CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

# The Role of Bacteria in the Caries Process: Ecological Perspectives

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## ABSTRACT

Dental biofilms produce acids from carbohydrates that result in caries. According to the extended caries ecological hypothesis, the caries process consists of 3 reversible stages. The microflora on clinically sound enamel surfaces contains mainly non-mutans streptococci and *Actinomyces*, in which acidification is mild and infrequent. This is compatible with equilibrium of the demineralization/remineralization balance or shifts the mineral balance toward net mineral gain (dynamic stability stage). When sugar is supplied frequently, acidification becomes moderate and frequent. This may enhance the acidogenicity and acidurance of the non-mutans bacteria adaptively. In addition, more aciduric strains, such as 'low-pH' non-mutans streptococci, may increase selectively. These microbial acid-induced adaptation and selection processes may, over time, shift the demineralization/remineralization balance toward net mineral loss, leading to initiation/progression of dental caries (acidogenic stage). Under severe and prolonged acidic conditions, more aciduric bacteria become dominant through acid-induced selection by temporary acid-impairment and acid-inhibition of growth (aciduric stage). At this stage, mutans streptococci and lactobacilli as well as aciduric strains of non-mutans streptococci, *Actinomyces,* bifidobacteria, and yeasts may become dominant. Many acidogenic and aciduric bacteria are involved in caries. Environmental acidification is the main determinant of the phenotypic and genotypic changes that occur in the microflora during caries.

**KEY WORDS:** acidogenicity, acidurance, acid-induced adaptation, acid-induced selection, *Actinomyces*, *Bifidobacterium*, caries-associated bacteria, caries process, extended caries ecological hypothesis, *Lactobacillus*, mutans streptococci, non-mutans streptococci.

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## INTRODUCTION

The supragingival dental biofilm constitutes an ecosystem of bacteria that exhibits a variety of physiological characteristics. In particular, the acid production resulting from carbohydrate metabolism by these bacteria and the subsequent decrease in environmental pH are responsible for the demineralization of tooth surfaces (Marsh and Nyvad, 2008). However, other physiological traits of the biofilm bacteria, such as base formation, may partly dampen the demineralization processes. Therefore, Kleinberg (2002) suggested that it is the proportions and numbers of acid-base-producing bacteria that are the core of dental caries activity.

Much research has identified mutans streptococci (MS) as the major pathogens of dental caries. This is because, first, MS are frequently isolated from cavitated caries lesions; second, MS induce caries formation in animals fed a sucrose-rich diet; third, MS are highly acidogenic and aciduric (Hamada and Slade, 1980; Loesche, 1986); and fourth, MS are able to produce surface antigens I/II and water-insoluble glucan, which promote bacterial adhesion to the tooth surface and to other bacteria (Hamada and Slade, 1980). A systematic literature review by Tanzer *et al.* (2001) confirms a central role for the MS in the initiation of dental caries on enamel and root surfaces.

However, several well-designed studies have revealed that the level of MS is not necessarily high in caries-associated biofilms, especially the microflora associated with non-cavitated stages of lesion formation (van Houte *et al.,* 1991a; Sansone *et al.,* 1993). Instead, it is proposed that non-mutans acidogenic and aciduric bacteria, including non-mutans streptococci and *Actinomyces* (Sansone *et al.,* 1993; van Houte, 1994; van Houte *et al.,* 1996), are more closely involved with the initiation of caries. In addition, van Ruyven *et al.* (2000) have detected non-mutans aciduric bacteria other than non-mutans streptococci and *Actinomyces* from dental biofilms covering white-spot lesions. They found that these bacteria consisted of various species, including lactobacilli and *Bifidobacterium.*

Given these circumstances, the authors reconsidered the caries process from a microbiological, biochemical, ecological, and clinical perspective, and proposed an extension of the ecological plaque hypothesis (Takahashi and Nyvad, 2008) to explain the relation between dynamic changes in the phenotypic/genotypic properties of plaque bacteria and the demineralization/remineralization balance of the caries process (Fig. 1). In this hypothesis, dental plaque is a dynamic microbial ecosystem in which non-mutans bacteria (mainly non-mutans streptococci and *Actinomyces*) are the key players for maintaining dynamic stability, *i.e.,* a natural pH cycle (dynamic stability stage). Microbial acid-induced adaptation and subsequent acid-induced selection of 'low-pH' non-mutans bacteria play a critical role in destabilizing the homeostasis of the plaque by facilitating a shift of the demineralization/remineralization balance from 'net mineral gain' to 'net mineral loss' (acidogenic stage). Once the acidic environment has been established, MS and other

aciduric bacteria may increase and promote lesion development by sustaining an environment characterized by 'net mineral loss' (aciduric stage).

From the perspective of microbial ecology, dental diseases may be considered a model system of amphibiosis (Ruby and Goldner, 2007), a term invented by the microbial ecologist Theodore Rosebury about 50 years ago (Rosebury, 1962). Amphibiosis is the dynamic adaptation that occurs in response to changing environmental conditions between two dissimilar organisms living together. Under 'normal' conditions, micro-organisms in the oral cavity live in a symbiotic relationship with the host, characterized by mutualism (beneficial to both). However, the nature of a particular symbiosis may shift under changing conditions in a reciprocal manner, with mutualism becoming parasitism (beneficial to one and detrimental to the other) and *vice versa* (Stanier *et al.,* 1970). This dynamic adaptation is the basic principle of endogenous disease processes, including dental caries, and is congruent with the ecological caries hypothesis (Marsh, 1994; Takahashi and Nyvad, 2008). In the present article, the authors will focus on recent microbiological findings about caries-associated bacteria, and re-assess the role of these bacteria in the caries process from an ecological perspective.

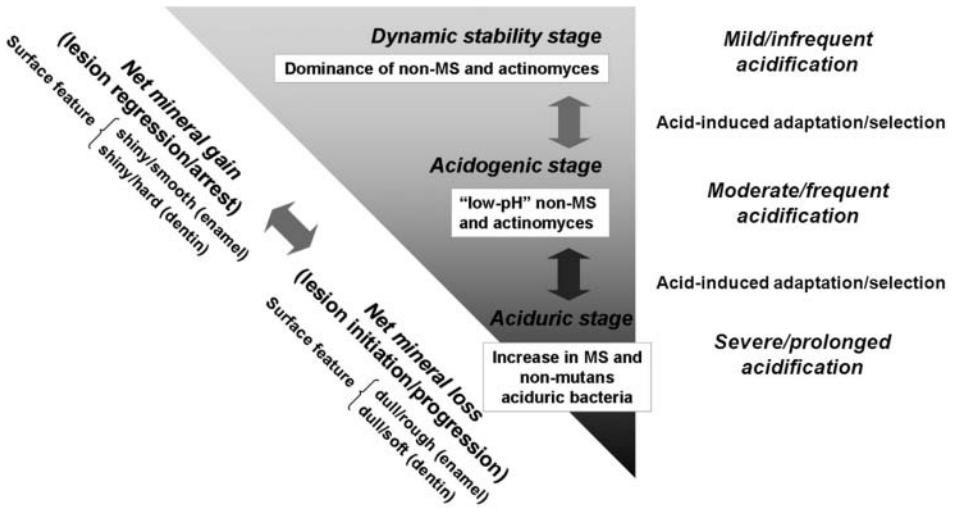


**Figure 1.** The caries process according to an extended caries ecological hypothesis (modified from Takahashi and Nyvad, 2008).

## BACTERIAL MEMBERS IN THE CARIES PROCESS: MICROFLORA OF DENTAL PLAQUE ON CLINICALLY SOUND ENAMEL SURFACES, WHITE-SPOT LESIONS, AND CAVITATED DENTIN LESIONS

Studies have shown that the initial colonizers of newly cleaned tooth surfaces constitute a highly selected part of the oral microflora, mainly *S. sanguinis, S. oralis,* and *S. mitis* 1 (Nyvad and Kilian, 1987), but other genera, such as *Actinomyces,* are also present (Li *et al.,* 2004; Dige *et al.*, 2009). Surprisingly, MS comprise only 2% or less of the initial streptococcal population, regardless of the caries activity of the individual (Nyvad and Kilian, 1990). These observations emphasize that the vast majority of the early colonizers on teeth belong to the 'mitis group'. These bacteria, as well as other viridans group streptococci, are often referred to as the 'non-mutans streptococci', which are genetically distinct from the MS that belong to the 'mutans group' (Kawamura *et al.,* 1995). As the microflora ages, the composition shifts from *Streptococcus*-dominant to *Actinomyces*-dominant (Syed and Loesche, 1978; van Palenstein Helderman, 1981). The predominant genera in mature smooth-surface plaque therefore belong to *Actinomyces* and *Streptococcus,* most of which are non-mutans streptococci (Ximénez-Fyvie *et al.,* 2000). MS are present in very low numbers (Bowden *et al.,* 1975).

The proportion of MS in plaque covering white-spot enamel lesions is often higher than that at clinically healthy sites (van Houte *et al.,* 1991b). Yet, non-mutans streptococci still remain the major bacterial group in white spots (Sansone *et al.,* 1993; van Houte *et al.,* 1996). In fact, it has been shown that, in the absence of MS and lactobacilli, the dissolution of enamel can be produced by members of the early microflora, exclusively (Boyar *et al.,* 1989).

In cavitated lesions in dentin, including rampant caries, MS constitute about 30% of the total flora (Loesche *et al.,* 1984; Milnes and Bowden, 1985; Boue *et al.,* 1987), suggesting that MS are associated with progressive stages of caries. By contrast, MS are encountered less frequently at the advancing front of dentin caries, where lactobacilli, Prevotellae, and *Bifidobacterium* are more prevalent (Edwardsson, 1974; Becker *et al.,* 2002; Martin *et al.,* 2002; Munson *et al.,* 2004; Chhour *et al.,* 2005; Aas *et al.,* 2008; Mantzourani *et al.,* 2009a).

All these findings clearly show that the microflora on the tooth surface changes with caries lesion development, from dominance of non-mutans streptococci and *Actinomyces* to dominance of MS and other non-mutans bacteria, including lactobacilli and *Bifidobacterium.* Recent molecular identification methods have also revealed that the microflora of clinically sound and carious surfaces is much more diverse and comprises hundreds of predominant species, of which 50-60% are not cultivable (Aas *et al.,* 2005, 2008). Nevertheless, these studies suggest, again, that bacterial species other than *S. mutans, e.g.*, *Lactobacillus, Bifidobacterium, Propionibacterium,* non-mutans streptococci, and *Actinomyces,* likely play important roles in the caries process.

**Table.** Acidogenicity of Representative Caries-associated Bacteria

| Bacteria | Final pH | Reference |
|---|---|---|
| Non-mutans streptococci | 4.2-5.2 [a] | 1 |
| *Actinomyces* | 4.3-5.7 [a] | 2 |
| Mutans streptococci | 4.0-4.4 [a] | 1 |
| Lactobacillus | 3.6-4.0 [a] | 1 |
| *Bifidobacterium* | 3.9-4.0 [b] | 3 |

[a]Final pH when grown in batch culture containing glucose.
[b]Final pH when incubated in glucose solution.
1 Holt, 1984.
2 Johnson *et al.,* 1990.
3 Haukioja *et al.,* 2008.

## BACTERIAL METABOLIC PROPERTIES RELEVANT TO CARIES AND SURVIVAL IN THE ORAL CAVITY

Most bacteria in supragingival plaque metabolize various sugars and produce acids through a common glycolytic pathway, the Embden-Meyerhof-Parnas pathway. When sugar is supplied in excess, oral streptococci, including MS and non-mutans streptococci (van Houte *et al.,* 1970; Hamilton, 1976; Takahashi *et al.,* 1991) and *Actinomyces* (Hamilton and Ellwood, 1983; Komiyama *et al.,* 1988), can store the extra sugars as intracellular polysaccharides (IPS), and they can utilize the IPS as an energy source to produce acids when sugar is limited, as occurs between meals. The final pH values of non-mutans streptococci, *Actinomyces,* MS, lactobacilli, and *Bifidobacterium,* when grown or incubated with glucose, are shown in the Table (Holt, 1984; Johnson *et al.,* 1990; Haukioja *et al.,* 2008). In general, on the basis of final pH values, MS, lactobacilli, and *Bifidobacterium* are more acidogenic and aciduric than non-mutans streptococci and *Actinomyces.* It should be realized, however, that the final pH values of non-mutans streptococci and *Actinomyces* can be lower than pH 5.5, the 'critical' pH for the demineralization of enamel.

In addition, non-mutans streptococci and *Actinomyces* have a variety of extracellular glycosidases (Schaal, 1984; Beighton and Whiley, 1990; Whiley and Beighton, 1998; Paddick *et al.,* 2005) that can liberate sugars and amino-sugars from glycoproteins such as the mucin contained in saliva. Studies have identified sialidases in many species, including *Streptococcus oralis, Streptococcus mitis,* and *Actinomyces naeslundii* (Beighton and Whiley, 1990; Bradshaw *et al.,* 1994). *S. oralis* also expresses *N*-acetyl-β-D-glucosaminidase and β-D-galactosidase, in addition to α-1-fucosidase and mannosidase activity (Byers *et al.,* 1999). Furthermore, mannosidase production has been identified within the viridans group streptococci (Homer *et al.,* 2001), and all non-mutans streptococci grow on amino-sugars (Byers *et al.,* 1996; Whiley and Beighton, 1998). This is an advantage for non-mutans streptococci and *Actinomyces* to survive in the oral cavity, where salivary glycoproteins are always available. However, most MS and lactobacilli do not have these metabolic features, except that fucosidase activity has been shown in *Lactobacillus rhamnosus* (Bradshaw *et al.,* 1994). Furthermore, most non-mutans streptococci can utilize arginine/arginine-containing peptides available in saliva through the arginine deiminase system, which degrades the arginine molecule to ammonia and carbon dioxide with production of ATP. Overall, this metabolic pathway produces alkali and neutralizes the intracellular and the environmental pH (Burne and Marquis, 2000). The arginine deiminase system is helpful for non-mutans streptococci not only to utilize arginine as an energy source, but also to survive under the acidic conditions in the oral cavity.

The *Actinomyces* have a unique glycolytic system (Takahashi *et al.,* 1995), in which they utilize high-energy polyphosphate and pyrophosphate compounds for synthesis of hexokinase and phosphofructokinase, respectively, acting as phosphoryl donors instead of ATP. This means that the *Actinomyces* are able to exploit a surplus ATP to synthesize polyphosphate as an energy reservoir, and salvage energy from pyrophosphate, a high-energy phosphoryl-bond-containing by-product from the metabolism of polymers such as nucleic acids and glycogens. In addition, the *Actinomyces* are often ureolytic (Kleinberg, 2002; Liu *et al.,* 2006) and can utilize lactic acid as a carbon source for growth (Takahashi and Yamada, 1996). These diverse physiological characteristics of *Actinomyces* seem to be advantageous to survival and domination in supragingival plaque (Takahashi and Yamada, 1999b).

## THE ROLE OF CARIES-RELATED BACTERIA IN THE CARIES PROCESS ACCORDING TO THE EXTENDED CARIES ECOLOGICAL HYPOTHESIS

### Dynamic Stability Stage

Many micro-organisms in dental plaque formed on clinically sound surfaces can produce acids from sugary foods, and the acids can demineralize the dental hard tissues. However, if the acidification episodes are mild and infrequent, homeostatic mechanisms in the plaque (Marsh and Martin, 1999) may easily restore the mineral balance toward net mineral gain in favor of 'remineralization' (Manji *et al.,* 1991). This dynamic environment brings the microflora to a stable stage, with dominance of non-mutans streptococci and *Actinomyces* (dynamic stability stage, Fig. 1). A chemostat study with 9 representative oral bacterial strains (Bradshaw and Marsh, 1998) revealed that 10 times the daily glucose supply (glucose pulse) at pH 7.0 established a stable microbial composition characterized by dominance of non-mutans streptococci and *Actinomyces,* which resembles that of oral biofilms on clinically sound enamel surfaces (Fig. 2A). In the chemostat, the growth medium contained a relatively high level of hog gastric mucin and a limited level of glucose, as in the oral cavity between meals, and a glucose pulse gave a temporary increase of glucose in the environment, which mimics mealtimes. In a person with healthy eating habits, sugar is always limited in the oral cavity (Carlsson, 1997), except for regular mealtimes, resulting in mild and low frequencies of acidification (dynamic stability stage, Fig. 1). Both non-mutans streptococci and *Actinomyces* have an ability to utilize glycoproteins and amino acids supplied continuously in saliva, along with dietary sugars provided at infrequent meals, supporting their co-existence with other bacteria in a nutritionally fluctuating environment.

## Acidogenic Stage

### Acid-induced Adaptation: Phenotypic Changes of Microflora

When sugar is supplied frequently or salivary secretion is too scarce to neutralize the acids produced, the pH decreases in the plaque become more severe and frequent. This change in the environment may enhance the acidogenicity and acidurance of the non-mutans bacteria adaptively. Takahashi and Yamada (1999a) have shown that when non-mutans streptococci, including *S. sanguinis, S. oralis, S. gordonii,* and *S. mitis,* were exposed to an acidic environment, they increased their acidogenicity. These bacteria were grown first at pH 7.0, and afterward at pH 5.5 for 0.5, 1, and 1.5 hrs, respectively (Fig. 3). The bacteria were then harvested, washed, and incubated with glucose, and the final pH values were measured as a marker of acidogenicity. Their acidogenicity expressed as final pH values varied (pH 4.04-4.33) without incubation at pH 5.5, but after incubation at pH 5.5 for 0.5, 1, and 1.5 hrs, all the bacteria increased their acidogenicity (pH 3.96-4.24 after 0.5 hr, pH 3.93-4.12 and pH 3.90-4.19 after 1.5 hrs, respectively). These bacteria were also able to increase their acidurance adaptively (Fig. 3). Bacteria initially grown at pH 7.0 were killed by acid stress in a strain-dependent manner following exposure to pH 4.0 for 1 hr (survival rate: 0.0009-71%), but after pre-acidification at pH 5.5 for 1 hr, all the bacteria increased their acidurance (survival rate: 0.4-81%). The biochemical mechanisms underlying the acid-induced adaptation are thought to involve the following mechanisms (Quivey *et al.,* 2000): (1) an increase in proton impermeability of the cell membrane; (2) induction of proton-translocating ATPase ($H^+$-ATPase) activity that expels proton from cells; (3) induction of the arginine deiminase system that produces alkali from arginine or arginine-containing peptides; and (4) induction of stress proteins that protect enzymes and nucleic acids from acid denaturation. In non-mutans streptococci, the increase in activities of $H^+$-ATPase and arginine deiminase and expression of stress proteins (homologues of heat-shock protein, Hsp60 and Hsp70) were observed following incubation at pH 5.5 (Takahashi and Yamada, 1999a).

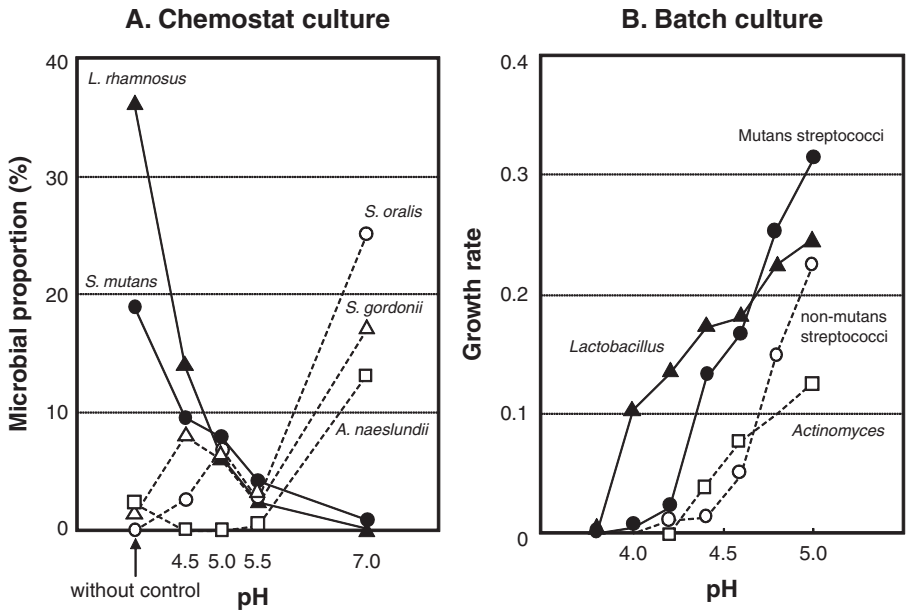


**Figure 2.** Growth ability at different pH values of representative oral bacteria. (**Panel A**) Results of chemostat study, modified from the data by Bradshaw and Marsh (1998). The culture pH was allowed to fall to either a preset value of 7.0-4.5 or without pH control. Five bacterial strains are shown in the panel, while the other 4 strains (*Neisserria subflava, Veillonella dispar, Prevotella nigrescens,* and *Fusobacterium nucleatum*) are omitted. (**Panel B**) Results of batch culture study, modified from the data by Horiuchi *et al.* (2009). Bacteria were grown at various pH values with pH control by the periodic addition of alkaline, and bacterial growth rates were calculated from the logarithmic growth phase. Data are given by means of 2 strains of MS, 2 strains of non-mutans streptococci, and 2 strains of *Actinomyces*.

### Acid-induced Selection of 'Low-pH' Non-mutans Bacteria: Genotypic Change of Microflora

Acidification of dental plaque microflora due to frequent sugar intake or poor salivary secretion can be a driving force to enhance the acidogenicity and acidurance of the non-mutans bacteria, resulting in establishment of a more acidic environment. Even if acid-induced adaptation occurs, non-mutans bacteria such as non-mutans streptococci and *Actinomyces* are still so heterogeneous with respect to acidurance (van Houte *et al.,* 1991b, 1996) that the population of more aciduric strains, *i.e.,* 'low-pH' non-mutans bacteria, will increase selectively in this environment. Microbial acid-induced adaptation (phenotypic change of the microflora) as well as acid-induced selection (genotypic change of the microflora) will cause a shift in the acidogenic potential of the microflora, which, provided the demineralization/remineralization balance is disturbed over an extended period of time, may lead to initiation/progression of dental caries (acidogenic stage, Fig. 1).

## Aciduric Stage

### Acid-induced Selection of Aciduric Bacteria by Temporary Acid-impairment

Although 'low-pH' non-mutans bacteria can increase their acidurance and acidogenicity, and take over the dominant position in supragingival plaque, MS and lactobacilli are more competitive under severely acidic conditions. Following a rapid exposure to pH 4.0, as often observed in mature dental plaque after a sugar exposure, non-mutans streptococci and *Actinomyces* partially lost their viability, while MS and lactobacilli were able to survive (Fig. 4A) (Horiuchi *et al.,* 2009). Furthermore, when non-mutans streptococci and *Actinomyces* initially treated at pH 4.0 in growth media were returned to pH 7.0, they started to

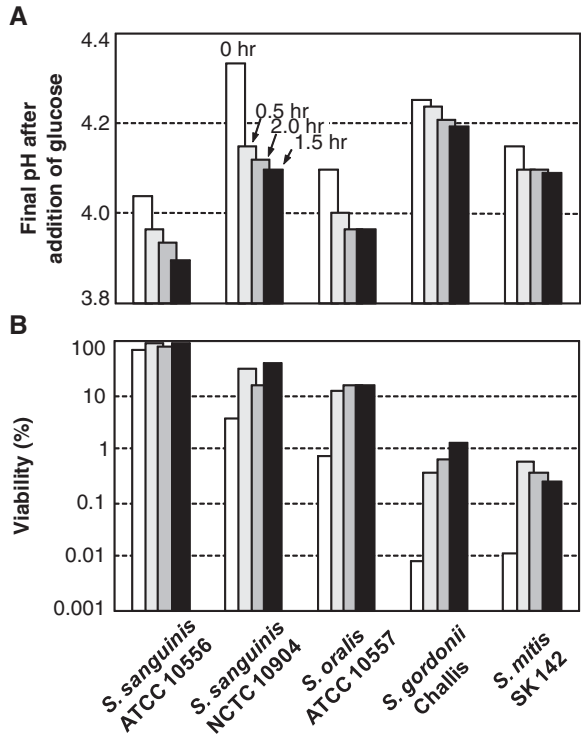


**Figure 3.** Acid-induced adaptation of non-mutans streptococci. The data were modified from Takahashi and Yamada (1999). **(Panel A)** Acidogenicity (final pH values) after acidification at pH 5.5. **(Panel B)** Acidurance (survival after 1 hr at pH 4.0) after acidification at pH 5.5.

grow again. However, the bacterial growth (actual growth curve) was much slower than that expected from the number of surviving bacterial cells (expected growth curve) (Fig. 4B) (Horiuchi *et al.,* 2009). Delay of the growth after acidification was common among non-mutans streptococci and *Actinomyces,* and ranged from 0.00 to 1.51 hrs after 0.5-hour acidification, from 1.54 to 2.44 hrs after one-hour acidification, and 2.41< hr after two-hour acidification. It should be noted that some non-mutans streptococci and *Actinomyces* strains did not start to grow by 10 hrs after a two-hour acidification, indicating that they required a considerable time for growth to start again, although the cultures contained a significant number of viable bacteria (Fig. 4A). No viability loss and delay were observed in MS and lactobacilli, clearly indicative of their high acidurance. These observations suggest that acidification impairs bacterial growth ability temporarily in a strain-dependent manner, and that the acid-impaired bacteria need a considerable time to recover their growth ability. Correspondingly, Takahashi *et al.* (1997) have shown that a transient acidification temporarily inactivated the glycolytic enzymes, which returned to original levels after the pH had returned to neutral, although the mechanisms have not yet been clarified.

Under these conditions, non-mutans streptococci and *Actinomyces,* probably except for some aciduric strains of non-mutans streptococci and *Actinomyces* (Nyvad and Kilian, 1990; Aas *et al.,* 2008), will be eliminated and replaced by more aciduric bacteria, such as MS and lactobacilli (aciduric stage, Fig. 1), leading to a pronounced net mineral loss and rapid lesion progression. Since *Bifidobacterium* is also acidogenic and aciduric, similar to lactobacilli and more so than MS (van Houte *et al.,* 1996; Haukioja *et al.,* 2008), as shown in the Table, they may also overcome the competition and increase their proportion of the microflora.

### Acid-induced Selection of Aciduric Bacteria by Prolonged Acidification

A chemostat experiment with 9 representative oral bacteria (Bradshaw and Marsh, 1998) showed that when the pH was allowed to fall to a preset value of 5.0, MS and lactobacilli became dominant, while non-MS and *Actinomyces* started to be excluded from the consortium (Fig. 2A). When pH was further allowed to fall to 4.5 and without control (final pH = 3.83), MS and lactobacilli increased dramatically. Similar results were obtained from a batch-culture experiment (Horiuchi *et al.,* 2009); at pH ≤ 5.0, MS and lactobacilli were able to grow faster than non-mutans streptococci and *Actinomyces* (Fig. 2B). At pH ≤ 4.6, lactobacilli grew faster than MS, consistent with the chemostat results that the proportion of lactobacilli became higher than that of MS at pH ≤ 4.5. Given these observations, it is suggested that prolonged acidic conditions around pH 5 may cause the emergence of MS and lactobacilli in the microbial flora, and that more severe acidic conditions around pH 4 may exclude the non-mutans streptococci and *Actinomyces.* In the oral cavity, prolonged acidic conditions (pH ≤ 5) can occur in carious cavities (Dirksen *et al.,* 1962; Hojo *et al.*, 1994), where clearance of acids is disturbed. This may be the reason MS and lactobacilli are frequently isolated from established carious cavities. It is noticeable that all caries lesions with pH < 5 were designated as 'active' lesions and contained lactic acid exclusively (Hojo *et al.,* 1994).

At the aciduric stage, acid-induced selection by acid-impairment and growth competition are the major reasons for the shift in the composition of the microflora. However, acid-induced adaptation may still occur in aciduric bacteria, such as MS and lactobacilli (Belli and Marquis, 1991; Ma *et al.,* 1997; Svensäter *et al.,* 1997), in which both the acidogenicity and acidurance are enhanced under severe and prolonged acidic conditions. The basic biochemical reactions in response to acid stress are therefore similar to those described above in the acidogenic stage.

## CLINICAL MICROBIOLOGICAL OBSERVATIONS IN SUPPORT OF THE EXTENDED ECOLOGICAL CARIES HYPOTHESIS

### Early Childhood Caries (ECC) and MS: Which Comes First, the MS or Poor Eating Habits/Low Socio-economic Status?

ECC refers to any dental caries in the primary dentition. ECC can destroy the primary dentition of toddlers and small children, and, if left untreated, it can lead to pain, acute infection, nutritional insufficiencies, and learning and speech problems (AAPD, 2008). In its less severe stage, ECC is characterized by smooth-surface lesions of the primary teeth and is called 'rampant caries' (Milnes, 1996).

In ECC lesions, the MS have been frequently isolated, and their proportion of the microflora was high (van Houte *et al.,* 1982; Milnes and Bowden, 1985). In addition, it has been reported that both the detection frequency of MS in saliva and the proportion of MS in plaque were associated with the severity

of ECC, suggesting that the MS are a major pathogen of ECC. A systematic review has confirmed that the presence of MS, in both plaque and saliva of young caries-free children, appears to be associated with a considerable increase in ECC risk (Thenisch *et al.,* 2006). These findings remind us of 'the mutans story', in which the MS were claimed to be the major pathogen in caries because of insoluble glucan formation and excessive acid production in response to a sucrose-containing diet.

However, recent studies have reported that eating habits and socio-economic status of children and their caregivers are good predictors of ECC (Nunn *et al.,* 2009). In addition, oral health promotion programs based on repeated preventive guidance initiated during the mother's pregnancy were successful in reducing the incidence of severe ECC in young children (Plutzer and Spencer, 2008). In this context, ECC also follows the steps of the extended ecological plaque hypothesis. Frequent acidification of plaque by poor eating habits, such as frequent intake of sugared beverages and snacks, increases the acidogenic/aciduric bacteria and subsequently leads to dominance of the MS, with progression of caries lesions. Likewise, the detection of lactobacilli and *Bifidobacterium* in ECC lesions (Becker *et al.,* 2002; Aas *et al.,* 2008) is in accord with the extended ecological hypothesis, since both bacterial genera are aciduric enough to colonize and proliferate in acidic caries lesions (Nakajo *et al*., in press).

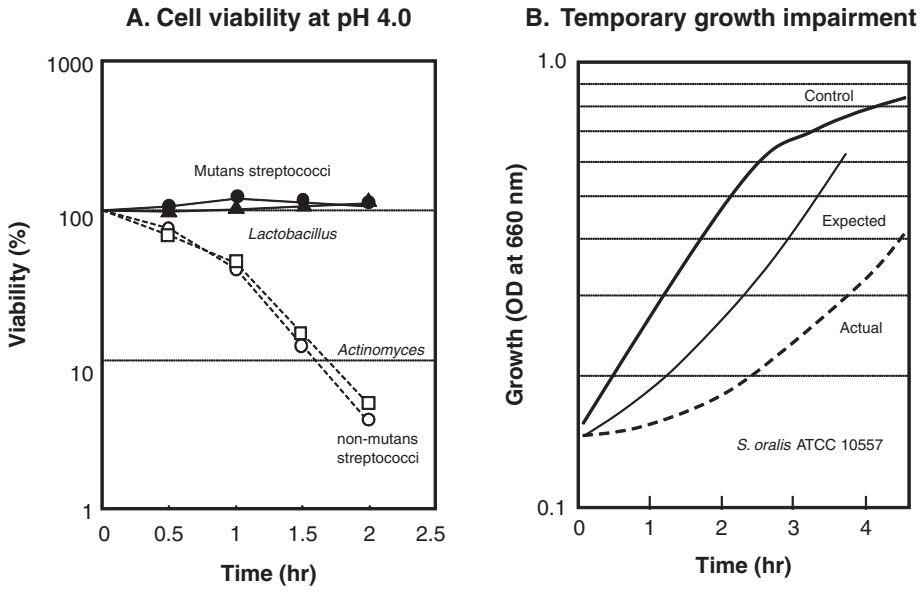


**Figure 4.** Effect of severe acidification on representative oral bacteria. The data were modified from Horiuchi *et al.* (2009). **(Panel A)** Cell viability at pH 4.0. The bacterial cells grown at pH 7.0 were exposed to pH 4.0 for 0, 0.5, 1, 1.5, and 2 hrs in buffer solution. The treated bacterial cells were plated on blood agar and counted for colony-forming units after anaerobic incubation. **(Panel B)** Temporary growth impairment at pH 4.0. Bacteria (*Streptococcus oralis* ATCC 10557) grown at pH 7.0 were exposed to pH 4.0 for 1 hr in growth medium, and then incubated in growth medium at pH 7.0. Actual = Actual growth curve determined by measurement of optical density of culture medium. Expected = Expected growth curve calculated from surviving cell numbers after the exposure at pH 4.0 for 1 hr. Control = growth without acid-exposure.

## Ecology of the Microflora in Patients with a Dry Mouth

Acidification of the biofilm could also happen because of hyposalivation, which reduces clearance of sugars and acids after carbohydrate consumption. Therefore, patients with a dry mouth run a higher risk of caries, particularly if their oral hygiene practices are poor. Hyposalivation can be caused by head and neck irradiation for the treatment of cancer, autoimmune diseases such as Sjögren's syndrome, hormonal disorders, neurological disorders, or psychogenic illness, but the most common reason for decreased salivation is medication. Radiation treatment for head and neck cancer produces a particularly aggressive form of dry mouth. In patients with permanent hyposalivation due to radiation treatment, acidification of plaque after a sugar challenge was significantly higher and more prolonged than in control patients (Eliasson *et al.,* 2006). These patients were also colonized by higher numbers of lactobacilli, MS, and *Candida* species in approximal plaque, suggesting that the acidic environment created by severe hyposalivation can be attributed to the propagation of aciduric bacteria. *Candida* species are known to be acidogenic and aciduric (Samaranayake *et al.,* 1986; Klinke *et al.,* 2009), but it could not be excluded that acquired suppression of immune defense mechanisms as a result of cancer therapy may partly explain the emergence of these species (Budtz-Jörgensen, 1990).

Longitudinal analyses of the microflora in patients receiving radiation treatment (Brown *et al.,* 1976) have demonstrated a rapid increase in the proportion of MS parallel with the onset of rampant caries. Increases of *Lactobacillus* species were observed to lag behind those of the MS, suggesting that the acidic environment created by hyposalivation severely destabilized the homeostasis of the microflora. In this case, the MS may be associated with the onset of caries, while lactobacilli are opportunists favored by the environmental change created by lesion initiation, because lactobacilli are more aciduric than MS (Fig. 2).

Interestingly, Brown and co-workers (Brown *et al.,* 1976) showed that deletion of dietary sucrose in the irradiated patients suppressed the emergence of MS and lactobacilli, and the levels of these bacteria remained considerably lower than in irradiated patients on an unrestricted diet. These longitudinal observations are congruent with the reciprocal adaptive microbial changes described in the extended caries ecological hypothesis.

### Root-surface Caries

Root surface caries was, for a long time, thought to be induced specifically by *Actinomyces* (Jordan and Hammond, 1972; Sumney and Jordan, 1974). This idea was probably ascribed to the sampling technique combined with the selective culturing techniques applied in these studies, Thus, it is to be expected that samples of softened carious dentin have a higher content of Gram-positive pleomorphic rods compared with samples containing superficial layers of plaque, because of the selective invasion of *Actinomyces*-like bacteria into demineralized root tissue (Nyvad and Fejerskov, 1990). Recent molecular studies have confirmed the abundance of *Actinomyces* in carious root dentin (Preza *et al.,* 2009).

*S. mutans* was detected in only half of the root caries lesions (Preza *et al.,* 2009). Furthermore, as with enamel caries, MS may comprise only a small proportion of the microflora of root-surface caries lesions. van Houte *et al.* (1996) reported that non-MS and *Actinomyces* spp. were dominant in dental plaque covering root-surface caries and that the isolated *Actinomyces* strains were heterogenous with respect to acidogenicity: Strains isolated from root-surface caries were more acidogenic than those from clinically sound root surfaces. Brailsford *et al.* (2001) observed a similar phenomenon in individuals with root-surface caries. These authors found that aciduric bacteria able to grow at pH 4.8 comprised 21.6% of the total microflora in root-surface caries lesions (lactobacilli and *Actinomyces* were dominant), whereas aciduric bacteria comprised 10.7% in clinically sound root surfaces (*Actinomyces*-dominant). However, in individuals without root-surface caries, aciduric bacteria comprised only 1.4% of total microflora in clinically sound root surfaces. These findings point to an association between acidogenic/aciduric *Actinomyces, i.e*., 'low-pH' *Actinomyces,* and root-surface caries.

Recently, Mantzourani *et al.* (2009b) demonstrated that the family *Bifidobacteriaceae,* including *Bifidobacterium, Scardovia,* and *Parascardovia,* was associated with cavitated root caries lesions, together with MS, lactobacilli, and yeasts (*Actinomyces* were not examined), indicating that the acidic environment of the lesions provided a suitable habitat for the proliferation of these aciduric micro-organisms. Collectively, the information obtained so far supports the contention (Bowden, 1990; Nyvad, 1993) that the ecological succession of the microflora in root-surface caries follows the same pattern as that observed for cavitated dentin caries.

## CONCLUSIONS AND FUTURE DIRECTIONS FOR RESEARCH

Our review of the literature supports the concept that dental caries is an endogenous disease, which is caused by a change from mutualistic symbiosis to parasitic symbiosis in the microbial ecosystem, *i.e.,* a microbial shift from dynamic stability *via* acid-induced adaptation and selection to an aciduric stage, according to the extended ecological plaque hypothesis. In this hypothesis, the entire consortium of acidogenic/aciduric bacteria, not only the MS, contributes to the caries process—a view that is compatible with the mixed-bacteria ecological approach proposed by Kleinberg (2002).

Acid production is the direct causative factor in the demineralization of tooth surfaces, but acid production is also an environmental determinant that influences both the phenotypic and genotypic properties of the oral microflora through acid-induced adaptation and selection. It is important to appreciate, however, that the enrichment of acidogenic/aciduric bacteria is a result of microbial acid formation during the caries processes—not the causative factor *per se*—and thus the removal of specific aciduric bacterial species such as the MS, through vaccination, gene therapy, or antimicrobial treatment, may not be an effective approach for long-term caries control. Rather, environmental control of the microflora should be achieved by avoiding acidification of the dental biofilm. Practical solutions to this strategy may include mechanical plaque control, reduction/substitution of the intake of sugary foods, and/or application of pH-neutralizing techniques such as saliva stimulation.

The caries process usually progresses rather slowly because of alternating de- and remineralization episodes in the biofilm. However, if the local environment changes—for example, in response to frequent sugar intake or low salivary secretion combined with insufficient oral hygiene—the equilibrium between the de- and remineralization episodes may favor a net mineral loss. These processes may lead to rampant caries, of which ECC and radiation caries are classic examples. Even so, it is salutary to know that the caries processes can be reversed, depending on the local environmental conditions. Therefore, it is important to learn how we can stimulate a mutualistic microflora to sustain clinically healthy conditions. Future microbiological studies of caries should therefore focus on a better understanding of the physiological mechanisms that serve to maintain the dynamic stability in dental biofilms. In this context, because of their association with mildly acidogenic environments, the non-mutans streptococci and *Actinomyces* may be interesting candidates for further analysis of their acid-base metabolic processes (Burne and Marquis, 2000; Kleinberg, 2002).

In recent years, investigators have advanced molecular identification methods in the attempt to resolve the microbiological foundation of caries. Hence, several elegant molecular studies have tried to elucidate the microbiological differences between clinically healthy and carious conditions (Becker *et al.,* 2002; Corby *et al.,* 2005; Aas *et al.,* 2008). While these studies have concluded that the microflora involved in caries is much more complex than hitherto anticipated, the analyses have not always shown a clear-cut pattern between health and disease. In addition to inter-individual differences (Aas *et al.,* 2005; Preza *et al.,* 2009), this may partly be because of methodological shortcomings. In some studies, the bacteria were pooled from several surfaces/lesions in the same person. Because of intra-oral environmental variability (Kleinberg and Jenkins, 1964; Fejerskov *et al.,* 1994; Haffajee *et al.,* 2009), pooled plaque samples cannot be expected to give a clear picture of the microbiome at a site. Another shortcoming may relate to the fact that refined gene technology does not match the crude style of lesion classification commonly used. Most studies of caries microbiology do not take into account novel knowledge about the dynamic metabolic processes in caries, and as a result, lesion activity is seldom defined. There is evidence that site-specific sampling of

well-defined surfaces and lesions may reveal microbial patterns of particular ecological interest. It has thus been reported that the overall composition of the microflora may change from a state of diversity to a state dominated by smaller numbers of aciduric species with increasing lesion activity of root caries (Nyvad and Kilian, 1990; Preza *et al.,* 2009). Therefore, for verification of the extended ecological plaque hypothesis, including the role of the non-mutans bacteria in the caries process, future molecular studies need to apply a refined caries lesion classification (Nyvad *et al.,* 1999) that has been validated for lesion activity (Nyvad *et al.,* 2003).

In view of our current philosophy, stressing the importance of environmental acidification, we propose that, to fully understand the ecological processes in caries, prospective molecular studies should involve not only bacterial identification and quantification, but also metabolic characterization of, *e.g.,* lesion pH *in vivo* (Fejerskov *et al.,* 1992), and/or the use of more sophisticated methodologies, such as metabolome analysis (metabolomics) and metagenome analysis (metagenomics), for reconstruction of metabolic networks in the microflora. Metabolomics can monitor a remarkable spectrum of metabolites in the microflora with the combination of chromatographic/electrophoretic separation and mass spectrometry-aided identification of metabolites (Takahashi *et al.,* in press), while metagenomics can identify metabolically functional genes, such as genes coding metabolic enzymes, comprehensively (Tringe *et al.,* 2005; Gianoulis *et al.,* 2009). We believe that such comprehensive approaches to analyzing the composition and function of well-defined microbial communities may offer new insight into the microbial ecosystem in caries, although these methodologies cannot give information about the spatial organization of the predominant genera and species (Dige *et al.,* 2009; Zijnge *et al.,* 2010).

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