United States (including uncounted voters in Florida). These microbial communities are very complex. About 500–600 bacterial taxa have been detected in samples from the oral cavity. This estimate is based on the detection of about 350 cultivated taxa and detection of over 200 uncultivated taxa (Floyd Dewhirst and Bruce Paster, personal communication). Uncultivated species have been detected by sequencing 16S rRNA fragments that were amplified directly from plaque samples and cloned into *E. coli*. Fortunately, the number of species may top out between 550 and 600 in that new taxa are infrequently found in studies pursuing uncultivated species in plaque samples (Paster and Dewhirst, personal communication). In any given plaque sample, it is not uncommon to detect 30 or more bacterial species. Thus, the biofilms that colonize the tooth surface

may be among the most complex biofilms that exist in nature. This complexity is due in large part to the non-shedding surface of the tooth, which permits persistent colonization and the opportunity for very complex ecosystems to develop. In addition, the relatively high nutrient abundance as well as the remarkable ability of oral species to coaggregate with one another, as discussed earlier, may facilitate this complexity.

Fig. 5 is a section of human supragingival dental plaque grown on an epon crown in a human volunteer (122–124). The section demonstrates many of the features of biofilms outlined earlier. Bacterial species adhered to the solid surface, multiplied and, in this section, formed columnar microcolonies. The heterogeneity of colonizing species is evident even at a morphological level and would be emphasized if the cells within the section had been characterized by cultural or molecular techniques. The surface of the biofilm exhibits morphotypes that are not evident in deeper layers and emphasizes the role that coaggregation plays in the development of biofilms. Not evident in this section are the water channels in biofilms described earlier. This might be due to preparation or fixation artifacts (40) or it might be because the plaque is typical of the "dense" bacterial model. Water channels have been observed in plaque grown in the human oral cavity by confocal microscopy (216). This dental biofilm has all of the properties of biofilms in other habitats in nature. It has a solid substratum, in this case an epon crown, but more typically a tooth, it has the mixed microcolonies growing in a glycocalyx and it has the bulk fluid interface provided by saliva.

A second biofilm ecosystem is shown in Fig. 6. This is a section of human subgingival plaque. The section is at lower magnification than Fig. 5 to permit visualization of regions within the biofilm. The plaque attached to the tooth surface is evident in the upper left portion of the section. This tooth-associated biofilm is an extension of the biofilm found above the gingival margin and may be quite similar in microbial composition. A second, possibly epithelial cell–associated biofilm, may be observed lining the epithelial surface of the pocket. This biofilm contains primarily spirochetes and gram-negative bacterial species (122–124). *P. gingivalis* and *T. denticola* have been detected in large numbers in periodontal pocket, epithelial cell–associated biofilms by immunocytochemistry (96). It might be surmised that the third member of the "red complex" (174), *Bacteroides forsythus*, might also be numerous in this zone, particularly since high levels of this spe-

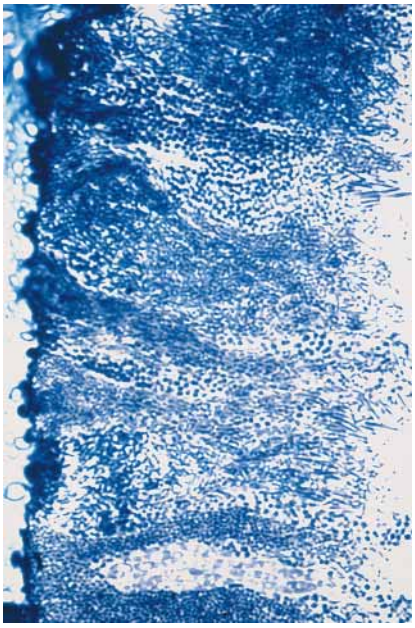


Fig. 5. Histological section of human supragingival plaque stained with toluidine blue–methylene blue. The supragingival plaque was allowed to develop for 3 days on an epon crown in a human volunteer. The crown surface is at the left and the saliva interface is towards the right (courtesy of Max Listgarten, University of Pennsylvania).

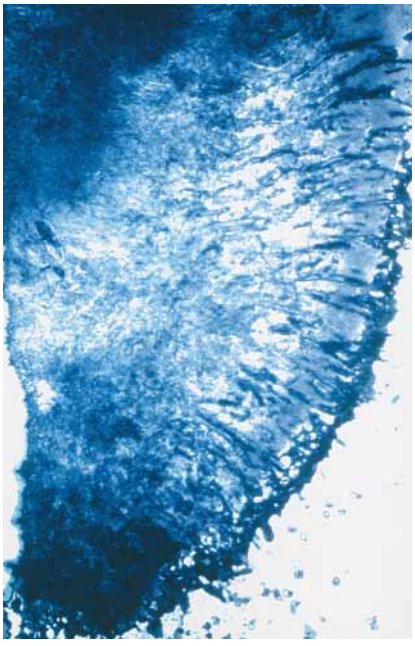
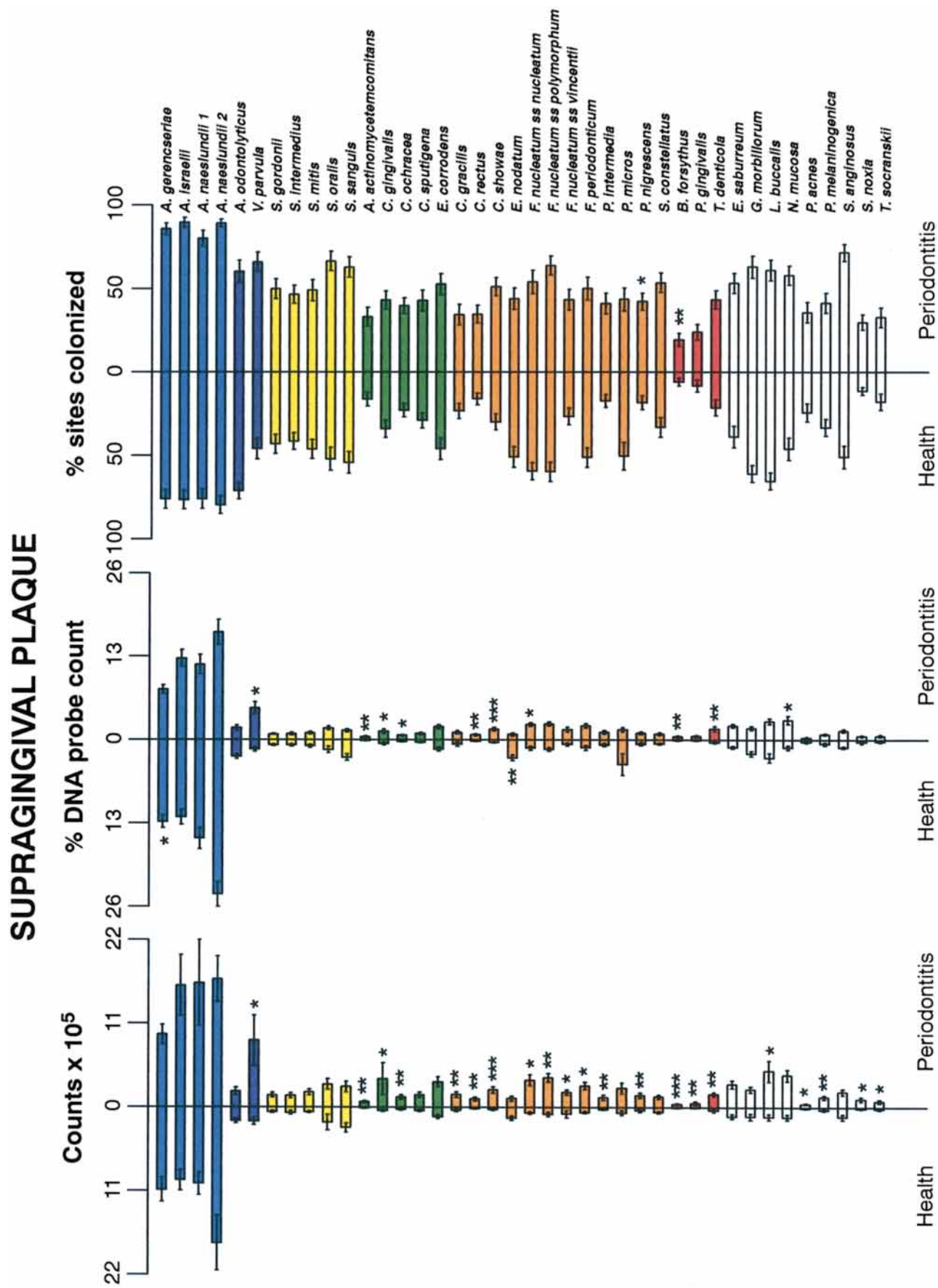


Fig. 6. Histological section of human subgingival dental plaque stained with toluidine blue–methylene blue. The tooth surface is to the left and the epithelial lining of the periodontal pocket is to the right. Bacterial plaque attached to the tooth surface is evident towards the upper left of the section, while a second zone of organisms can be observed lining the periodontal pocket wall (courtesy of Max Listgarten, University of Pennsylvania).


SUPRAGINGIVAL PLAQUE
Counts x 10^5
% DNA probe count
% sites colonized
Health
Periodontitis
A. gerencseriae
A. israelii
A. naeslundii 1
A. naeslundii 2
A. odontolyticus
V. parvula
S. gordonii
S. intermedius
S. mitis
S. oralis
S. sanguis
A. actinomycetemcomitans
C. gingivalis
C. ochracea
C. sputigena
E. corrodens
C. gracilis
C. rectus
C. showae
E. nodatum
F. nucleatum ss nucleatum
F. nucleatum ss polymorphum
F. nucleatum ss vincentii
F. periodonticum
P. intermedia
P. micros
P. nigrescens
S. constellatus
B. forsythus
P. gingivalis
T. denticola
E. saburreum
G. morbillorum
L. buccalis
N. mucosa
P. acnes
P. melaninogenica
S. anginosus
S. noxia
T. socranskii

cies have been detected, using DNA probes, in association with the epithelial cells lining the periodontal pocket (54). Between the tooth-associated and epithelial cell–associated biofilms, a less dense zone of organisms may be observed. These organisms may be "loosely attached" or they might be in a planktonic state. The critical feature of Fig. 6 is that there appear to be tooth-associated and epithelial cell–associated regions in subgingival plaque as well as a possible third weakly or unattached zone of microorganisms. It is strongly suspected that these regions differ markedly in microbial composition, physiological state and their response to different therapies.

Microbial composition of supragingival and subgingival biofilms

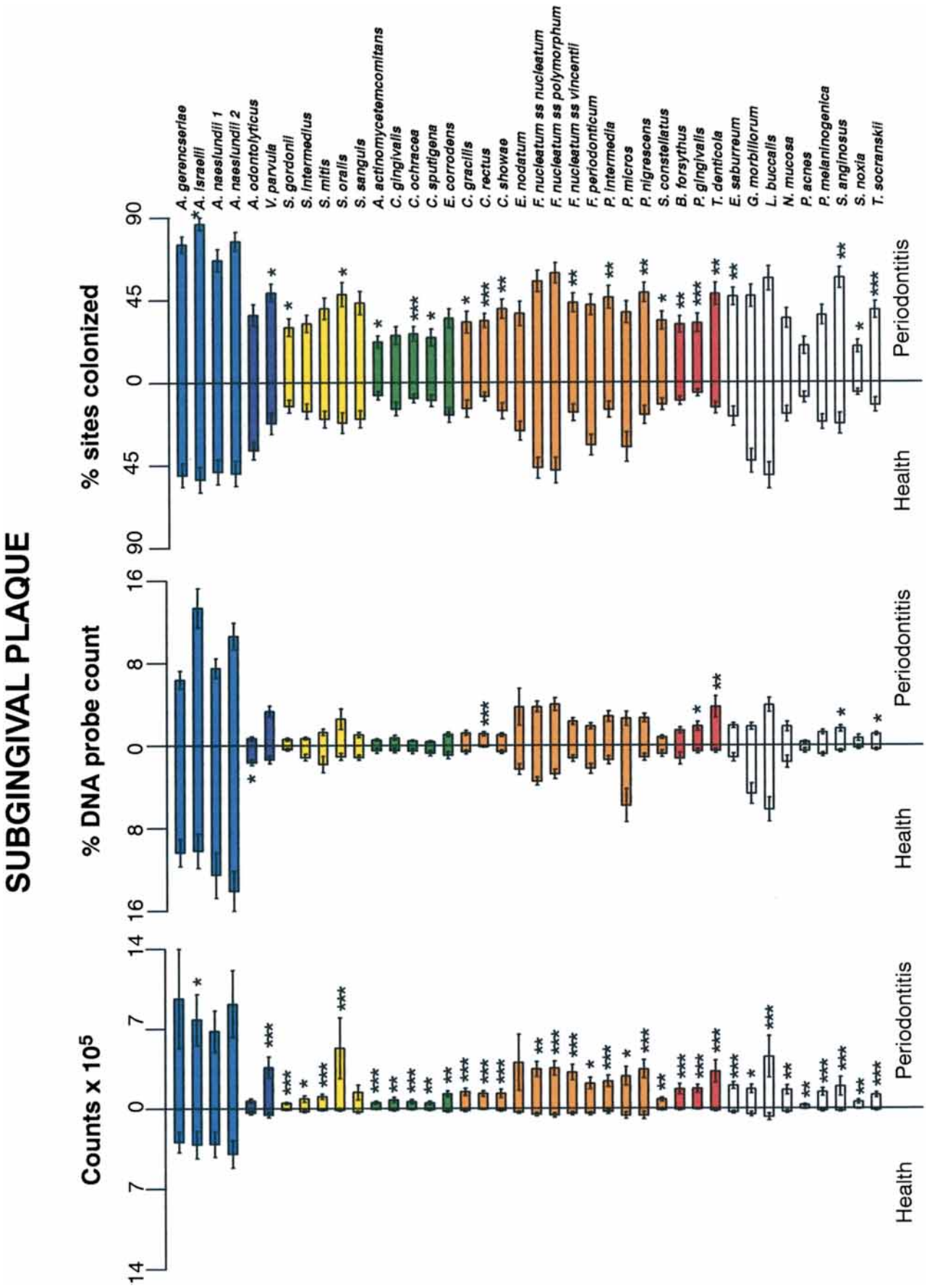
The bacteria associated with periodontal diseases reside within biofilms both above and below the gingival margin. The supragingival biofilm is attached to the tooth surface and is predominated by *Actinomyces* species in most plaque samples. Fig. 7 provides the counts, proportions and prevalence (percentage of sites colonized) of 40 taxa grouped according to microbial complexes (174) in supragingival plaque samples from periodontally healthy and periodontitis subjects (217). *Actinomyces* species predominate in both health and disease irrespective of the method of enumeration. Further, all taxa examined could be found (on average) in both health and disease, although the counts and proportions of periodontal pathogens were significantly higher in the periodontally diseased subjects.

As described above, the nature of subgingival biofilms is more complex with both a tooth-associated and tissue-associated biofilm separated by loosely bound or planktonic cells. Fig. 8 presents the counts, proportions and prevalence of 40 taxa in subgingival plaque samples from periodontally diseased and periodontally healthy individuals (217). Similar to supragingival plaque, the dominant species subgingivally are *Actinomyces*, but significantly higher counts, proportions and prevalence of red and orange complex species were found in the samples from the periodontitis subjects. Data for the red complex species are provided in Fig. 9, which highlights the increased levels, proportions and prevalence of these species in both supragingival and subgingival plaque of periodontitis subjects when compared with similar samples from periodontally healthy individuals. Fig. 10 summarizes the major differences in microbial complexes between supragingival and subgingival plaque in health and periodontitis. As one moves from the supragingival to the subgingival environment and from health to disease, there is a significant decrease in the *Actinomyces* species and an increase in the proportion of members of the red complex (*B. forsythus*, *P. gingivalis* and *T. denticola*). Knowledge of the differences between health and disease should help the therapist to define microbial endpoints in the treatment of periodontal infections.

Fig. 7. Bar charts of the counts ($\times 10^5$), proportions and percentage of sites colonized in supragingival plaque samples taken from 22 periodontally healthy and 23 subjects with adult periodontitis. The bars represent the mean values and the whiskers the SEM. Supragingival plaque samples were taken from the mesial surface of each tooth, excluding third molars and individually processed for their content of 40 bacterial species using checkerboard DNA-DNA hybridization. The left bars represent health and the right bars disease. The species are arranged within microbial complexes described by Socransky et al. (174) and are color coded accordingly. Significance of difference between health and disease was determined using the Mann-Whitney test and adjusted for multiple comparisons (175).

Periodontal pathogens

The complexity and nature of subgingival dental plaques have been emphasized in preceding sections. The natural question is which of these many species are the causative agents of periodontal diseases that lead to loss of the supporting tissues. For over 100 years, periodontal microbiologists have been searching for the causative agents of periodontal diseases. These studies have been reviewed extensively (70, 222) and the data will not be re-reviewed here. However, studies using predominantly cultural techniques and microscopy to identify bacterial species have resulted in the establishment of a group of species thought to play a role in destructive periodontal diseases. The studies compared the microbiotas at periodontally healthy and diseased sites, actively progressing lesions and nonprogressing lesions as well as successfully and unsuccessfully treated sites after different forms of periodontal therapy. Three species, *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus*, were strongly associated with periodontal disease status, disease


SUBGINGIVAL PLAQUE
Counts x 10^5
% DNA probe count
% sites colonized
Health
Periodontitis
A. gerencseriae
A. israelii
A. naeslundii 1
A. naeslundii 2
A. odontolyticus
V. parvula
S. gordonii
S. intermedius
S. mitis
S. oralis
S. sanguis
A. actinomycetemcomitans
C. gingivalis
C. ochracea
C. sputigena
E. corrodens
C. gracilis
C. rectus
C. showae
E. nodatum
F. nucleatum ss nucleatum
F. nucleatum ss polymorphum
F. nucleatum ss vincentii
F. periodonticum
P. intermedia
P. micros
P. nigrescens
S. constellatus
B. forsythus
P. gingivalis
T. denticola
E. saburreum
G. morbillorum
L. buccalis
N. mucosa
P. acnes
P. melaninogenica
S. anginosus
S. noxia
T. socranskii

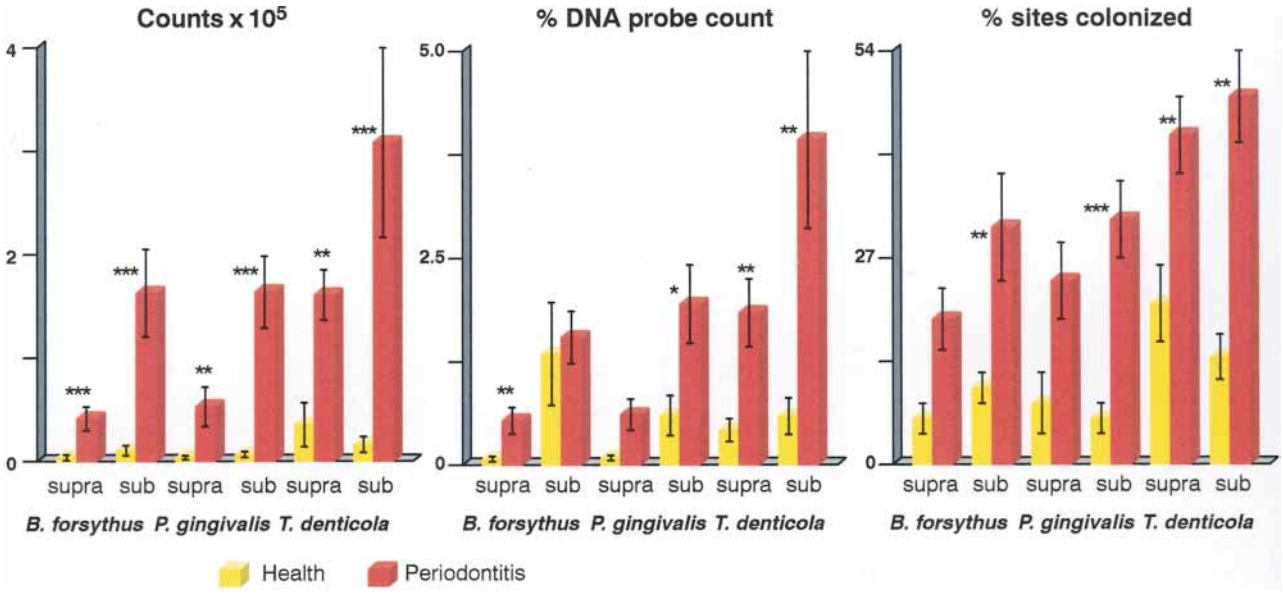


Fig. 9. Bar charts of the counts (×10^5), proportions and percentages of sites colonized by the red complex species, *B. forsythus*, *P. gingivalis* and *T. denticola* in supragingival and subgingival plaque samples taken from 22 periodontally healthy subjects and 23 subjects with adult periodontitis. The significance of differences between health and disease was determined using the Mann-Whitney test and adjusted for multiple comparisons (175).

progression and unsuccessful therapy. As such, these species were designated as periodontal pathogens at the 1996 World Workshop of Periodontology (30, 222). Other species such as *F. nucleatum*, *Campylobacter rectus*, *P. intermedia*, *P. nigrescens*, *Eubacterium nodatum*, *P. micros* and various spirochetes have also been implicated in causing periodontal diseases, although the evidence for their causative role is less extensive (70). More recently, viruses including cytomegalovirus, Epstein-Barr virus, papillomavirus and herpes simplex virus have been proposed to play a role in causing periodontal diseases, possibly by changing the host response to the local subgingival microbiota (31–35, 77, 149, 192, 202).

Recent studies have extended the data that support the role of many of these species in periodontal diseases. For example, the use of DNA-DNA hybridization permitted the examination of large numbers of species in large numbers of plaque samples (177). These data were analyzed in terms of levels, proportions and prevalence (percentage of sites colonized) of various taxa in subjects with different periodontal status. Although all three measures of microbial colonization are valuable, the prevalence of species appears to relate particularly to disease status and to disease susceptibility. Fig. 11 presents the percentage of sites colonized and counts of 40 subgingival taxa in subjects with adult periodontitis, refractory periodontitis, periodontal health and in well-maintained elder subjects (68). Clearly, the major difference among groups was the increased prevalence of *B. forsythus*, *P. gingivalis* and *T. denticola* in subjects with periodontal disease. In addition, other putative periodontal pathogens including *F. nucleatum* subsp. *vincentii*, *C. rectus* and *P. intermedia* were also more prevalent in periodontitis and refractory subjects.

Fig. 8. Bar charts of the counts (×10^5), proportions and percentages of sites colonized in subgingival plaque samples taken from 22 periodontally healthy and 23 subjects with adult periodontitis. The format is as described for Fig. 7.

Treatment of periodontal biofilms

The information presented above indicates that the biofilms that colonize tooth surfaces and oral soft tissues are complex and that the resident bacteria have intriguing interactions with each other and with the surfaces that they colonize. The data indicate that organisms in biofilms are "worthy" adversaries for control and/or eradication. Of concern to the therapist in treating dental biofilm infections is the fact that the pathogenic species exist in very large numbers, are widely distributed within the oral cavity and exist in community structures that provide protection

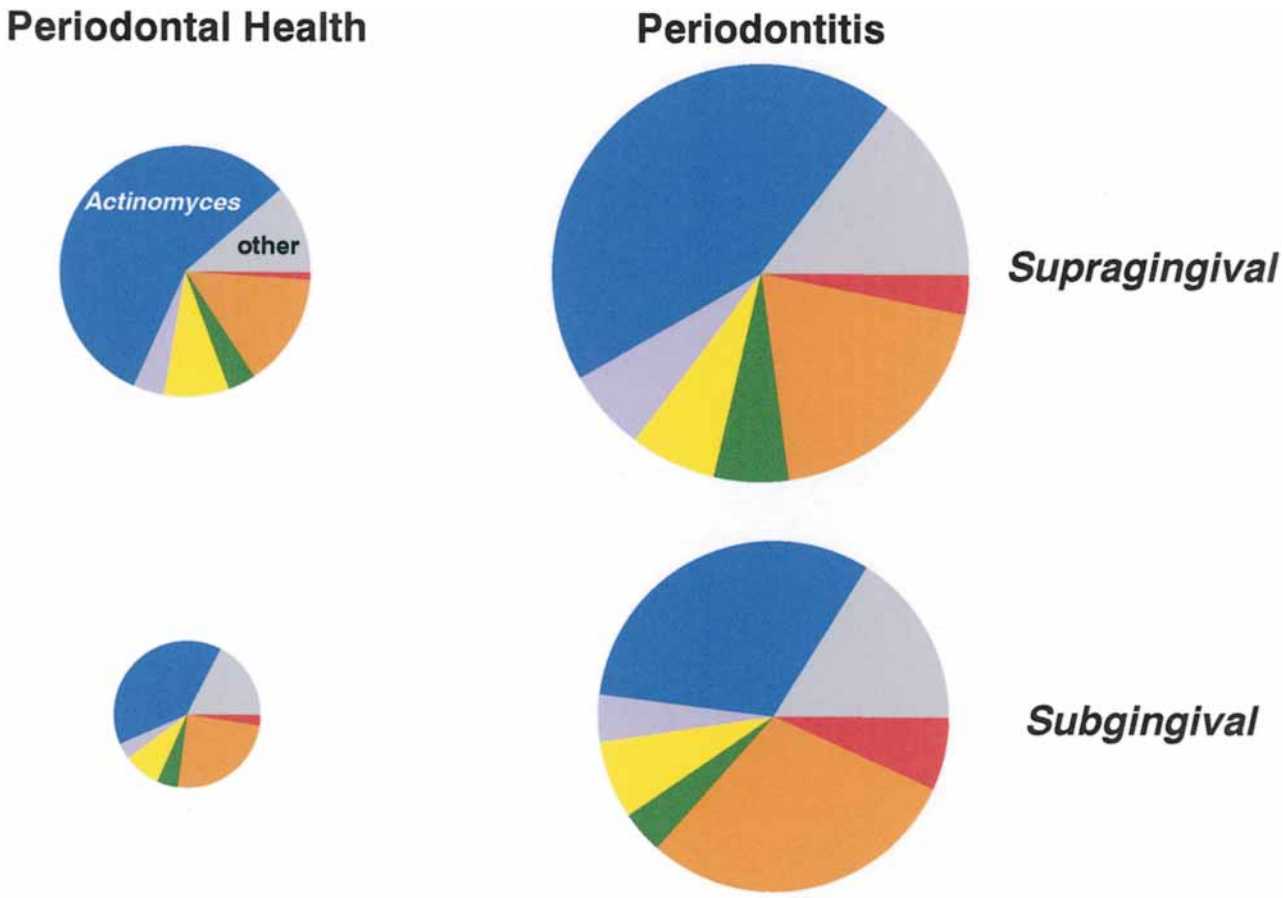
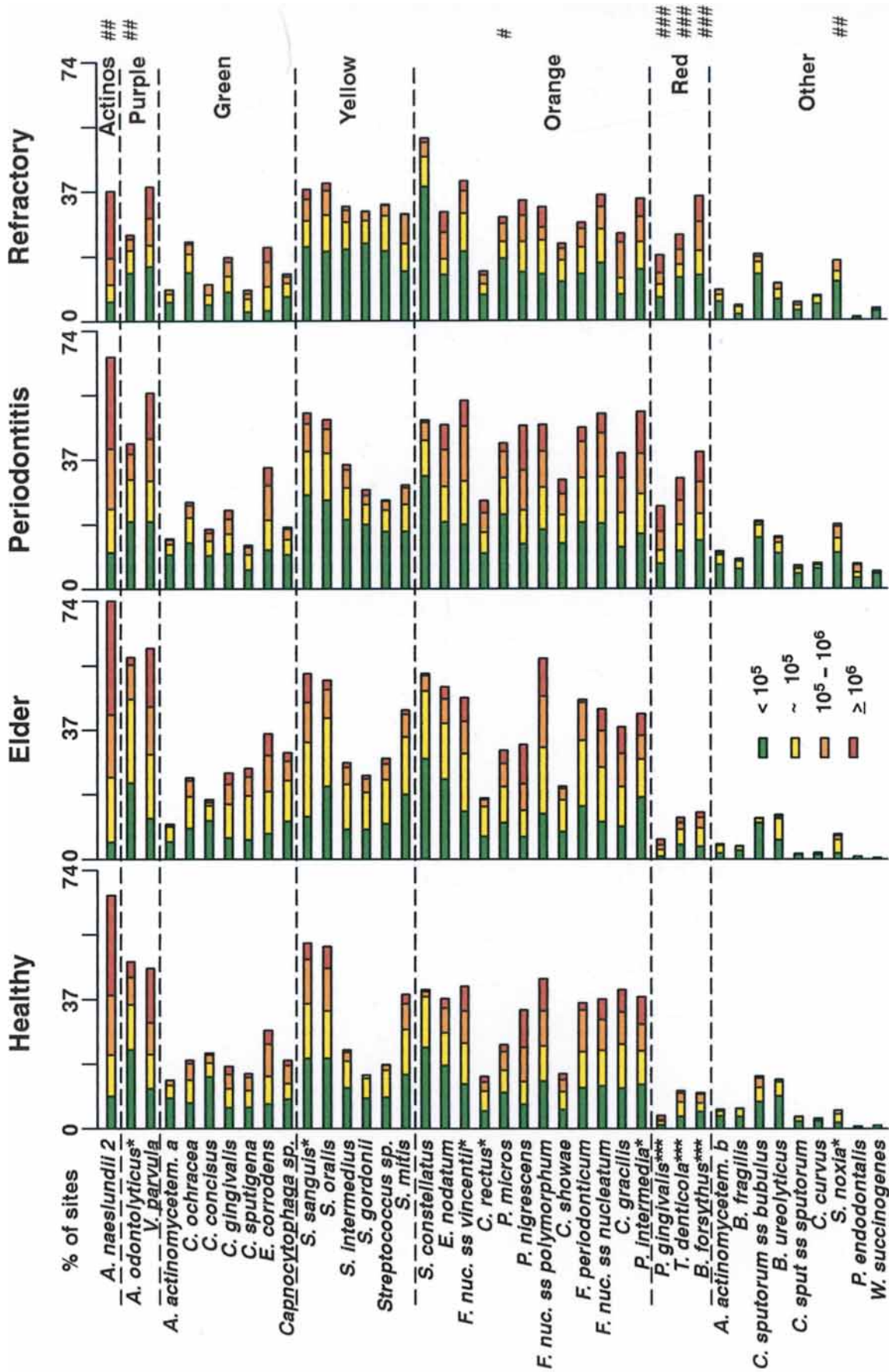


Fig. 10. Pie charts of the mean percentage DNA probe count of microbial groups in supragingival and subgingival plaque samples from 22 periodontally healthy and 23 periodontitis subjects. The species were grouped into seven microbial groups based on the description of Socransky et al. (174). The areas of the pies were adjusted to reflect the mean total counts at each of the sample locations. The significance of differences in mean percentages of the supragingival and subgingival complexes in health and disease was tested using the Kruskal-Wallis test. The "red", "orange" and *Actinomyces* species were significantly different at $P<0.001$, and the "green" complex species differed at $P<0.05$ after adjusting for 7 comparisons. The "other" category represents probes to species that did not fall into a complex as well as probes to new species whose relationships with other species have not yet been ascertained. Reprinted with permission of the *Journal of Clinical Periodontology* (Ximenez-Fyvie et al. (217)).

against host defense mechanisms and antimicrobial agents. Further, the organisms can multiply like hell and have a talent for attaching to new surfaces of the host or other organisms that are already attached to the host; thus, spread and re-colonization are a persistent threat. Given this formidable opponent, it is worth reminding ourselves that periodontal therapies, by and large, have a beneficial effect in terms of slowing or stopping progression of periodontal diseases and maintaining the periodontium. The "by and large" is a phrase that certainly indicates that therapies are not always successful and that alternative methods or adjunctive methods are sometimes essential. The purpose of this section is to examine some of the changes that are brought about by therapies of different types alone and in combination.

Fig. 11. Stacked bar charts of the mean prevalence (percentage of sites colonized) and levels of 40 subgingival species evaluated in 27 periodontally healthy, 35 well-maintained elder, 115 untreated periodontitis and 36 refractory periodontitis subjects. The species were ordered according to the microbial complexes described by Socransky et al. (174). The percentage of sites colonized at different levels by each of the 40 species examined was computed for each subject and then averaged across subjects in the four groups. Significance of differences in mean counts and prevalence among groups was evaluated using the Kruskal-Wallis test. For counts: $*P<0.05$; $**P<0.01$; $***P<0.001$; for prevalence, $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ after adjusting for multiple comparisons (175). Reprinted with permission of the *Journal of Clinical Periodontology* (Socransky et al. (176)).


Healthy
Elder
Periodontitis
Refractory
% of sites
0
37
74
A. naeslundii 2
A. odontolyticus*
V. parvula
A. actinomycetem. a
C. ochracea
C. concisus
C. gingivalis
C. sputigena
E. corrodens
Capnocytophaga sp.
S. sanguis*
S. oralis
S. intermedius
S. gordonii
Streptococcus sp.
S. mitis
S. constellatus
E. nodatum
F. nuc. ss vincenti*
C. rectus*
P. micros
P. nigrescens
F. nuc. ss polymorphum
C. showae
F. periodonticum
F. nuc. ss nucleatum
C. gracilis
P. intermedia*
P. gingivalis***
T. denticola***
B. forsythus***
A. actinomycetem. b
B. fragilis
C. sputorum ss bubulus
B. ureolyticus
C. sput ss sputorum
C. curvus
S. noxia*
P. endodontalis
W. succinogenes
Actinos
Purple
Green
Yellow
Orange
Red
Other
##
##
#
###
###
###
##
< 10^5
~ 10^5
10^5 – 10^6
≥ 10^6

Greater detail on these and other forms of therapy will be presented throughout this volume.

Antimicrobial periodontal therapies at the present time can be grouped in three broad categories; those that physically remove microorganisms, often called mechanical debridement, those that attempt to kill or affect the metabolism of the organism such as antiseptics and antibiotics and those that affect the environment of the organisms. Other types of therapy are on the horizon, such as possible vaccines against oral pathogens or replacement therapy in which a species is introduced to the biofilm in order to control potentially pathogenic microorganisms. These two approaches will not be discussed further, although their addition to the therapist's armamentarium will certainly be welcome.

The physical removal of microorganisms – mechanical debridement

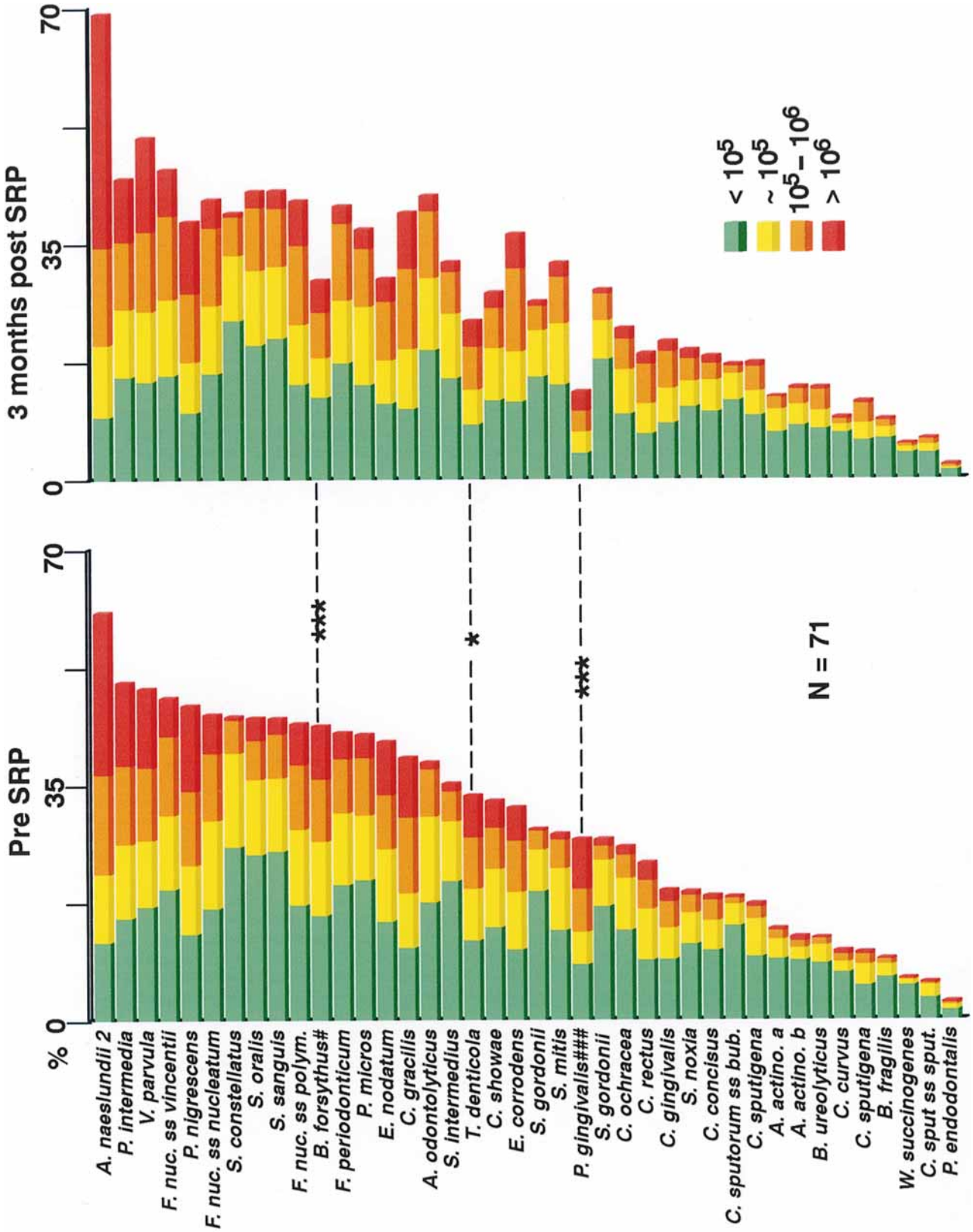
Given the remarkable resistance of organisms in biofilms to host defense mechanisms and antimicrobial agents, the logical first step in the control of these organisms would be their removal by physical means. Fortunately, biofilms in the oral cavity, unlike many other biofilms, are readily accessible allowing their physical removal. Indeed, the most common form of periodontal therapy is the removal of supra and subgingival plaque by procedures such as self performed oral hygiene, scaling and root planing or periodontal surgery. The following data provide an example of the effect of scaling and root planing on clinical parameters and the composition of the subgingival plaque. In this study, an extension of the study described by Haffajee et al. (67), 71 subjects with adult periodontitis were monitored clinically at six sites per tooth. Subgingival plaque samples were taken from the mesiobuccal surface of each tooth excluding third molars and individually analyzed for their content of 40 bacterial species using checkerboard DNA-DNA hybridization. Clinical measurements and microbial samples were taken at baseline and 3 months after treatment which consisted of full-mouth scaling and root planing accompanied by oral hygiene instruction. Fig. 12 presents the mean levels and mean prevalence of the 40 test species before and after scaling and root planing. The data indicate that, on average, the majority of species did not change significantly. However, three species of the "red" complex, *B. forsythus*, *P. gingivalis* and *T. denticola*, were significantly decreased in both counts and percentages of sites colonized 3 months after scaling and root planing when adjusted for multiple comparisons. These microbial changes were accompanied by a significant decrease in full-mouth mean pocket depth and attachment level (Fig. 13).

The data of this investigation are of interest in the light of the previous discussion on biofilms. The mechanical procedures undoubtedly removed most organisms that colonized the tooth surface. Perhaps 90% or even somewhat higher proportions were removed. However, given the rapid multiplication rates of bacteria, it is not surprising that the majority of taxa examined returned to almost baseline levels at 3 months. Data in the literature suggest that the return to baseline total counts might occur within 4–8 days (60, 170). However, certain taxa were affected by this procedure. These taxa might have been diminished by the mechanical procedures and returned more slowly, in part, because of their fastidious nature, and, in part, because the environment presented by the tissues may have been changed. A decrease in inflammation and an improvement of the epithelial barrier within the pocket might diminish the nutrient availability to these taxa, slowing, but not completely controlling their return. Other mechanical debridement procedures, such as periodontal surgery, have similar or even greater effects on the red complex and also affect members of the orange complex (115).

Antibiotics in the treatment of periodontal infections

Since periodontal diseases are infections, it is not surprising that antibiotics have been used and continue to be used in their treatment. Given the discussion on increased antibiotic resistance that occurs in organisms growing in biofilms as well as the difficulty of some antibiotics to effectively penetrate

Fig. 12. Stacked bar charts of the prevalence and levels of 40 subgingival species prior to and 3 months after scaling and root planing in 71 subjects (no. of samples=3546). The left panel summarizes pre-therapy values and the right panel post-therapy values. The total length of each bar stack indicates the percentage of sites colonized (prevalence of the species). The different shadings within each stack indicate the percentage of sites colonized by different levels of the species. The significance of differences in mean counts and prevalence between pre and post-therapy visits was determined using the Wilcoxon signed-ranks test. For prevalence: *$P<0.05$; ***$P<0.001$; for counts, $^{\#}P<0.05$; $^{\#\#\#}P<0.001$ after adjusting for multiple comparisons (175).


3 months post SRP
Pre SRP
% 0
35
70
0
< 10^5
~ 10^5
10^5 – 10^6
> 10^6
N = 71
***
*
***
A. naeslundii 2
P. intermedia
V. parvula
F. nuc. ss vincentii
P. nigrescens
F. nuc. ss nucleatum
S. constellatus
S. oralis
S. sanguis
F. nuc. ss polym.
B. forsythus#
F. periodonticum
P. micros
E. nodatum
C. gracilis
A. odontolyticus
S. intermedius
T. denticola
C. showae
E. corrodens
S. gordonii
S. mitis
P. gingivalis###
S. gordonii
C. ochracea
C. rectus
C. gingivalis
S. noxia
C. concisus
C. sputorum ss bub.
C. sputigena
A. actino. a
A. actino. b
B. ureolyticus
C. curvus
C. sputigena
B. fragilis
W. succinogenes
C. sput ss sput.
P. endodontalis

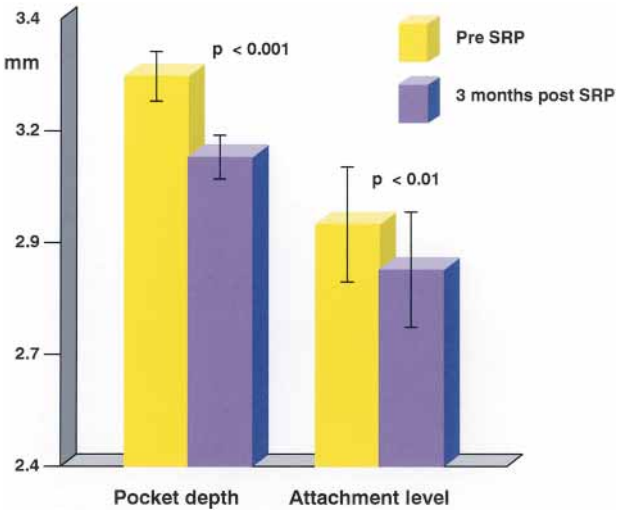


Fig. 13. Bar chart of the mean pocket depth and attachment level (±SEM) at baseline and 3 months after scaling and root planing (no. of subjects=71). The significance of differences between pre and post-therapy visits was determined using the Wilcoxon signed-ranks test. Note that the *y*-axis does not start at 0.

a biofilm, one might question the use of these agents in the treatment of periodontal diseases. However, antibiotics have been successfully employed as adjuncts in the treatment of these infections (7, 57, 69, 89, 91–93, 121, 127–131, 146, 147, 178, 196, 199). The expectation that one might have of the results of treatment by a systemically administered antibiotic might vary widely. On the one hand, the expectation might be that the agent would kill all the sensitive species and leave insensitive species (hopefully host compatible) to emerge. On the other hand, from the earlier discussion one might surmise that the antibiotic might have virtually no effect even on species found to be susceptible in a planktonic state due to the barrier and changed resistance of organisms grown in biofilms. The "truth" lies somewhere in between. Systemically administered antibiotics do have certain effects on segments of the subgingival microbiota, but usually do not completely eliminate the sensitive bacterial species. The following study provides examples of two popularly employed systemic antibiotics, amoxicillin and metronidazole, used individually as adjuncts to scaling and root planing (59). After baseline clinical monitoring and microbial sampling the 17 adult periodontitis subjects received full-mouth scaling and root planing. Subjects were then randomly assigned to treatment groups receiving either systemically administered amoxicillin (500 mg three times daily) or systemically administered metronidazole (250 mg three times daily) for 14 days. Post-therapy, full-mouth clinical monitoring and sampling was performed at 3, 6 and 12 months. Additional subgingival plaque samples were taken at 3, 7 and 14 days during antibiotic administration as well as at 3, 7 and 14 days after completion of the antibiotic therapy from pairs of randomly selected posterior teeth. Both therapies produced a significant improvement in clinical parameters (Fig. 14). In particular, full-mouth mean pocket depth, attachment level and the percentage of sites with bleeding on probing were significantly decreased in both groups, whereas amoxicillin therapy also produced a significant decrease in the percentage of sites exhibiting plaque. Fig. 15 and 16 present the counts of the 40 taxa examined at baseline, 3, 6 and 12 months in the two treatment groups. Both amoxicillin and metronidazole produced significant decreases in counts of many of the taxa particlarly red and orange complex species. The decrease in counts of red complex species was particularly marked, and the initial decreases were maintained to 12 months, most noticeably in the subjects treated with metronidazole.

The different effects of the antibiotics are highlighted by their effect on the proportions and levels of the various microbial complexes described earlier. Fig. 17 demonstrates that the proportions of *Actinomyces* and red complex species decreased during amoxicillin administration accompanied by an increase in proportions of yellow complex species during this interval. After 90 days, the proportions tended to revert to baseline levels, but the *Actinomyces* spp. were still at reduced levels. In contrast, the major effect of metronidazole was on the red complex species, and to a lesser extent, orange complex species, which were markedly reduced during antibiotic administration (Fig. 17). The red complex species remained at lower levels throughout the study. The proportions of *Actinomyces* and yellow complex species were less affected by this agent.

The proportion of organisms that were resistant to the two agents before, during and after their administration are shown in Fig. 18. More that half of the cultivable organisms were resistant to metronidazole at baseline. This figure increased to about 81% after 14 days of metronidazole administration and decreased to baseline levels at 90 days. Approximately 0.5% of the isolates were resistant to amoxicillin at baseline. The resistant proportion rose to about 41% at the end of amoxicillin administration and declined to close to baseline levels at 90 days. Thus, 19% of the organisms in the biofilms were sensitive to metronidazole and 59% were sensitive to amoxicillin even when the subjects had taken the pre-

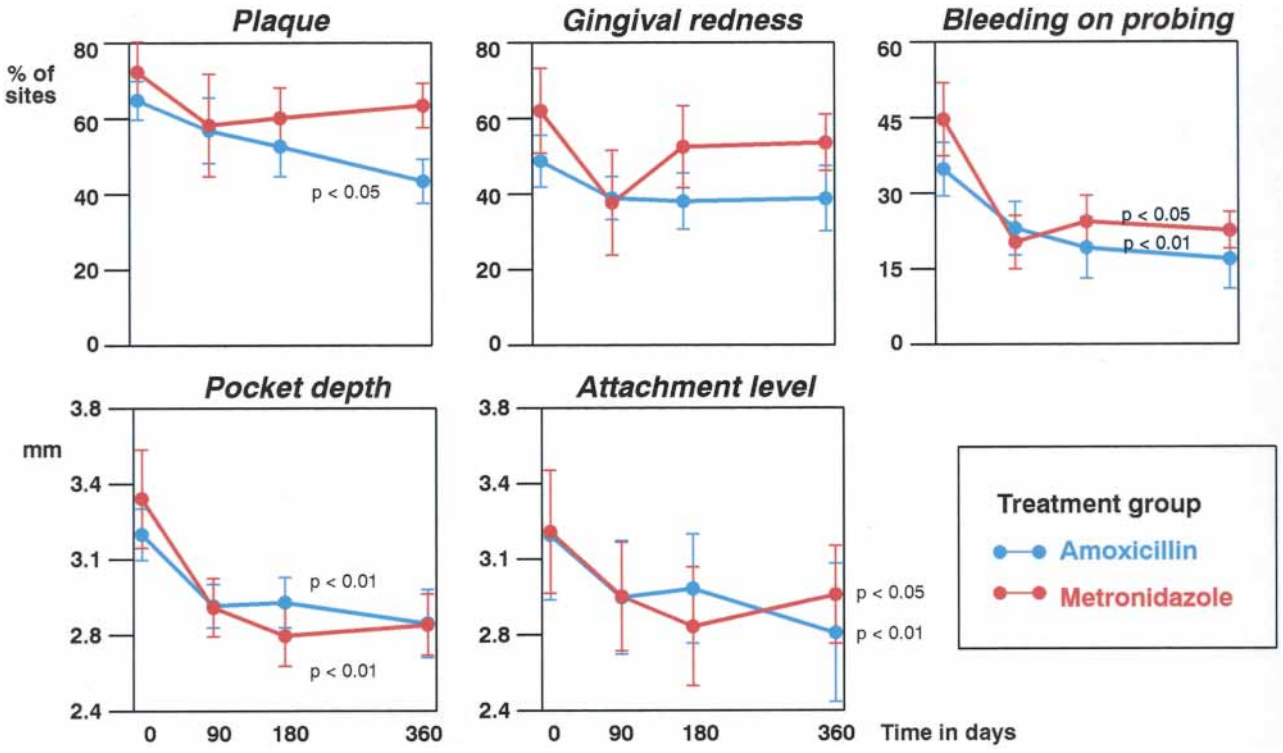


Fig. 14. Plots of the full-mouth mean values (±SEM) for clinical parameters at baseline, 90, 180 and 360 days for subjects treated with scaling and root planing and metronidazole or scaling and root planing and amoxicillin. The circles represent the mean values, and the whiskers represent the standard error of the mean. The values for each parameter were measured at up to 168 sites in each subject, averaged within a subject and then averaged across subjects in each treatment group for each time point. The significance of differences over time was tested using the Quade test. The significance of differences between groups at each time point was tested using ANCOVA. Reprinted with permission of the *Journal of Clinical Periodontology* (Feres et al. (59)).

scribed agent for 14 days. These figures attest to the protection afforded organisms in biofilms that was discussed above. The microbial count data and the antibiotic resistance data suggest the possibility that the systemically administered antibiotics may have affected, primarily, the organisms in the epithelial cell associated biofilms and the loosely adherent adjacent cells. These organisms might be more accessible to the administered agents, in part, because of their proximity to the host tissues and, in part, because of a less developed glycocalyx. Conceivably, organisms in this area were reduced to very low levels. However, the same species may also have been resident in the tooth-associated biofilms but at much lower levels. The species in the tooth-associated biofilms may have been more resistant to the antibiotics due to mechanisms described earlier, and thus the potential for re-growth from this source was perpetuated. It might be surmised that physical removal of the tooth-associated biofilms prior to or during antibiotic administration might minimize this re-growth.

The data from the study outlined above indicate that systemically administered antibiotics do affect microorganisms located within biofilms. Many of the test species were significantly reduced in numbers even up to 1 year after the initial therapy, although no species, on average, was eliminated. It was also clear that different agents have different effects on the subgingival microbiota. Although both agents reduced, at least initially, the red complex, metronidazole appeared to have a more pronounced and long-lasting effect. Further, amoxicillin produced a significant decrease in the proportion of *Actinomyces* species, with a concomitant increase in the proportion of yellow complex species. This potentially undesirable effect was not seen in the subjects treated with metronidazole. It was also apparent, within the limitations of the study, that short-term use of these two agents did not affect the proportion of antibiotic-resistant species in the long term. The effect of an antibiotic can go beyond its direct effects on individual species. For example, the counts of *Actinomyces* species were found to be decreased 3

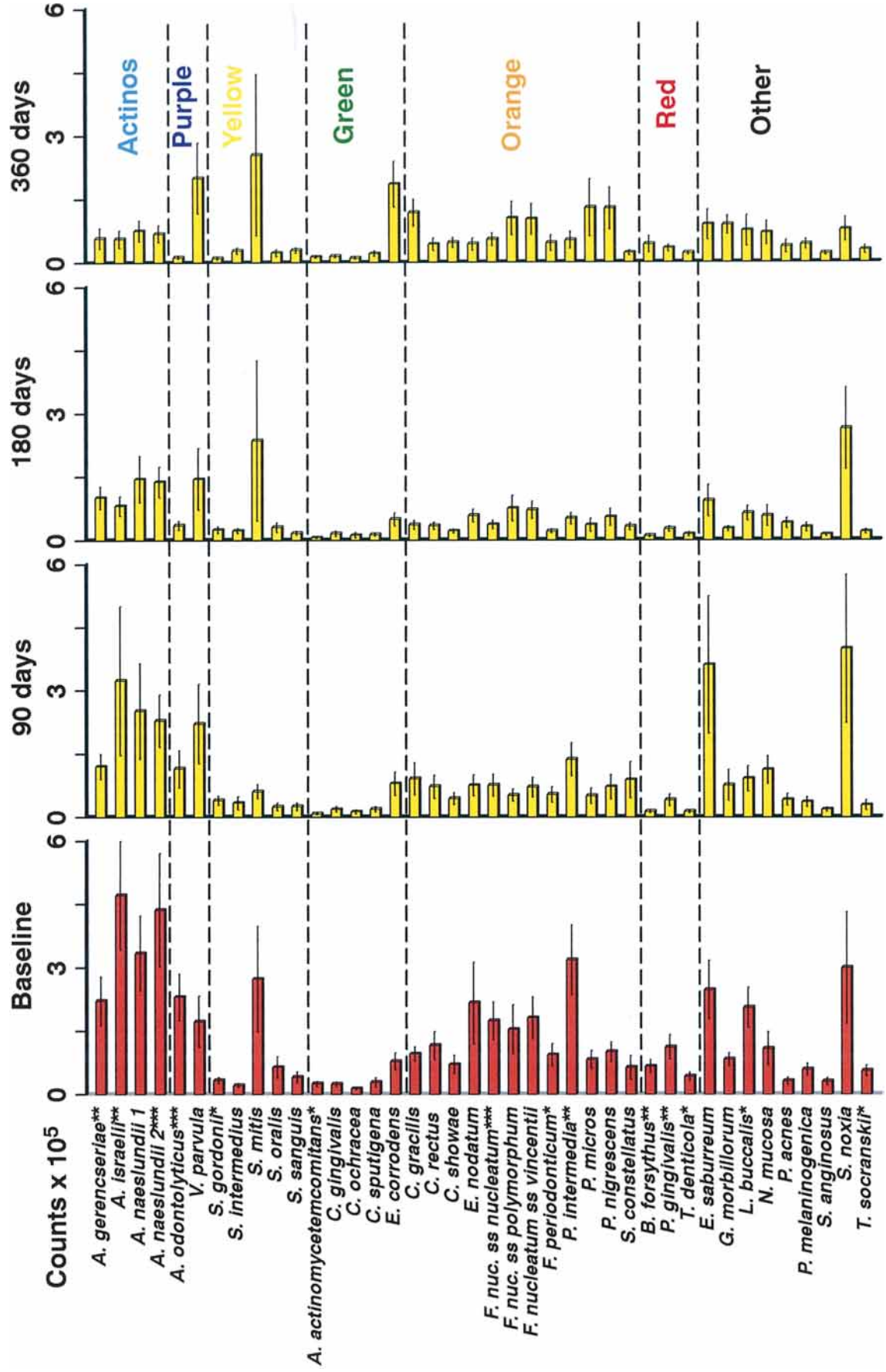
months after metronidazole administration. *Actinomyces* species are not sensitive to metronidazole, suggesting that this reduction was due to a decrease in other taxa that affected the inflammatory status of the habitat which, in turn, lowered levels of all colonizing species (160, 161). A dental biofilm is a consortium of organisms, and alteration of part of this consortium will affect both habitat and the remaining taxa.

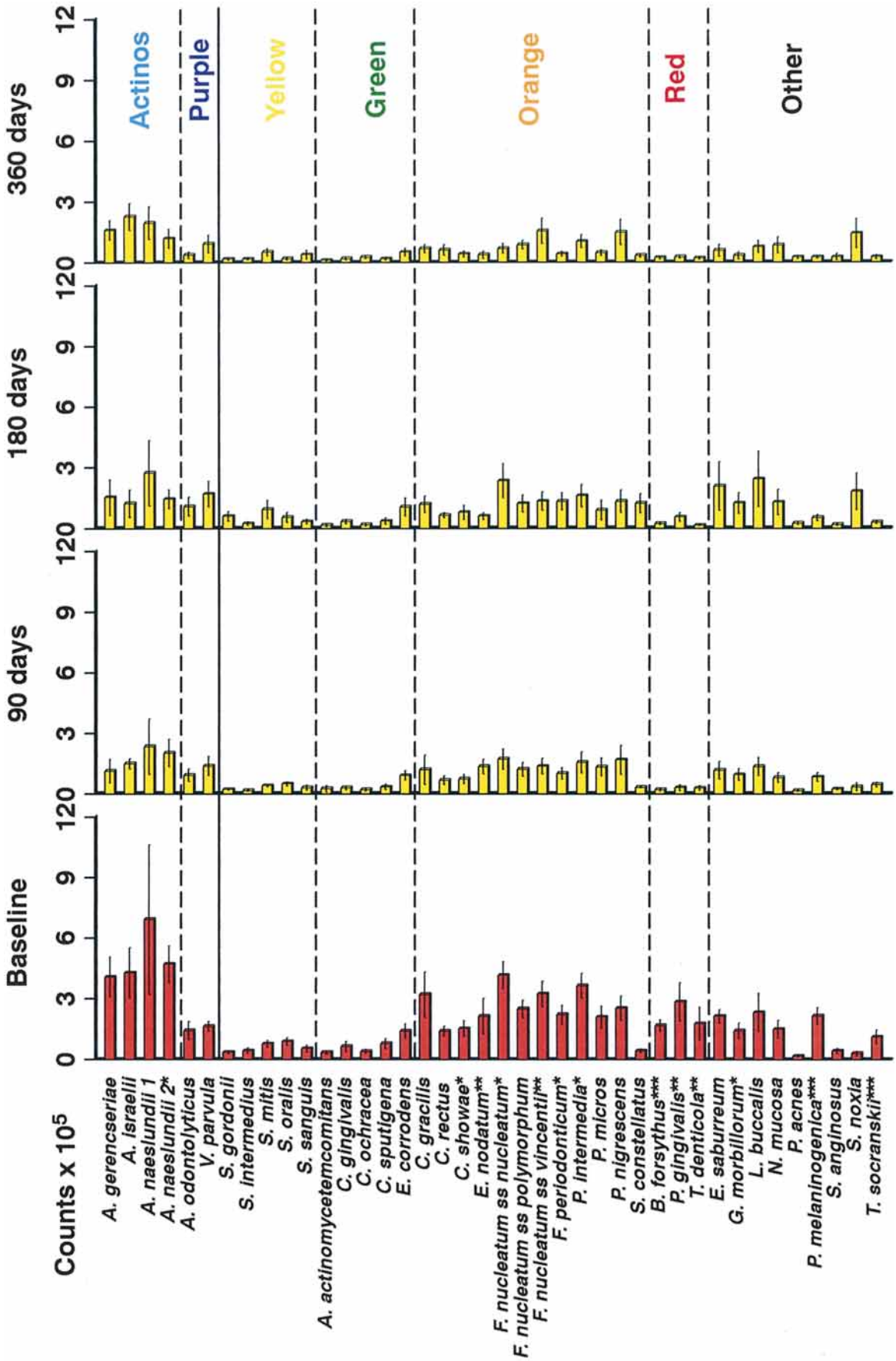
Therapies that affect the microbial environment – supragingival plaque removal

Although it is widely recognized that bacteria can affect the tissues that they colonize, it is less appreciated that the environment of the bacteria has a major effect on their growth and activities. It is axiomatic in microbial ecology that colonizing species affect the habitat and the habitat affects the colonizing organisms. This is clearly the case in the oral environment, where species have specific tissue tropisms, with certain taxa flourishing on certain surfaces and others in other locations. The subgingival environment is clearly one in which many species including those of the red and orange complex flourish. This habitat provides an environment conducive to their multiplication and perhaps to their ability to lead to tissue destruction. If the environment surrounding the subgingival microbiota were altered, then changes in numbers, proportions and prevalence of species would be likely to be affected. The two major environmental factors that affect subgingival plaque are the tissues of the periodontal pocket and the supragingival plaque. Changes in either of these factors are likely to lead to changes in the composition of the subgingival plaque. This is indeed the case. It has been recognized for a long period of time that meticulous removal of supragingival plaque leads to an improvement in the parameters associated with gingival inflammation. Indeed, clinicians have urged and continue to urge patients to carefully and regularly remove supragingival deposits. What is less appreciated is the effect of regular supragingival plaque removal on subgingival plaque composition. A few studies have suggested that supragingival plaque removal can decrease putative periodontal pathogens (1, 44, 82, 173, 190). These effects were examined further in a study of 18 adult periodontitis subjects who were in a periodontal maintenance program (218). After baseline clinical and microbiological examination, the subjects received full-mouth scaling and root planing followed by weekly professional removal of supragingival plaque for 3 months. The subjects performed their normal home care procedures for the 12 months of the study. The subjects were monitored at 3, 6 and 12 months, at which time points they also received maintenance subgingival scaling. Fig. 19 presents the mean total counts of supragingival and subgingival plaque at baseline, 3, 6 and 12 months. Total counts of both supragingival and subgingival plaque decreased significantly at 3 months, immediately after completion of the professional supragingival plaque removal phase. It was of interest, however, that the counts continued to decrease at the 6- and 12-month visits, even though professional cleaning had not been employed for 3 and 9 months respectively. Similar findings were seen in both supragingival and subgingival samples for the individual taxa examined. Fig. 20 presents the mean counts of the 40 test taxa at baseline, 3, 6 and 12 months in 1804 subgingival plaque samples. Thirty-four species were significantly reduced over time. These included periodontal pathogens such as *B. forsythus, P. gingivalis* and *A. actinomycetemcomitans*. The 12-month microbial profile was remarkably similar to that seen in 22 periodontally healthy subjects evaluated microbiologically in the same way (Fig. 21). Although there were multiple significant differences in counts between the baseline and 12-month data in the treated subjects, there were no significant differences between the 12-month data and those for the healthy subjects. This suggests that meticulous removal of supragingival plaque after initial periodontal therapy, such as scaling and root planing, can lead to a microbiota that is remarkably similar to that observed in periodontal health.

In controlling infectious diseases, there is usually a two-pronged effort. The first is to control the level of the organism in the environment, usually by sanitary procedures. The second is to target the organism in an infected host who is exhibiting disease. On a population basis, the first might be the more important in that epidemic disease has been markedly

Fig. 15. Bar charts of the mean counts ($\times 10^5$,±SEM) of 40 subgingival species at baseline, 90, 180 and 360 days for the amoxicillin-treated subjects. The species are grouped according to the microbial complexes described by Socransky et al. (174). The mean levels of each species were computed for each subject and then averaged across subjects at each time point. Significance of differences over time was tested using the Quade test (*$P<0.05$; **$P<0.01$; ***$P<0.001$). Thirteen species were significantly reduced. Reprinted with permission of the *Journal of Clinical Periodontology* (Feres et al. (59)).


Baseline
90 days
180 days
360 days
Counts x 10^5
0
3
6
Actinos
Purple
Yellow
Green
Orange
Red
Other
A. gerencseriae**
A. israelii**
A. naeslundii 1
A. naeslundii 2***
A. odontolyticus***
V. parvula
S. gordonii*
S. intermedius
S. mitis
S. oralis
S. sanguis
A. actinomycetemcomitans*
C. gingivalis
C. ochracea
C. sputigena
E. corrodens
C. gracilis
C. rectus
C. showae
E. nodatum
F. nuc. ss nucleatum***
F. nuc. ss polymorphum
F. nucleatum ss vincentii
F. periodonticum*
P. intermedia**
P. micros
P. nigrescens
S. constellatus
B. forsythus**
P. gingivalis**
T. denticola*
E. saburreum
G. morbillorum
L. buccalis*
N. mucosa
P. acnes
P. melaninogenica
S. anginosus
S. noxia
T. socranski*


Baseline
90 days
180 days
360 days
Counts x 10^5
0
3
6
9
12
120
Actinos
Purple
Yellow
Green
Orange
Red
Other
A. gerencseriae
A. israelii
A. naeslundii 1
A. naeslundii 2*
A. odontolyticus
V. parvula
S. gordonii
S. intermedius
S. mitis
S. oralis
S. sanguis
A. actinomycetemcomitans
C. ginglvalis
C. ochracea
C. sputigena
E. corrodens
C. gracilis
C. rectus
C. showae*
E. nodatum**
F. nucleatum ss nucleatum*
F. nucleatum ss polymorphum
F. nucleatum ss vincenti**
F. periodonticum*
P. intermedia*
P. micros
P. nigrescens
S. constellatus
B. forsythus***
P. gingivalis**
T. denticola**
E. saburreum
G. morbillorum*
L. buccalis
N. mucosa
P. acnes
P. melaninogenica***
S. anginosus
S. noxia
T. socranski***

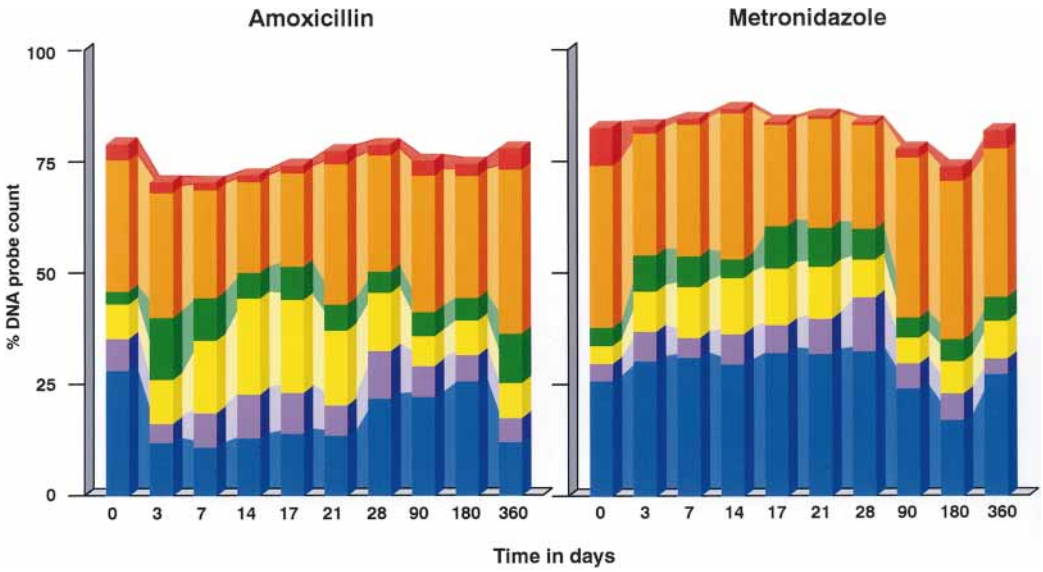


Fig. 17. Stacked bar charts describing the mean proportion of microbial complexes at different time points for the nine subjects in the amoxicillin group and the eight subjects in the metronidazole group. The percentage of DNA probe counts for each species were determined at each site, averaged within a subject and then averaged across subjects at each time point. Species in the complexes were summed and the proportions that each complex comprised was determined. The "other" group, composed of species that did not fit any complex or represented new DNA probes to species whose relationship to existing complexes have not yet been determined, made up the difference to 100%.

reduced in industrialized countries by improvements in sewage systems, water supplies, food handling and other public health measures. Supragingival plaque control may be thought of as a "sanitary" procedure that lowers the levels of potentially pathogenic species that colonize the individual and that colonize the community. As such, this reduction in the reservoir of potentially pathogenic organisms is of major importance in lowering the risk of new disease or recurrence of disease in infected individuals. However, supragingival plaque removal has an added benefit in that it appears to affect the numbers and composition of the subgingival microbiota. Clearly, the removal of the biofilm from the supragingival area affects the composition of the subgingival biofilm on the same surface. This may be due to a direct effect of the supragingival colonizers on subgingival organisms or an effect on the adjacent periodontal tissues, which might lead to reduction in subgingival species. Most likely the effect is due to both phenomena. Supragingival organisms and also the adjacent periodontal tissues provide both nutrients and physical and chemical environments for proliferation of the subgingival taxa. Removal of the supragingival colonizers would diminish this source, while diminished inflammation and improved barrier function of the epithelium would reduce a second source of growth requirements (160, 161). In essence, the subgingival biofilm numbers decrease because the essential requirements for growth have been cut off or decreased. Alteration of the habitat may be one of the most important mechanisms for the long-term control of subgingival pathogens. If our treatments or preventions diminish nutrient availability and maintain epithelial barrier function, then the numbers of organisms in the subgingival environment will diminish and the proportions of organisms that appear to be pathogenic will decrease. Further, decreasing reservoirs of pathogenic species by systematic removal from tooth surfaces, perhaps accompanied by suppression on soft tissue surfaces, should lead to long-term stability in the majority of periodontal patients.

Fig. 16. Bar charts of the mean counts ($\times 10^5$,±SEM) of 40 subgingival species at baseline, 90, 180 and 360 days for the metronidazole-treated subjects. The presentation of the data and significance testing was as described in Fig. 15. Thirteen species were significantly reduced. Reprinted with permission of the *Journal of Clinical Periodontology* (Feres et al. (59)).

Combined antimicrobial therapies

The use of combined therapies has been shown to be effective in the treatment of several medically im-

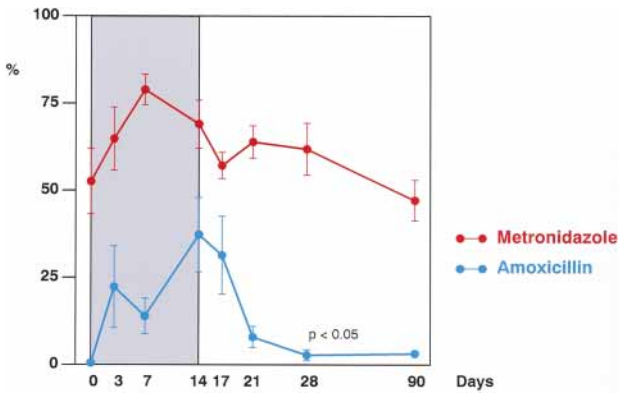


Fig. 18. Percentage of isolates resistant to amoxicillin or metronidazole in the plaque samples from subjects receiving those agents. Samples were plated on enriched blood agar plates with or without one of: 2 μg/ml metronidazole or 2 μg/ml amoxicillin. Colonies were counted at 7 days. The data were averaged within a subject at each time point and then across subjects separately in the two groups. The circles represent the mean and the whiskers the standard error of the mean. The shaded area represents the period of antibiotic administration in the test subjects. Significant differences in the percentage of resistant organisms over time were determined by the Quade test.

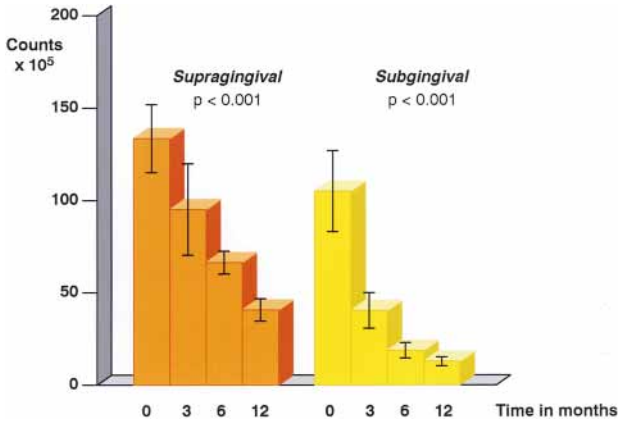
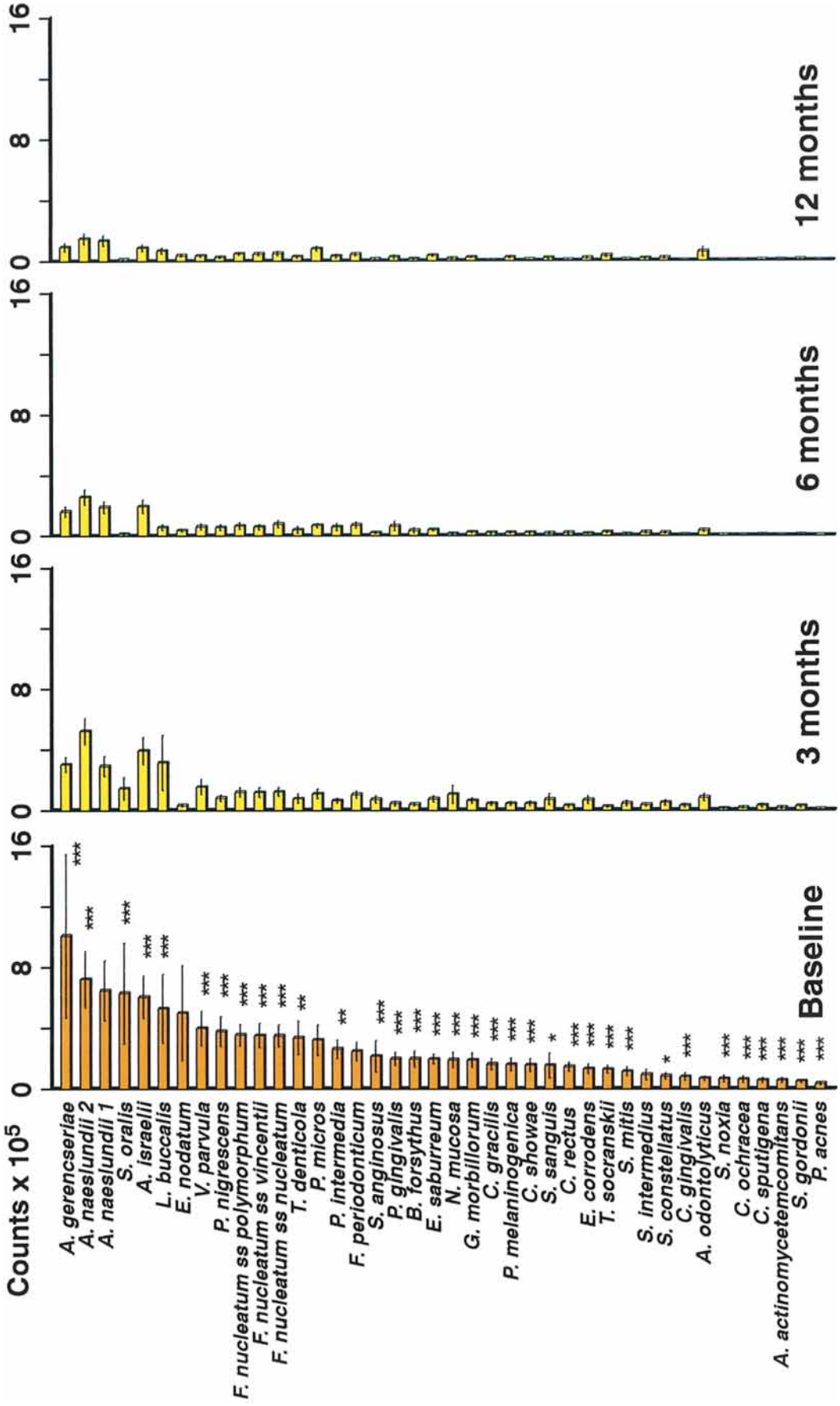


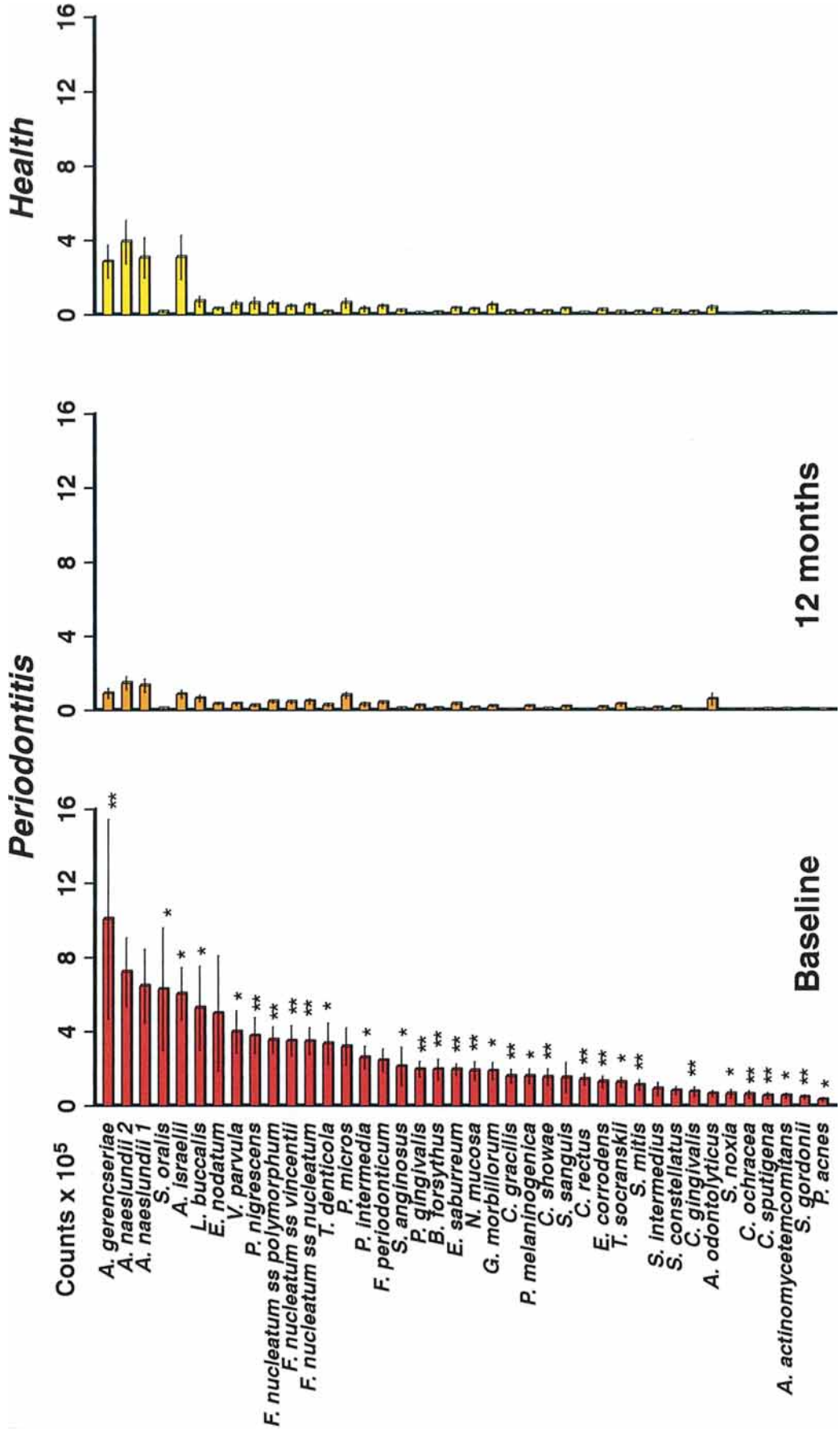
Fig. 19. Mean total DNA probe counts (×10^5,±SEM) in supra and subgingival plaque samples taken at baseline, 3, 6 and 12 months. Professional supragingival plaque control was performed between baseline and 3 months. Mean counts were computed for a subject for each visit and then values were averaged across the 18 subjects at each time point. The whiskers indicate the SEM. Significance of differences over time was sought using the Quade test.

portant infections. For example, the treatment of HIV infections currently employs two or three therapeutic agents providing better outcomes than the single therapies employed previously. Treatment of stomach ulcers caused by *Helicobacter pylori* usually is best accomplished by the combined use of two or more agents such as metronidazole with amoxicillin and bismuth. The combination of systemically administered amoxicillin and metronidazole for the treatment of certain periodontal infections has been quite effective (14, 150, 166, 200, 201, 213). For example, this treatment in combination with scaling and root planing significantly reduced the detection of *A. actinomycetemcomitans* in subjects with *A. actinomycetemcomitans*–associated adult periodontitis (150), localized juvenile periodontitis, as well as rapidly progressive and refractory periodontitis (200, 201). Other studies have shown that the combination of these two agents was also effective in controlling the levels of other pathogens such as *P. gingivalis*, *B. forsythus* and *P. intermedia* (14, 132, 150, 166, 213).

In an earlier study (29), it was found that certain subjects exhibited a poor clinical response to the sequential use of scaling and root planing, surgery and systemically administered antibiotics. These subjects showed mean full-mouth attachment loss or more than three sites with greater than 2.5 mm of attachment loss within 1 year after each therapy. Examination of the changes in the subgingival microbiota of these subjects suggested that the suspected periodontal pathogens were decreased comparably to the reduction observed in successfully treated subjects. However, in spite of a reduction in microbial load, the individuals continued to exhibit disease progression. One hypothesis for this continued disease progression might be that the subjects had reduced ability to cope with periodontal infections than other individuals. A second hypothesis might be that the pathogens were of more virulent clonal types than those found in subjects who responded well to therapy. This led to the hypothesis that, although a reduction in pathogenic species occurred as a result of conventional therapy, this reduction was not sufficient in this group of refractory subjects to prevent disease progression. Thus, an approach was designed that would employ combinations of antimicrobial agents that might further

Fig. 20. Bar charts of the mean counts (×10^5,±SEM) of the 40 test species in subgingival plaque samples taken at baseline, 3, 6 and 12 months. Professional supragingival plaque control was performed between baseline and 3 months. Mean counts for each species were computed for a subject for each visit and then values were averaged across the 18 subjects at each time point. The whiskers indicate the SEM. The significance of differences over time was sought using the Quade test. *$P<0.05$; **$P<0.01$; ***$P<0.001$ after adjusting for multiple comparisons. Reprinted with permission of the *Journal of Clinical Periodontology* (Ximenez-Fyvie et al. (218)).


Counts x 10^5
0
8
16
Baseline
3 months
6 months
12 months
A. gerencseriae
A. naeslundii 2
A. naeslundii 1
S. oralis
A. israelii
L. buccalis
E. nodatum
V. parvula
P. nigrescens
F. nucleatum ss polymorphum
F. nucleatum ss vincentii
F. nucleatum ss nucleatum
T. denticola
P. micros
P. intermedia
F. periodonticum
S. anginosus
P. gingivalis
B. forsythus
E. saburreum
N. mucosa
G. morbillorum
C. gracilis
P. melaninogenica
C. showae
S. sanguis
C. rectus
E. corrodens
T. socranskii
S. mitis
S. intermedius
S. constellatus
C. gingivalis
A. odontolyticus
S. noxia
C. ochracea
C. sputigena
A. actinomycetemcomitans
S. gordonii
P. acnes


Health
Periodontitis
Baseline
12 months
Counts x 10^5
0
4
8
12
16
A. gerencseriae
A. naeslundii 2
A. naeslundii 1
S. oralis
A. israelii
L. buccalis
E. nodatum
V. parvula
P. nigrescens
F. nucleatum ss polymorphum
F. nucleatum ss vincentii
F. nucleatum ss nucleatum
T. denticola
P. micros
P. intermedia
F. periodonticum
S. anginosus
P. gingivalis
B. forsythus
E. saburreum
N. mucosa
G. morbillorum
C. gracilis
P. melaninogenica
C. showae
S. sanguis
C. rectus
E. corrodens
T. socranskii
S. mitis
S. intermedius
S. constellatus
C. gingivalis
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C. sputigena
A. actinomycetemcomitans
S. gordonii
P. acnes

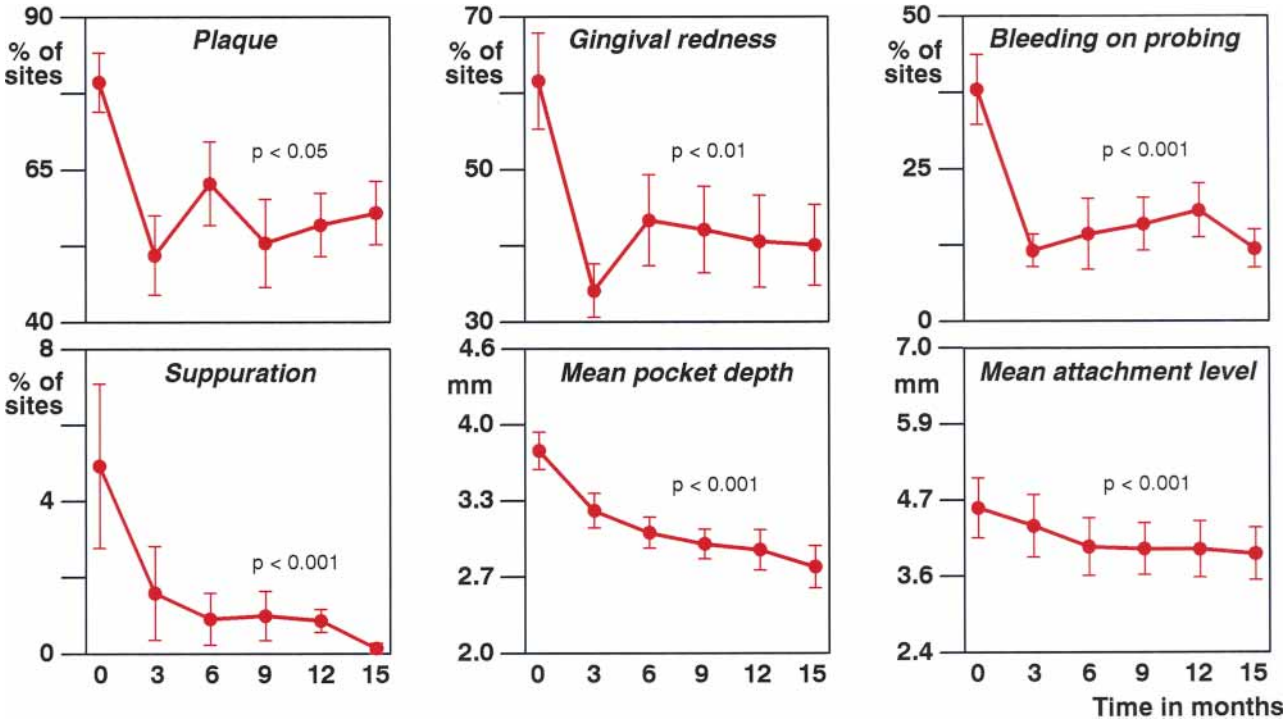

Fig. 22. Plots of the full-mouth mean values (±SEM) for clinical parameters at baseline, 3, 6, 9, 12 and 15 months for eight refractory subjects. The circles represent the mean values and the whiskers represent the standard error of the mean. Values for each parameter were measured at up to 168 sites in each subject, averaged within a subject and then averaged across subjects for each time point. The significance of differences over time was tested using the Quade test.

reduce pathogen load and lead to more sustained clinical stability. The therapy consisted of full-mouth scaling and root planing, systemically administered metronidazole and amoxicillin, local delivery of tetracycline at sites with pocket depth >4 mm and professional removal of supragingival plaque weekly for 3 months. The notion was that the scaling and root planing would physically remove organisms, that the tetracycline fibers would dramatically decrease the pathogens in the deeper pockets and that systemically administered amoxicillin and metronidazole would affect sensitive species that remained after reduction by physical removal and local antibiotics. Finally, repeated supragingival plaque removal would be employed in order to attempt to affect the habitat of the colonizing organisms and thus minimize the extent and level of recolonization. In this ongoing study, eight refractory periodontitis subjects have been treated and followed for at least 15 months. Fig. 22 presents the mean clinical values for the percentage of sites exhibiting visible plaque, gingival redness, bleeding on probing and suppuration as well as pocket depth and attachment level before and up to 15 months after therapy. All parameters were significantly decreased over time. Fig. 23 presents the baseline and 15-month data for each clinical parameter in each of the eight subjects. Every subject showed an improvement in the precentage of sites exhibiting bleeding on probing, suppuration, mean pocket depth and mean attachment level, while only one of eight subjects failed to show a decrease in the precentage of sites exhibiting

Fig. 21. Bar chart of mean counts ($\times 10^5$,±SEM) of individual species in subgingival plaque samples taken from the 18 periodontitis subjects at baseline and 12 months as well as from 22 periodontally healthy subjects at baseline (218). The bars represent the mean counts and the whiskers indicate the standard error of the mean. Professional supragingival plaque control was performed between baseline and 3 months in the periodontitis group. The mean counts for each species were computed for a subject for each visit and then values averaged across the subjects at each time point. The significance of differences between baseline and 12 months was sought using the Wilcoxon signed-ranks test. *$P<0.05$; **$P<0.01$, after adjusting for multiple comparisons. The significance of differences between the healthy and 12 month periodontitis samples was sought using the Mann-Whitney test. No significant differences were observed after adjusting for multiple comparisons. Reprinted with permission of the *Journal of Clinical Periodontology* (Ximenez-Fyvie et al. (218)).

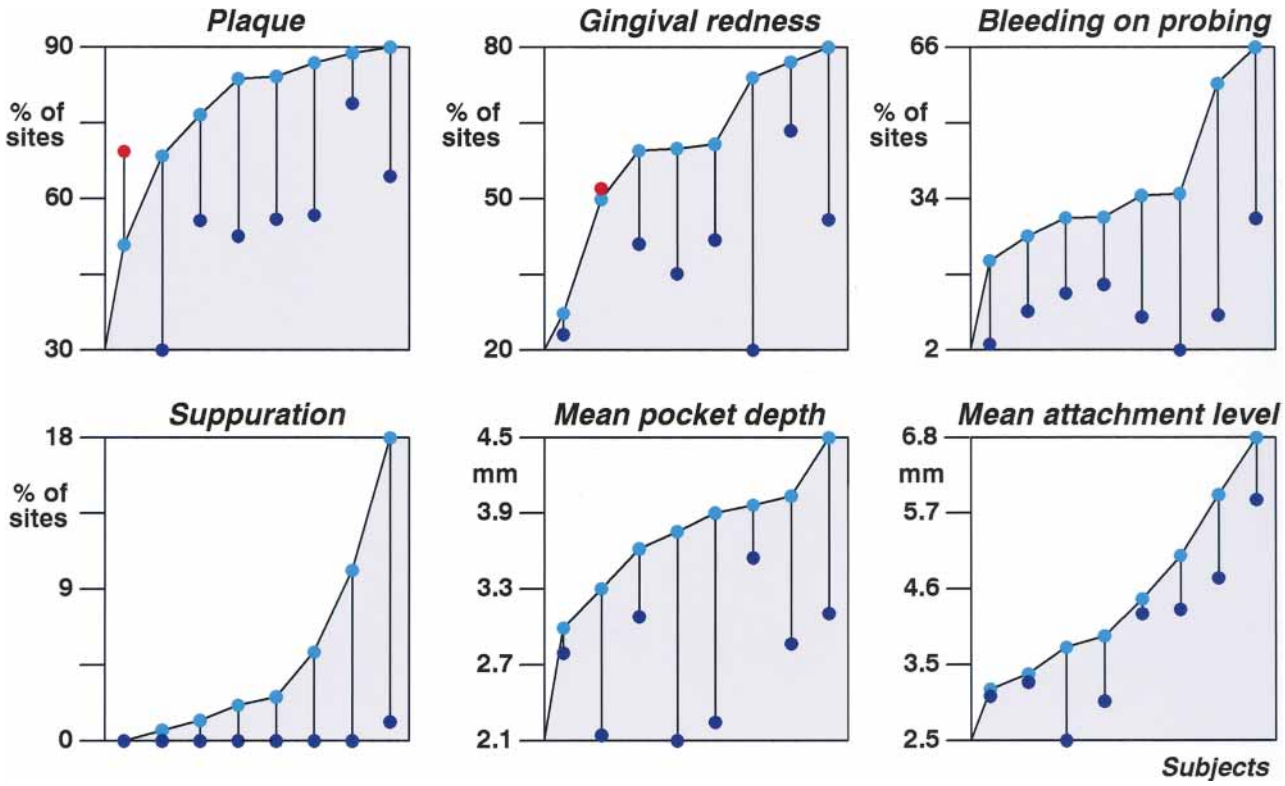

Fig. 23. Plots of the mean values for clinical parameters at baseline and 15 months in each subject. Each circle represents the mean value for an individual subject. The turquoise circles represent the mean values at baseline for each subject and the black or red circles represent the mean values for the same subject at 15 months. The black circles in the shaded area represent a decrease from baseline values, while the red circles in the unshaded area represent an increase from baseline values.

plaque or gingival redness. The mean full-mouth pocket depth and attachment level reductions were 0.99 and 0.69 mm respectively. These impressive clinical changes were accompanied by significant decreases in the counts and prevalence of subgingival species. In particular, counts of the red complex species, *B. forsythus* and *P. gingivalis*, and the orange complex species *E. nodatum*, *F. nucleatum* subsp. *vincentii* and *F. nucleatum* subsp. *nucleatum*, were significantly reduced (Fig. 24). It was interesting that the "*Streptococcus milleri*" species, *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*, which have been frequently implicated in refractory disease (29, 134), were also significantly reduced by the combined therapy. Many of the same species were also significantly reduced in prevalence (percentage of sites colonized) (Fig. 25). The counts and prevalence data for two species, *P. gingivalis* and *S. constellatus*, are presented in more detail in Fig. 26. These taxa that were at reduced levels due to earlier therapy were further reduced by the combined therapy utilized in this study.

The results of this ongoing study, albeit from a limited number of subjects, indicated that the combined therapies employed were able to improve or hold stable the clinical parameters examined. This is quite remarkable, in that previous therapies in this group of subjects had failed to prevent disease progression let alone lead to periodontal improvement. The second remarkable finding was that the combined therapy was able to significantly lower counts and proportions of many putative periodontal pathogens that were already at relatively low levels prior to the combined treatment. It should be pointed out that it is much easier to lower counts of organisms when they are at high levels prior to treatment. Clearly, the data of the present investigation together with those of van Winkelhoff and others suggest that combined therapies may be more effective in treating and/or preventing the recurrence of periodontal diseases. Individual therapies are often effective for subjects whose disease is readily controlled by any of several therapeutic approaches. This was demonstrated by the results of the studies presented in earlier sections of this chapter. However, a subset of subjects respond poorly to

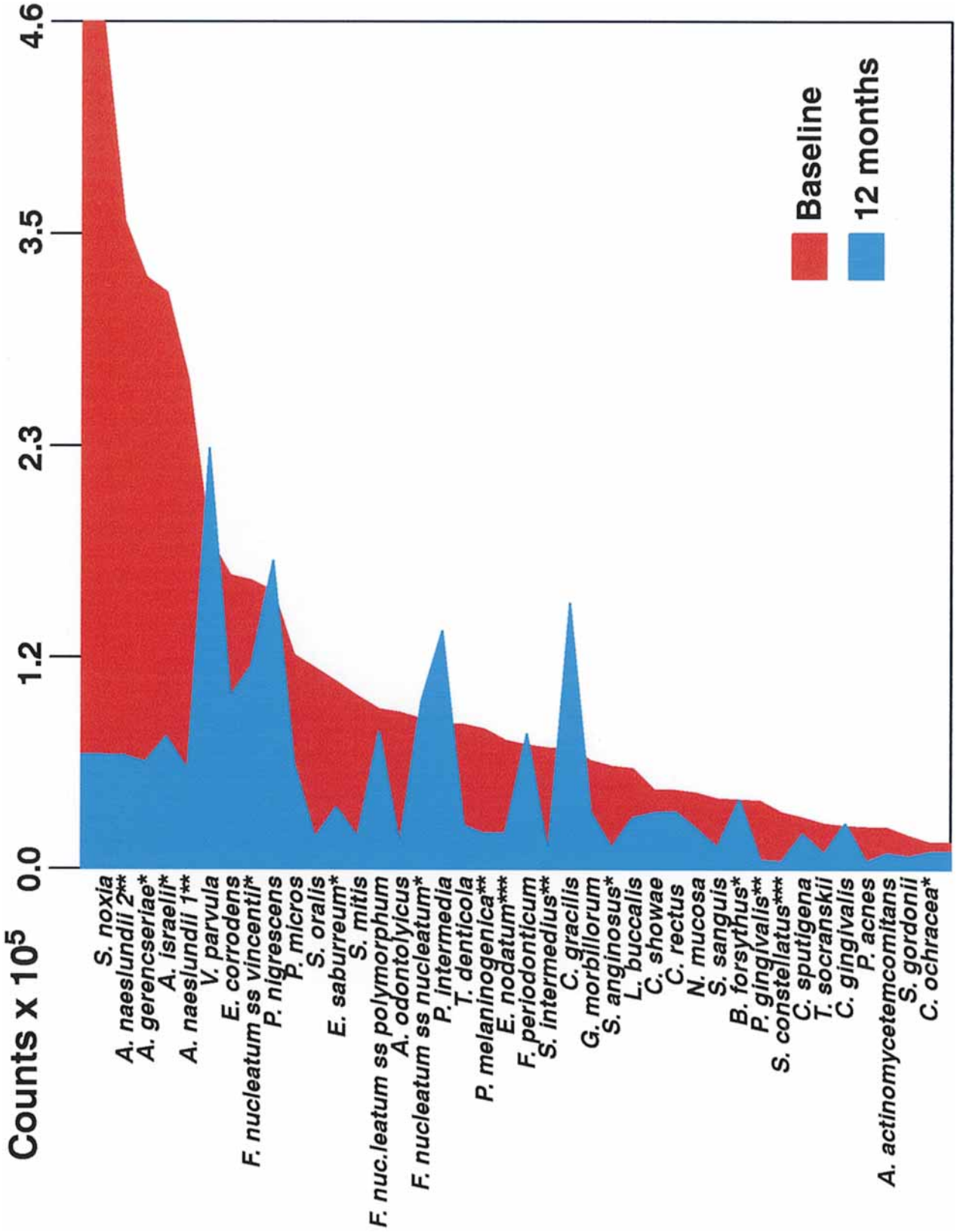

Fig. 24. Microbial profiles of the mean counts ($\times 10^5$) of 40 microbial taxa in subgingival plaque samples taken from the 8 refractory subjects at baseline and 12 months. The profiles represent the mean counts derived by averaging the counts of each species within a subject and then across subjects for both time points. The species are ordered according to the baseline mean counts. Significance of difference for each species between time points was determined using the Wilcoxon signed-ranks test.

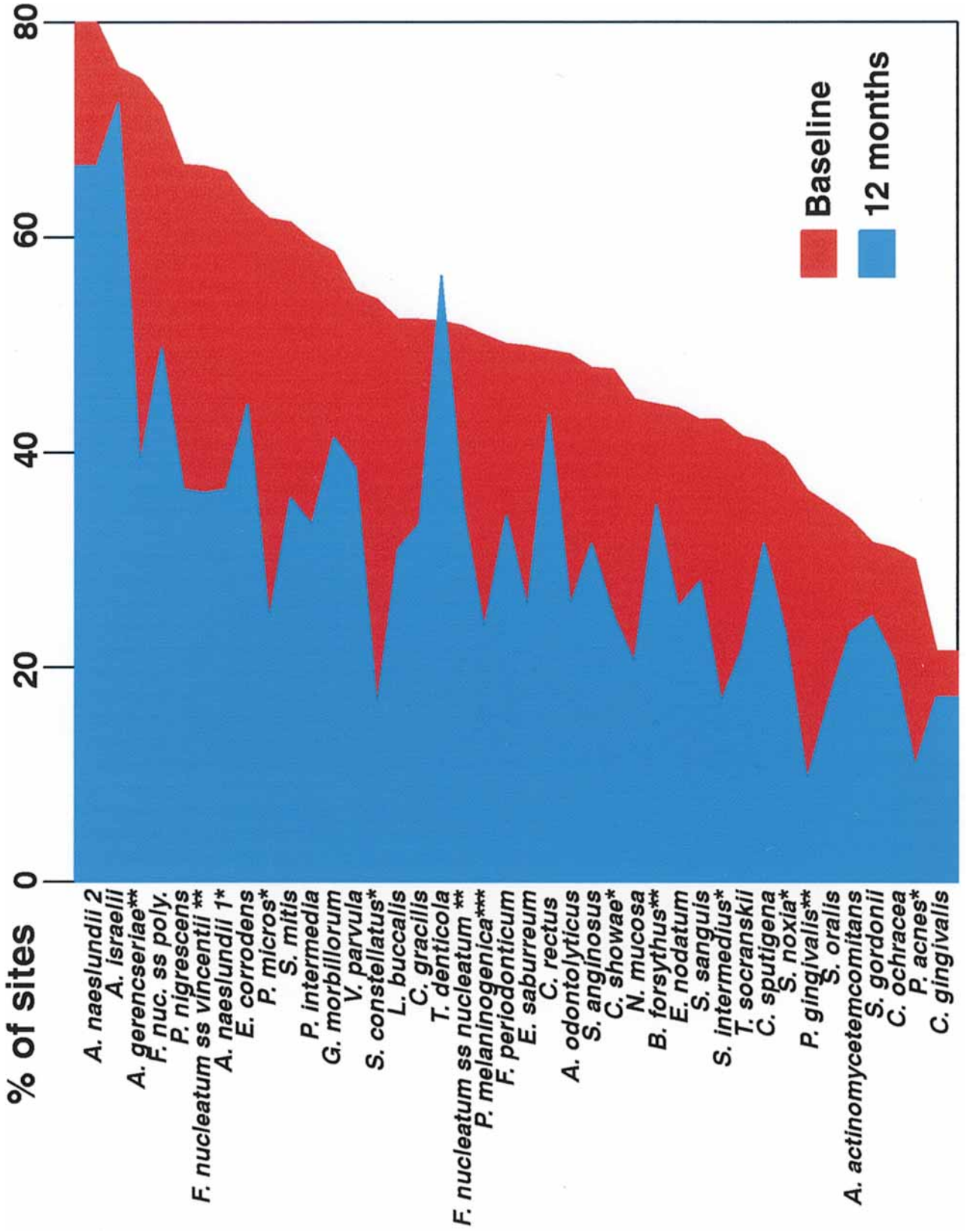

Fig. 25. Microbial profiles of the prevalence (percentage of sites colonized) of 40 microbial taxa in subgingival plaque samples taken from the eight refractory subjects at baseline and 12 months. The percentage of sites colonized in each subject at both time points was determined for each species and averaged across subjects at the different time points. Significance of difference for each species between time points was determined using the Wilcoxon signed-ranks test.

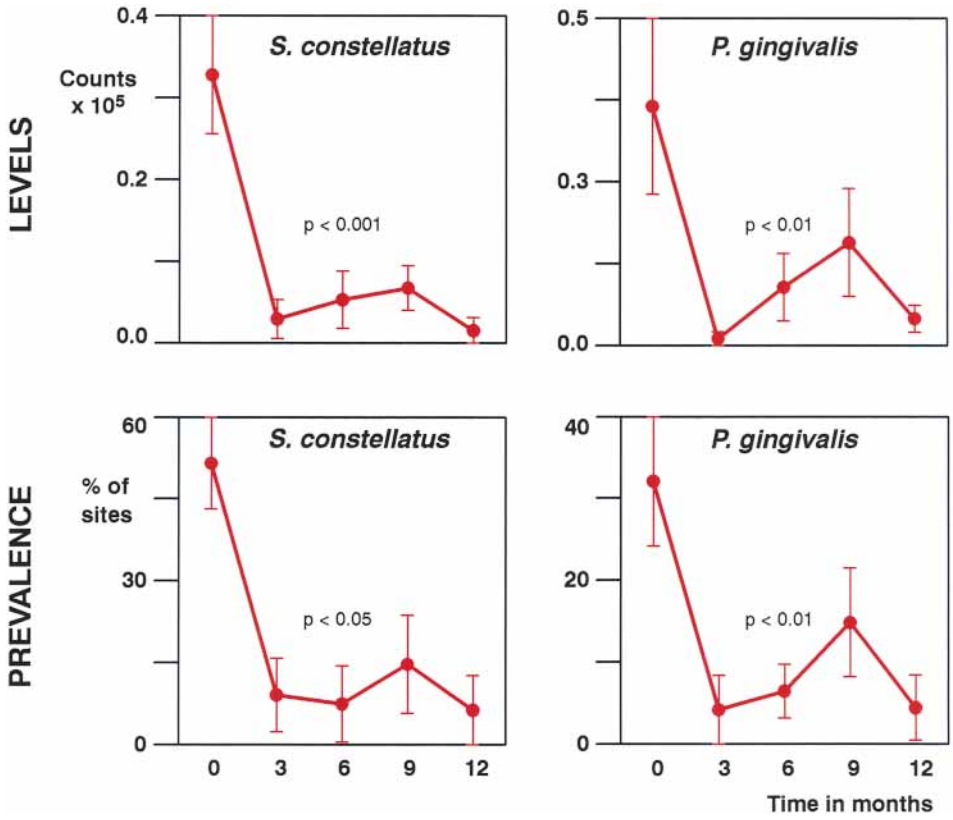

Fig. 26. Mean counts and prevalence of *P. gingivalis* and *S. constellatus* in refractory subjects at baseline, 3, 6, 9 and 12 months. For the upper panels, the counts were averaged for each species within a subject at each time point and then averaged across subjects for each time point separately. For the lower panels, the percentage of sites colonized in each subject at each time point was determined for each species and averaged across subjects at the different time points. The circles represent the means and the whiskers the SEM. Significance of differences over time was tested using the Quade test.

such therapies, and it is likely that combined therapies will prove effective for the control of periodontal infections in such individuals. Ideally, such therapies should provide different modalities of antimicrobial or host-affecting procedures.

Concluding thoughts

This chapter has attempted to provide a description of the nature of biofilms in general and oral biofilms in particular as well as approaches to their control. A picture of biofilms has emerged of a complex structure consisting of pure or mixed microcolonies surrounded by a glycocalyx primarily composed of exopolysaccharides produced by the resident bacteria. Recent technological advances have demonstrated the presence of water channels within biofilms that permit the passage of nutrients and waste products to and from the overlying bathing fluid. Apparently, cell-to-cell communication and transfer of genetic information is common within biofilms, permitting them to change in response to their environment. The biofilm structure provides a defense against host protective mechanisms as well as against antimicrobial agents. Organisms growing within biofilms often differ physiologically from those growing in a planktonic state, and physiological activity differs markedly from one site in a biofilm to another. The biofilm is an effective survival structure that protects the resident organisms from exogenous, potentially harmful factors and permits cooperative interactions between cells of the same or different species. Essential to the strategy of survival is a phase in biofilm colonization in which organisms are released from the biofilm to colonize other sites in the same individual or other individuals. The subgingival plaque may be unique in that there may be two biofilms in apposition to each other: one attached to the tooth surface and the other attached to the epithelial cells lining the periodontal pocket/gingival sulcus. These biofilms may

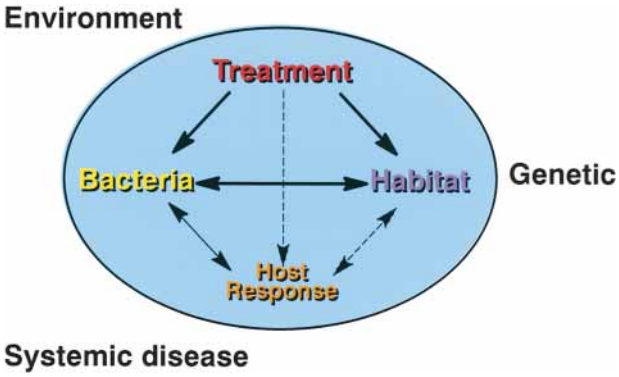

Fig. 27. Diagrammatic representation of the effect of therapy on colonizing bacteria, the host and the habitat. Treatment can affect the composition of the bacterial plaque directly, can affect the host response or alter the habitat. Alterations of any of these factors can impact on the remaining factors in this triad. As indicated, treatment effects are influenced by the genetic background of the subject, environmental influences such as smoking and the systemic well-being of the patient.

be bridged by a zone of loosely attached or unattached species. The cells attached to the tooth and to the epithelial cell surface probably differ in physiological state and certainly differ in the proportion of different bacterial species.

Many different biofilms exist in nature; some are useful (to the human), and others are associated with potentially harmful effects. Dental plaque is a naturally occurring biofilm that has the potential to cause disease. Dental plaques have many properties in common with biofilms found in other locations. However, they have certain characteristics that are important in terms of control of disease. They are easily accessible and thus allow direct removal and application of antimicrobial agents. However, they are microbiologically very complex. This complexity helps the therapist in one way and presents problems in another. The complexity helps to assure the therapist that treatment will usually lead to the return of a relatively similar, diverse microbial plaque; hopefully with pathogenic species reduced or eliminated. If treatment virtually eliminated all or most species, the potential for colonization by even more harmful organisms would be very high. On the other hand, the complexity can present difficulties for the therapist. The first is knowing which of several potential pathogens in an individual is causing that individual's disease. The second is that the network provided by the community structure may help to "rescue" a suppressed species by providing the essential factors needed for rapid recolonization. Nevertheless, as indicated in this chapter and as described in greater detail in other chapters in this volume, dental biofilms can be altered by various therapies providing a beneficial outcome to the patient. Treatment can affect bacteria directly by physical removal and/or chemotherapeutic agents (Fig. 27). Treatment can also affect the habitat, for example, by eliminating or by meticulously removing supragingival plaque. As discussed earlier, the bacteria affect their habitat and the habitat affects the bacteria so that the elimination of pockets or the removal of supragingival plaque will provide a less favorable environment for the growth of subgingival species, particularly those associated with disease. Treatment can also affect host response, possibly by "vaccination" during mechanical debridement procedures or using anti-inflammatory or host-modifying local or systemic agents. Modification of host response affects the habitat and also affects the colonizing microbiota. Thus, the therapist can potentially affect periodontal infections at several levels improving the possibility for long-term periodontal stability. For certain periodontal patients, combinations of therapy may be required in order to control the infection via different antimicrobial strategies.

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