α-amylase inhibitors such as acarbose and maltotriitol are capable of moderating starch cariogenicity in humans.

The acarbose inhibition in *S. sanguinis* was weaker than for the other streptococci. This could possibly be attributed to the starch-degrading activity of *S. sanguinis* (fig. 1), which might tolerate acarbose. Meanwhile, xylitol inhibited the starch metabolism by the mutans streptococci through a direct inhibition of bacterial glycolysis. It has been reported that xylitol is incorporated into mutans streptococci as xylitol 5-phosphate and inhibits several intracellular glycolytic enzymes including glucose phosphate isomerase and phosphofructokinase, which leads to inhibition of glycolysis [Trahan, 1995; Miyasawa, 2003]. Xylitol did not inhibit starch metabolism of the non-mutans streptococci, as xylitol has no effect on either the glycolysis of the non-mutans streptococci [Vadeboncoeur et al., 1983] or the activity of α-amylase as shown in the present study.

In conclusion, the present study clearly showed that cooked starch in the presence of salivary α-amylase was a potential source of acids that could be produced by oral streptococci. In addition, non-mutans streptococci as well as mutans streptococci can be significant acid producers from cooked starch since they have similar acidogenicity. The acidogenicity might be controlled by α-amylase inhibitors and xylitol.

Because of the adhesiveness of starchy foods, they are easily retained in the dentition [Lingström and Birkhed, 1993], and thus a continuous supply of carbohydrate derived from salivary starch digestion could help to maintain a low plaque pH for a long time [Lingström and Birkhed, 1993; Kashket et al., 1994]. To address the adhesiveness of starchy foods as well as the diffusion of starch and acidic end products within dental plaque, research using biofilm models could be valuable [Kashket et al., 1994; Duarte et al., 2008].

In modern society, there are many opportunities to consume starchy foods not only as staple foods, but also as snacks. Therefore, people with root surfaces exposed to the oral cavity, those with hyposalivation and those who frequently consume starchy foods such as snacks need to pay particular attention to the acidogenic potential of these cooked starch products.

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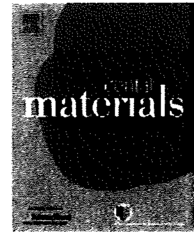

Fluoride released from glass-ionomer cement is responsible to inhibit the acid production of caries-related oral streptococci

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ABSTRACT

Objectives. Glass-ionomer cements (GICs) are known to have inhibitory effects on bacterial growth, but the biochemical mechanism of this property has not been fully understood. This study aimed to evaluate inhibitory effects of GIC on the acid production of caries-related oral streptococci, and to identify the components responsible for the inhibition.

Methods. An eluate was prepared by immersing set GIC in phosphate-buffered saline at 37 °C for 24 h. Fluoride and other elements in the eluate were quantified by fluoride ion electrode and atomic absorption photometry, respectively. *Streptococcus mutans* NCTC 10449 and *Streptococcus sanguinis* NCTC 10556 were used to evaluate the pH fall and the rate of acid production after the addition of glucose in the presence or absence of the eluate. Acidic end products from glucose were also assayed by carboxylic acid analyzer.

Results. The eluate contained silicon (1.24 ± 0.26 mM), fluoride (0.49 ± 0.02 mM) and aluminum (0.06 ± 0.00 mM), and inhibited the pH fall and the acid production rate of both streptococci at acidic pH, with a concomitant decrease in lactic acid production. These effects were comparable to those of a potassium fluoride solution containing the same concentration of fluoride as the eluate.

Significance. These results indicate that the GIC eluate inhibits the acid production of caries-related oral streptococci at acidic pH and that the effect is due to fluoride derived from the GIC. Thus, adjacent to GIC fillings, bacterial acid production and the subsequent bacterial growth may decrease, establishing a cariostatic environment.

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1. Introduction

Fluoride-releasing restorative materials, including conventional glass-ionomer cements (GICs), are frequently used for restorations of permanent or deciduous teeth for the patients with high caries-risk because of their biocompatibility and cariostatic properties.

The eluate of GICs has been found to have no effect on the viability of mouse odontoblast-like cells [1], a weak inhibitory effect on the protein synthesis of human gingival fibroblasts

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[2] and a weak damaging effect on the morphology of human gingival fibroblasts [3]. In addition, GICs are free of cytotoxic components such as 2-hydroxyethyl methacrylate (HEMA), usually contained in resin-modified glass-ionomer [4]. Thus, the available evidence indicates that GICs are less toxic to the cells compared with resin-based materials.

Furthermore, enamel and dentin surfaces adjacent to GIC fillings are protected from acid attack [5,6], indicating that GICs can inhibit acid-induced demineralization of teeth. Furthermore, the demineralized dentin layer adjacent to GIC fillings turns acid resistant with an increase in mineral content such as calcium, and becomes harder [7], suggesting that GICs can promote tooth remineralization [8]. It is considered that these cariostatic properties are due to the fluoride released from GICs; the fluoride can be incorporated in a hydroxyapatite crystal lattice and form fluoroapatite, which has a low acid-solubility [9].

GICs have also been found to have antibacterial effects. It was reported that the population of *Streptococcus mutans* on the surface of GIC fillings was lower than on composite fillings [10,11]. In addition, the pH fall after sucrose fermentation for 4 or 20 h by *S. mutans* cells layered on the surface of a GIC disc was smaller than that on the composite disc [12,13]. These results suggest that GICs inhibit the acid production of *S. mutans* and subsequently decrease the bacterial population. However, the identity and mechanism of this component of GICs is unclear. The observation that the pH of dental plaque decreases within a few minutes after sugar intake [14,15] suggests that the short-term measurement of pH is needed to evaluate the inhibitory effect on microbial acid production.

Therefore, the hypothesis tested was that GIC eluate has a short-term inhibitory effect on the pH fall and the acid production rate of caries-related oral streptococci, *S. mutans* and *Streptococcus sanguinis*, under anaerobic conditions mimicking those in a deep layer of dental plaque, where tooth demineralization occurs. An attempt was also made to identify the components of the GIC eluate that were responsible for these effects.

2. Materials and methods

2.1. *Preparation of GIC eluate and determination of composition*

The powder and liquid of GIC (Fuji IX, GC, Tokyo, Japan) were mixed at the ratio of 1:3.6 for 30 s, and placed into a cylindrical mold (height, 2 mm; diameter, 10 mm). The specimen was allowed to set for 30 min at 25 °C, and immersed in 5 mL of Dulbecco's phosphate-buffered saline containing 154 mM sodium ion, 4 mM potassium ion, 139 mM chloride ion, 10.75 mM hydrogen ion and 9.6 mM phosphate ion (D-PBS; Invitrogen Corp., Rockville, MD, USA) using multi-plates (6 wells). After storage at 37 °C for 24 h, the eluate was collected and the concentration of fluoride was measured using a fluoride ion electrode (F-53, Horiba, Ltd., Kyoto, Japan) after buffering the solution with total ionic strength adjustment buffer (TISAB; Orion Research Inc., Cambridge, MS, USA) at pH 5.3. The concentrations of silicon, aluminum, strontium, magnesium, and calcium in the eluate were determined using atomic absorption spectrometry (AA-6800F, Shimadzu Corp., Kyoto, Japan).

2.2. *Bacterial strains and growth conditions*

S. mutans NCTC 10449 and *S. sanguinis* ATCC 10556 were used. Basal culture medium contained 1.7% tryptone (Difco Laboratories, Detroit, MI, USA), 0.3% yeast extract (Difco Laboratories), and 0.5% NaCl. 0.5% glucose and 50 mM potassium phosphate buffer were added through a sterile membrane filter (pore size 0.22 μm, Millipore Corp., Bedford, Mass, NY, USA) to the autoclaved basal culture medium. This culture medium was kept in an anaerobic chamber (ANB-180L, Hirasawa Works, Tokyo, Japan: 80% N_2, 10% H_2 and 10% CO_2) for at least 3 days before use to remove oxygen. Each strain was pre-cultured in the medium at 37 °C overnight, transferred (5% inoculum size) to medium at pH 7.0 and incubated at 37 °C until the late-log growth phase (O.D. = 0.9–1.0 at 660 nm) in the anaerobic chamber. Bacterial cells were harvested and washed 3 times by centrifuging (21,000 × *g* for 7 min at 4 °C) in D-PBS. Double-sealed centrifuging tubes (Kubota Corp., Tokyo, Japan) were used to protect bacterial cells from air exposure. The cells were suspended in D-PBS in another anaerobic chamber (ANB-180L, Hirasawa Works: 90% N_2 and 10% H_2). The following experiments were performed in this chamber.

2.3. *pH fall by glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 10 at 660 nm), D-PBS and the GIC eluate or potassium fluoride (KF) solution. The final concentrations of fluoride in the reaction mixture ranged from 0 to 0.43 mM, which were equivalent to fluoride concentrations in the reaction mixtures for the GIC eluate. The maximum fluoride concentration in the reaction mixture for the GIC eluate containing 0.49 mM fluoride turned to 0.43 mM after mixing with bacterial cell suspension and glucose solution. The reaction mixture was adjusted to pH 7.0, and preincubated at 35 °C for 4 min with agitation by a magnetic stirrer. Then, 10 mM glucose was added to the mixture, and the pH fall was monitored for at least 30 min, using a pH electrode (GS-5015C, Toa Electronics, Tokyo, Japan).

2.4. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The reaction mixture contained the cell suspension (0.3 mL, O.D. = 3.5 at 660 nm), D-PBS and the GIC eluate or KF solution. The reaction mixture was maintained at pH 5.5 or 7.0 by titration with 60 mM KOH using pH stat (AUT-211S, Toa Electronics) with agitation by a magnetic stirrer, and preincubated at 35 °C for 4 min. The reaction was started by the addition of 10 mM glucose and the acid production was monitored for 10 min.

2.5. *Analysis of acidic end products*

Procedures for analysis of lactic, formic and acetic acids have been detailed previously [16]. At 10 min after the addition of glucose, the reaction mixture was mixed with 0.6N perchloric

Table 1 – Concentrations of elements in the GIC eluate.

Element	Mean concentration (S.D.)
Fluoride	0.49 (0.02) mM
Silicon	0.82 (0.26) mM
Aluminum	0.01 (0.02) mM
Strontium	<0.001 mM
Magnesium	<0.001 mM
Calcium	<0.001 mM

Data are given in mean (standard deviation) of 3 replicates.

acid. The resultant mixtures were brought out from the anaerobic chamber, filtered through a membrane (pore size 0.20 μm; polypropylene; Toyo Roshi Ltd., Tokyo, Japan) to remove cell debris, diluted with 0.2N HCl and assayed using a carboxylic acid analyzer (Eyela S-3000, Tokyo Rika Co. Ltd., Tokyo, Japan).

2.6. *Statistical analysis*

Statistical significance was assessed by the Dunnett test. A probability of $P < 0.05$ was considered to indicate significance.

3. Results

3.1. *Determination of composition in the eluate from GIC*

The eluate contained silicon, fluoride and aluminum with trace amounts of strontium, magnesium and calcium (Table 1).

3.2. *pH fall by glucose fermentation in the presence of the GIC eluate*

Serial pH falls by glucose fermentation of *S. mutans* are shown in Fig. 1. In all experiments using *S. mutans*, pH started to fall immediately after the addition of glucose. However, 10 min after the addition of glucose, the pH of reaction mixtures differed between the various concentrations of fluoride. At 30 min, in reaction mixtures containing eluate with a fluoride concentration of 0.43 mM or KF solution with a fluoride concentration of 0.43 mM, the pH stabilized at 4.93–5.00, which was significantly higher than the pH values of the control reaction mixture, the reaction mixture containing eluate with a fluoride concentration of 0.043 mM, and the reaction mixture containing KF solution with a fluoride concentration of 0.043 mM (Table 2). After 30 min, in the reaction mixture containing eluate with fluoride concentration of 0.043 mM or KF solution with a fluoride concentration of 0.043 mM, the pH continued to fall and approached the control gradually (date not shown). Similar results were obtained for *S. sanguinis* (Table 2).

3.3. *Acid production rate from glucose fermentation in the presence of the GIC eluate*

The rate of acid production of *S. mutans* at pH 5.5 was inhibited significantly by the GIC eluate in a dose-dependent manner, and decreased to about 10% of the control value at pH 5.5 in the presence of the eluate containing 0.43 mM fluoride (Table 3).

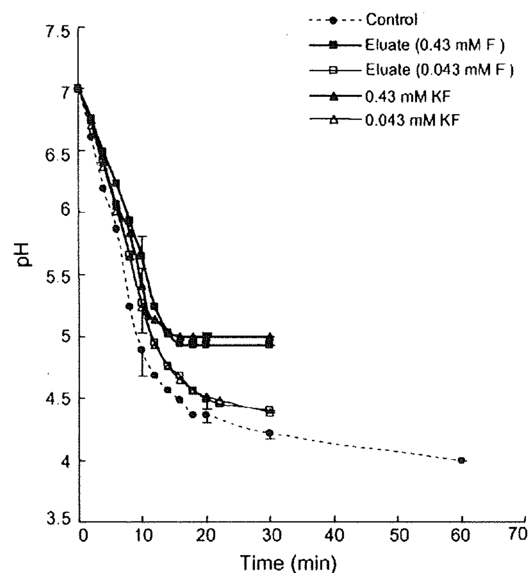

Fig. 1 – Effect of the GIC eluate or KF solution on the pH fall after addition of 10 mM glucose to the cell suspension of *S. mutans* NCTC 10449. The data are the means of 3 independent experiments. Vertical bars indicate standard deviations.

Only a weak inhibition was observed at pH 7.0. All KF solutions inhibited acid production to a degree similar to that of the eluate containing the same concentration of fluoride. There was no significant difference between the Eluate and KF solution containing the same concentration of fluoride. Similar results were obtained for *S. sanguinis* (Table 3).

3.4. *Analysis of acidic end products*

The profiles of acidic end products at pH 5.5 are shown in Fig. 2. As the concentration of fluoride in the eluate or KF solution increased, the proportion of lactic acid decreased, whereas the proportion of formic and acetic acids increased.

Table 2 – pH fall by *S. mutans* and *S. sanguinis* at 30 min after the addition of glucose.

In the presence of:	Mean pH (SD)	
	S. mutans	*S. sanguinis*
None (control)	4.21 (0.04)	4.34 (0.1)
Eluate (0.043 mM F^-)	4.35 (0.06)	4.44 (0.12)
Eluate (0.43 mM F^-)	4.93 (0.10)**, ##	4.79 (0.09)*
0.043 mM KF	4.38 (0.02)	4.46 (0.08)
0.43 mM KF	5.00 (0.03)**, #	4.98 (0.00)**, #

Data are given in mean (standard deviation) of 3 replicates. Significant difference (*$P < 0.05$, **$P < 0.01$) from the controls. Significant difference (#$P < 0.05$, ##$P < 0.01$) from the eluate (0.043 mM F^-) and KF solution (0.043 mM F^-).

Table 3 – Acid production rate by *S. mutans* and *S. sanguinis* for 10 min at pH 7.0 and 5.5.

pH	In the presence of	Percentage of acid production rate (S.D.)	
		S. mutans	*S. sanguinis*
7.0	None (control)	100	100
	Eluate		
	0.43 mM F⁻	93.4 (4.6)	93.8 (3.9)
	KF		
	0.43 mM	95.4 (4.6)	94.2 (6.2)
5.5	None (control)	61.3 (11.1)*	35.6 (5.4)*
	Eluate		
	0.043 mM F⁻	32.5 (5.2)*,**	32.5 (6.1)*
	0.086 mM F⁻	28.8 (1.5)*,**	33.4 (1.7)*
	0.215 mM F⁻	20.7 (2.6)*,**	15.6 (4.1)*,**
	0.43 mM F⁻	6.2 (1.3)*,**	5.4 (3.5)*,**
	KF		
	0.043 mM	31.6 (5.4)*,**	29.0 (8.2)*
	0.086 mM	28.4 (5.5)*,**	28.7 (4.5)*
	0.215 mM	14.4 (2.1)*,**	16.5 (2.2)*,**
	0.43 mM	6.5 (0.6)*,**	4.5(2.0)*,**

Data are given in mean (standard deviation) of 3 replicates. There was no significant difference between the Eluate and KF solution containing same concentration of fluoride.

* Significant difference ($P < 0.01$) from the control at pH 7.0.

** Significant difference ($P < 0.01$) from the controls at pH 5.5.

4. Discussion

In the present study, in addition to fluoride, silicon, aluminum, strontium, magnesium, and calcium were detected in the GIC eluate (Table 1). These elements have previously been reported to leach from GICs [17–19]. The concentrations of these elements are known to differ among GICs [18], probably due to differences in composition. In addition, their concentrations in eluates appear to vary according to eluting conditions. With Fuji IX (a filling cement used in the present study) and Fuji I (a luting cement similar in composition to Fuji IX), the proportion of silicon in the eluate is known to decrease under acidic conditions and increase under neutral pH conditions [17–19]. Consequently, in the present study, D-PBS at pH 7.3 was used for elution, and the proportion of Si in the eluate was relatively high.

The GIC eluate stopped the pH fall completely around pH 4.8–5.0 (Table 2 and Fig. 1) and markedly decreased the rate of acid production at pH 5.5 (Table 3). These findings suggest that streptococcal acid production in areas adjacent to GIC fillings may be inhibited at an acidic pH around the critical pH of tooth demineralization, resulting in a decrease in streptococcal cariogenicity. Furthermore, the present finding that the GIC eluate inhibited acid production to a degree similar to that of the KF solution with the same concentration of fluoride

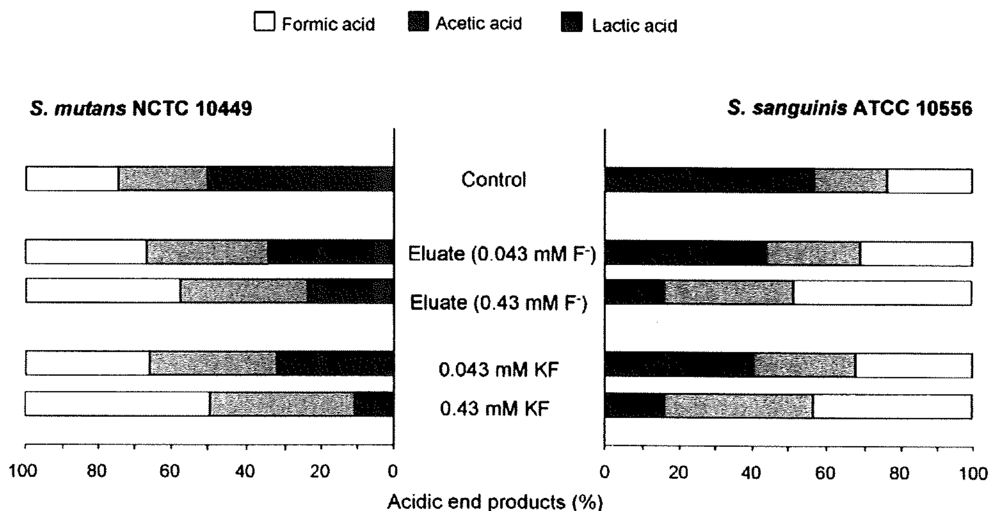

Fig. 2 – Effect of the GIC eluate or KF solution on the profiles of acidic end products for 10 min after addition of 10 mM glucose to the cells of *S. mutans* NCTC 10449 and *S. sanguinis* ATCC 10556 at pH 5.5. The data are the means of 3 independent experiments.

(Tables 2 and 3, Fig. 1) indicates that this effect of the eluate was due to the fluoride derived from the GIC.

The efficient inhibition of fluoride at acidic pH is due to the fact that hydrogen fluoride (HF) behaves according to the equilibrated reaction, $H^+ + F^- \leftrightarrows HF$ with a pKa value of 3.15. It is possible that fluoride is released from GIC mainly in the form of sodium fluoride (NaF) because the main cation released from Fuji IX is reportedly Na^+ [19], but NaF dissociates to sodium ion (Na^+) and fluoride ion (F^-) immediately at neutral pH. At acidic pH, F^- accepts a proton (H^+) and forms the undissociated form (HF) more easily, according to the equilibrated reaction where the reaction shifts toward the right as the environmental pH is lowered; the concentration of HF is calculated to be about 30 times higher at pH 5.5 than at pH 7.0. Because bacterial cell membranes are more permeable to HF than F^- [20], HF can enter the cells and dissociate into F^- and H^+ again in the relatively alkaline cytoplasm. This intracellular F^- inhibits enolase, a glycolytic enzyme, resulting in a decrease in acid production from glycolysis [21–23]. Thus, an acidic environment can promote entry of HF into bacterial cells and the subsequent inhibition of bacterial acid production via intracellular F^-. Furthermore, H^+ released from HF in the cell can acidify the intracellular pH, leading to a decrease in the entire glycolytic activity. F^- can also inhibit cell membrane-associated H^+-ATPase, a proton pump, resulting in decreased excretion of H^+ from the cells. This can also enhance the acidification of intracellular pH [24–26].

In addition to the inhibitory effect of fluoride, it has been reported that GICs such as Fuji IX are able to neutralize acidic conditions [27], suggesting that there is a buffering potential of released elements from GICs to neutralize acids produced by bacteria.

The present finding, that the proportion of lactic acid was decreased by both the eluate and KF solution (Fig. 2) supports that the eluate and KF solution exert this effect via the same mechanism. Previous researches indicate that this decrease in the proportion of lactic acid coincides with a slowdown in glycolysis through the enolase inhibition by F^- and the subsequent decrease in levels of intracellular glycolytic intermediates [28,29]. A slowdown in glycolysis leads to a decrease in the level of fructose 1,6-bisphosphate (a glycolytic intermediate that activates lactate dehydrogenase), resulting in a decrease in lactic acid production, while the concomitant decrease in levels of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (glycolytic intermediates that inhibit pyruvate formate-lyase), keeps formic and acetic acids production [30].

From the atomic absorption spectrometry, it is not possible to determine the form in which the elements were present in the eluate, i.e. compounds, complexes or ions. Therefore, it is difficult to evaluate the inhibitory effects of elements other than fluoride precisely. However, the most possible ions of the elements in the eluate, SiO_3^- and Al^{3+}, have no inhibitory effects on oral bacterial acid production at the concentrations determined in the present study [31,32]. Although Al^{3+} is known to enhance the inhibitory effects of fluoride on bacterial acid production [33] and H^+-ATPase [25], the concentration of Al^{3+} seemed to be too low to express the synergistic effect. It appears that the concentrations of the other elements were too low to demonstrate the influences on bacterial acid production.

The previous study indicated a possible retention within dental plaque. In the case of resin-modified GIC [8], the plaque fluoride levels increased from the saliva/plaque interface (0.1 mM) towards the plaque/GIC filling interface (1.6 mM). Although fluoride contained in cured GICs is expected to decrease gradually over time [12,34], GIC fillings can undergo a fluoride-recharge by absorbing fluoride from fluoride products such as dentifrices and mouth washes [35,36]. GICs may function as a biomaterial to reserve and release fluoride continuously in the oral cavity.

It is reported that fluoride is bacteriostatic and bactericidal to oral streptococci at concentrations of >15.8 and >160 mM, respectively [37,38]. However, in the current study as well as previous studies [29,39], bacterial acid production from sugar fermentation was inhibited by a lower fluoride concentration, especially at an acidic pH around the critical pH of tooth demineralization. Hallgren et al. [40] observed that dental plaque on the orthodontic brackets retained with GIC had a lower activity of lactic acid production by glucose fermentation as compared to composites. This result supports the antibacterial effect at a low concentration of fluoride as shown in the present study. In addition, they demonstrated that those plaque samples had a lower proportion of mutans streptococci in total viable count [41] and a higher content of fluoride [42].

The inhibition of bacterial acid production not only directly protect against dental caries, but also suppresses the growth of caries-related oral bacteria, which obtain most of the energy for their growth from sugar fermentation. Thus, adjacent to GIC fillings, the acid production and the subsequent growth are expected to decrease. It appears that the inhibition of acid production observed in the present study, together with the inhibition of demineralization and the promotion of remineralization at the tooth surface, would establish a cariostatic environment around GIC fillings *in vivo*. Further study is needed *in vivo* to confirm the expectation.

Acknowledgments

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ORAL MICROBIOLOGY AND IMMUNOLOGY

Transient acid-impairment of growth ability of oral *Streptococcus*, *Actinomyces*, and *Lactobacillus*: a possible ecological determinant in dental plaque

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Introduction: Dental plaque pH decreases to about 4 through bacterial fermentation of carbohydrates and this low pH is maintained for from several minutes to about an hour. Repeated acidification causes demineralization of the tooth surface, resulting in caries formation. The acidification also influences plaque bacteria. Severe acidification kills bacteria efficiently, while physiological acidification, the condition occurring in plaque, kills bacteria partially and may impair growth ability. We, therefore, investigated the effects of physiological acidification on representative caries-related bacteria.
Methods: *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguinis*, *Streptococcus oralis*, *Lactobacillus paracasei*, and *Actinomyces naeslundii* were used. Effects of physiological acidification at pH 4.0 on cell viability and growth ability, as well as the growth rate of these bacteria at pH 4.0–7.0, were investigated.
Results: Mutans streptococci and *Lactobacillus* grew at pH 4.0 but the growth of *S. sanguinis* and *S. oralis* ceased below pH 4.2 and pH 4.2–4.4, respectively. Acidification at pH 4.0 for 1 h killed 43–89%, 45% and 35–76% of *S. sanguinis*, *S. oralis*, and *Actinomyces*, respectively. Furthermore, assessment of bacterial growth curves revealed that the growth ability of the surviving cells of *S. sanguinis*, *S. oralis* and *Actinomyces* was impaired, but it was recovered within 2–5 h after the environmental pH had returned to 7.0. The acidification neither killed nor impaired the growth of mutans streptococci and *Lactobacillus*.
Conclusions: These results indicate that physiological and transient acidification is not sufficient to kill bacteria, but it causes a temporary acid-impairment of their growth ability, which may function as an ecological determinant for microbial composition in dental plaque.

Key words: acid impairment; acidification; *Actinomyces*; bacterial growth; cell viability; *Lactobacillus*; *Streptococcus*

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Dental plaque on the tooth surface consists of a variety of saccharolytic bacteria. After carbohydrate intake, the pH of dental plaque decreases rapidly to about 4 as the result of bacterial fermentation and this low pH is maintained for from several minutes to about an hour (12, 20). Repeated acidification causes demineralization of the tooth

surface, resulting in dental caries formation.

The acidification also influences bacterial physiology such as growth rate and cell viability. Harper and Loesche (10) demonstrated that when culture pH was lowered from 7.0 to 5.0 the growth rates of *Streptococcus mutans* and *Lactobacillus casei* decreased to 3–30% and 54%, respectively, while *Streptococcus sanguinis* ceased to grow. Experiments with mixed continuous cultures using representative oral bacteria indicated that the reduction of culture pH affected bacterial growth rate and subsequently modified bacterial composition. In the continuous cultures, *S. mutans* and *Lactobacillus rhamnosus* dominated the other bacteria, including non-mutans streptococci and *Actinomyces*, at a pH below 5.5 (3, 4), and the proportions of *S. mutans* and *L. casei* increased gradually after repeated acidification (5) or prolonged acidification (17) while those of the other bacteria decreased. These pH-driven modifications of bacterial composition observed in mixed continuous cultures were similar to the microbial shift from healthy site to carious site *in vivo* (2, 18, 19, 26), where environmental acidification is expected to occur. On the basis of these results, Marsh (15, 16) proposed the 'ecological plaque hypothesis' that environmental acidification causes a microbial shift towards a more acidogenic and aciduric population, resulting in a cariogenic microflora. Recently, Takahashi and Nyvad (24) expanded this hypothesis to 'microbial dynamics' of the caries process, in which the environmental acidification induces microbial acid-adaptation and subsequently increases microbial acidogenicity and acidurance, while the environmental acidification also triggers microbial shift through acid-selection of acidurant bacteria.

It is known that environmental acidification kills bacteria. Svensäter et al. (21) demonstrated that acidification at pH 2.3–4.5 for 3 h caused cell death of oral bacteria and that the acid-killing efficiency was dependent on bacterial species in the order of *Actinomyces* > non-mutans streptococci > mutans streptococci > *Lactobacillus*. In addition, Takahashi et al. (23) demonstrated that physiological and transient acidification (pH 4.0 for 1 h) occurring in dental plaque did not kill *S. sanguinis* efficiently but caused a temporary impairment of growth ability. They also showed that the impaired cells were capable of growing again after an 80-min incubation in the culture media at pH 7.0, along with the reactivation of glycolytic enzymes (23). However, no acid-impairment was observed in *S. mutans*. These observations suggest that acid-impairment of growth ability is an important ecological determinant of microbial composition in dental plaque, where repeated acidification occurs daily.

In the present study, we investigated the effects of physiological and transient acidification at pH 4.0 on cell viability and growth ability, as well as growth rate at pH 4.0–7.0, of representative oral bacteria, *Streptococcus*, *Actinomyces*, and *Lactobacillus*.

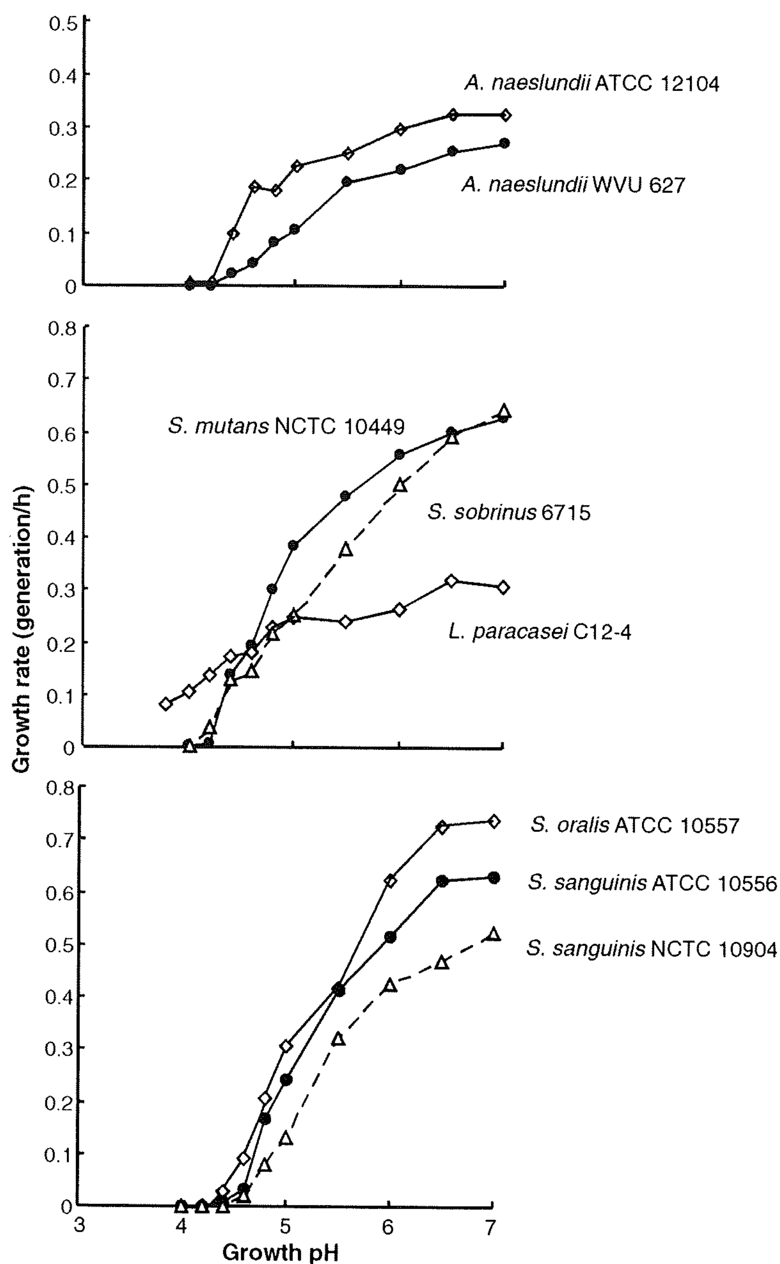

Fig. 1. Bacterial growth rate at various pH values.

Materials and methods

Bacterial strains

Actinomyces naeslundii genospecies 1 ATCC 12104, *A. naeslundii* genospecies 2 WVU 627, *Lactobacillus paracasei* subsp. *paracasei* (formerly *L. casei* subsp. *casei*) C12-4, *S. mutans* NCTC 10449, *S. oralis* ATCC 10557, *S. sanguinis* ATCC 10556, *S. sanguinis* NCTC 10904, and *Streptococcus sobrinus* 6715 were used in this study. These bacteria were maintained on blood agar plates. The purity of the culture was confirmed

by plating the culture on blood agar for all the strains and on mitis-salivarius agar (BBL Microbiology Systems, Cockeysville, MD, USA) for the streptococcal strains. These plates were incubated at 35°C for 2 days under anaerobic conditions.

Growth conditions

The bacteria were precultured in 5 ml trypticase soy broth (BBL Microbiology Systems) supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, MI, USA), 0.5% glucose, 0.1% L-cysteine hydrochloride monohydrate, and 0.04% sodium carbonate anhydrous (TSBYG-1) for 15 h at 35°C in an anaerobic chamber (Hirasawa Works, Tokyo, Japan; gas phase, 10% CO_2, 10% H_2, and 80% N_2). The cultures were transferred into 100 ml trypticase soy broth supplemented with 0.5% yeast extract, 0.5% glucose, 0.1% L-cysteine hydrochloride monohydrate, 0.01% ammonium bicarbonate, and 38 mM potassium phosphate buffer (pH 7.0) (TSBYG-2) and incubated at 35°C in an anaerobic chamber. The pH of the culture was maintained at 6.7–7.0 by regular manual addition of 6 M KOH The culture pH was checked using a handy pH meter (model pH BOY-P1, Sindengen Kogyo, Tokyo, Japan), which can measure the pH of a 30-μl sample. The following experiments were carried out in the same anaerobic chamber. To remove oxygen, all the media, solutions, and buffers were kept in the chamber for at least 3 days before use.

Bacterial growth rate at various pH values

At the exponential phase of growth, 2 ml of the culture was transferred into 5 ml of new TSBYG-2. Just before the experiments, the pH of the medium was adjusted to 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.5, 6.0, 6.5, or 7.0 by addition of 6 M HCl or KOH. The culture was then incubated at 35°C and the growth was monitored photometrically at 660 nm. The pH of the culture was maintained by periodical addition of 6 M KOH as describe above. The growth rate was calculated as generations per hour from initial inclinations of the growth curve.

Acidification of bacterial culture

At the exponential phase of growth [optical density at 660 nm (OD_{660}) 0.6–0.8], the culture (100 ml) was mixed with 100 ml prewarmed sterile TSBYG-2 medium and the pH of the culture was reduced to 4.0 within 3 min using 6 M HCl. The culture was then incubated at 35°C and optical density was monitored at 660 nm.

Survival of bacteria at low pH

After acidification at pH 4.0, the culture was sampled at 0, 0.5, 1, and 2 h. The samples were serially diluted with TSBYG-2 without glucose (pH 7.0) and spread on TSBYG-2 agar plates (pH 7.0). The plates were incubated at 35°C for 2 days and then colony-forming units were counted.

Growth ability of bacteria after acidification

After acidification at pH 4.0, 5 ml of the culture was sampled at 0, 0.5, 1, and 2 h and transferred into 5 ml of new TSBYG-2 (pH 7.0). The culture was then incubated at 35°C and the growth was monitored photometrically at 660 nm. The pH of the culture was maintained by occasional addition of 6 M KOH.

Statistical analyses

Difference in cell viability was analysed by the one-way repeated measures analysis of variance, and significance was examined using the Dunnet's test as *post hoc*. Statistical analysis was performed using STATFLEX software version 5.0 (Artech, Osaka, Japan). Differences were considered significant at the level $P < 0.05$.

Results

Bacterial growth rate at various pH values

All the bacterial strains in exponential growth continued to grow when they were transferred into new culture media. The growth rates of all the bacteria decreased as the pH of the growth media was lowered (Fig. 1). The growth rates of streptococci were higher than those of other bacteria above pH 5.5, although those of *S. sanguinis* and *Streptococcus oralis* declined sharply as the pH was lowered. The growth rate of these streptococci decreased to 4–12% at pH 4.6 and ceased completely at pH 4.2–4.4. In contrast, the growth rates of *S. mutans* and *S. sobrinus* decreased more gradually at acidic pH and remained at 22–31% at pH 4.6. However, no growth was found at pH 4.0. The growth rates of *Actinomyces* were about half the streptococcal growth rates at pH 7.0, but decreased gradually as the pH was lowered. The growth rates were maintained at 14–52% at pH 4.6 and no growth was observed at pH 4.2. The growth rate of *Lactobacillus* was as low as those of *Actinomyces* at pH 7.0, but the *Lactobacillus* strain maintained growth rates of 35 and 26% at pH 4.0 and pH 3.8, respectively.

Bacterial killing by acidification at pH 4.0

The acidification to pH 4.0 killed *A. naeslundii*, *S. sanguinis*, and *S. oralis*. The cell viability of these bacteria decreased significantly as the acidification was prolonged (Fig. 2), and decreased to 24–64%, 10–57% and 55% after 1 h of acidification,

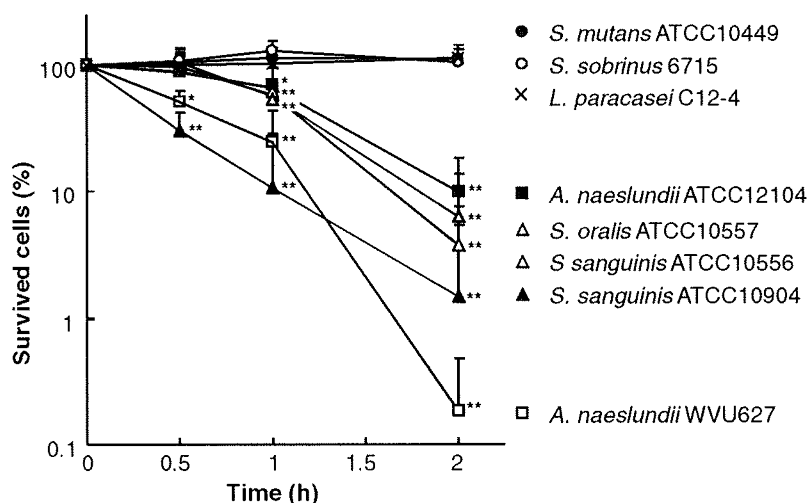

Fig. 2. The effect of acidification at pH 4.0 on cell viability. The mean values with standard deviations of three independent experiments are given. $*P < 0.05$, $**P < 0.01$.

respectively. In contrast, mutans streptococci and *Lactobacillus* maintained almost 100% viability during a 2-h acidification at pH 4.0.

Growth impairment

The bacterial cells acidified at pH 4.0 for 1 h were transferred into new TSBYG-2 media (pH 7.0), and their growth was monitored. At the beginning, the growth rates of *A. naeslundii*, *S. sanguinis*, and *S. oralis* (actual growth curve) were lower than those of control cultures, but they gradually returned to the original level (Fig. 3). There was no effect on mutans streptococci and *Lactobacillus*.

The expected growth curves of *A. naeslundii*, *S. sanguinis*, and *S. oralis* were also calculated from the proportion of viable cells (Fig. 2). For example, assuming that the surviving cells of *S. oralis* (55% of cells were viable after exposure to pH 4.0 for 1 h) started to regrow at the original growth rate at pH 7.0, the growth curve could be estimated by calculation using the following formulae:

1. OD value at n h (OD_n) = OD value contributed by dead cells (ODD) + OD value contributed by growing cells at n h (ODG_n)
2. ODD = $OD_0 \times (100 - VC\%)/100$
3. $ODG_n = (OD_{n-1} - ODD) \times 2^R$

where OD_0, VC% and R are initial OD value, viable cell % at 0 h and growth rate (i.e. generation/hour), respectively (indicated by open squares in Fig. 3).

In the same way, the expected growth curves of all the strains were obtained, except for mutans streptococci and *Lactobacillus* because of their high viability after acidification (Fig. 2). The actual growth curves of *A. naeslundii*, *S. sanguinis*, and *S. oralis* were always below the expected growth curves. The bacterial cells acidified at pH 4.0 for 0.5 or 2 h showed a similar result, though a 0.5-h acidification resulted in both actual and expected curves closer to control curves while 2-h acidification resulted in curves more separated from the control curves (data not shown).

Furthermore, the duration of the lag phase was calculated as follows (Fig. 4): when line a, the tangent line of the actual growth curve, became approximately parallel to the growth control curve at a logarithmic growth phase, A h was defined as the duration of the lag phase, namely a lag time. Similarly, the expected lag time was calculated from the expected growth curve (B h). The lag time increased as the acidification was prolonged (Table 1). The cells of *A. naeslundii* WVU 627 acidified for 2 h could not resume growth at pH 7.0 at 10 h. The expected lag time was always shorter than the actual lag time. No lag time was observed in mutans streptococci and *Lactobacillus* (Table 1).

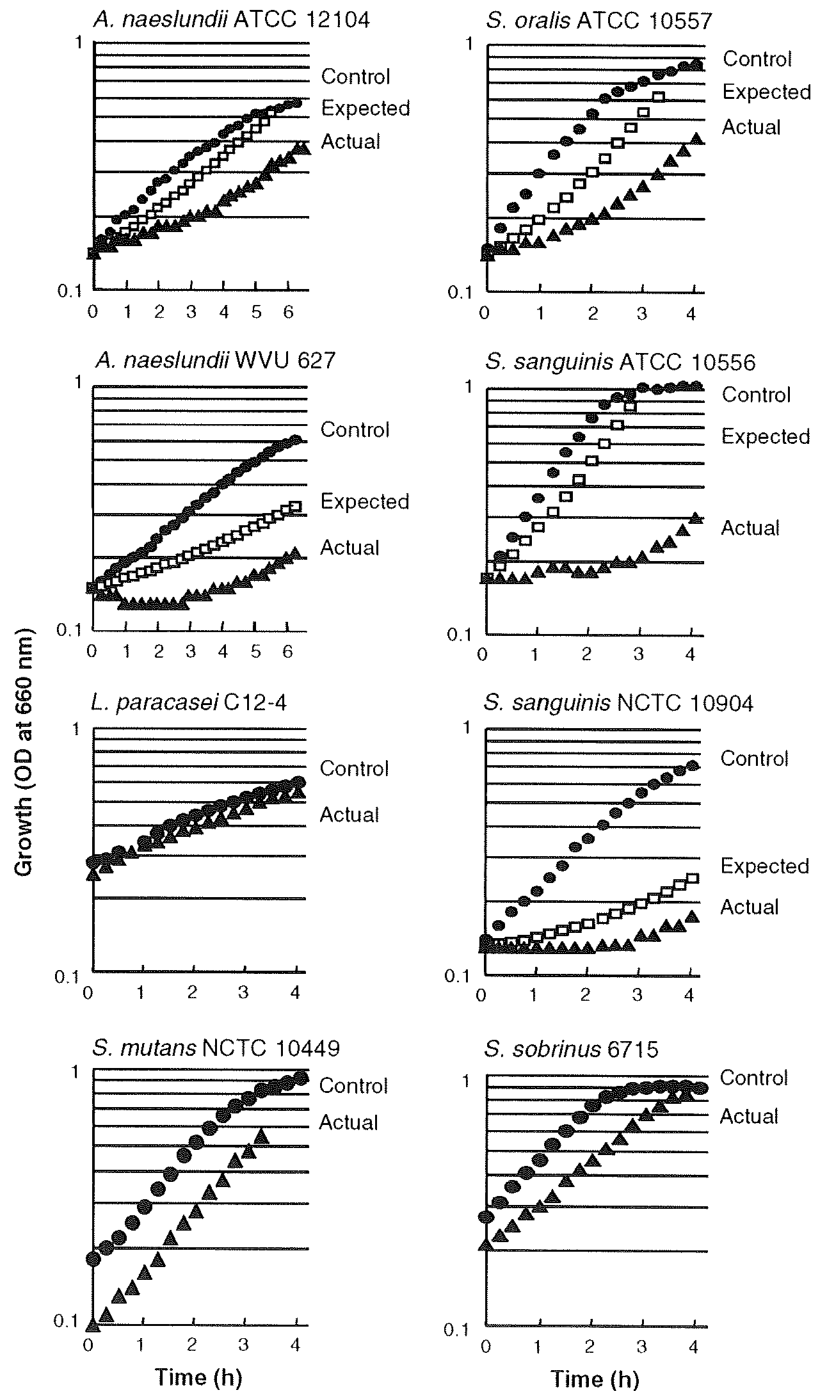

Fig. 3. Comparison between actual (▲) and expected (estimated from number of viable cells) (□) growth curves after acidification at pH 4.0 for 60 min. Control growth curves without acidification (•) are also shown. A representative growth curve from three independent experiments is given for each bacterial strain. Results from three independent experiments were similar.

Discussion

Around neutral pH, all the oral bacteria tested could grow efficiently, although the growth rates of *Actinomyces* and

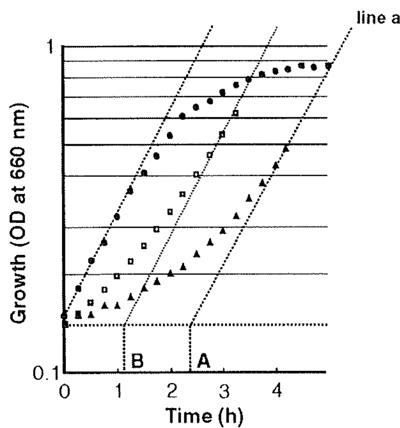

Fig. 4. The method of calculating lag time of growth after acidification. Control growth curve at pH 7.0 without acidification (•), expected growth curve estimated from number of viable cells after acidification at pH 4.0 for 60 min (□) and actual growth curve after acidification at pH 4.0 for 60 min (▲) are shown. Line a is a tangent line of the actual growth curve and is approximately parallel to that of the control growth curve. A, actual lag time; B, expected lag time.

Lactobacillus were about half those of oral streptococci under experimental conditions. As the environmental pH was lowered, however, the growth rates of mutans streptococci rose above those of *S. sanguinis* and *S. oralis* at pH 4.8 and above those of *Actinomyces* at pH 4.4. *Lactobacillus* showed a similar tendency but the growth rates became higher than those of mutans streptococci at pH 4.4. These results were consistent with those of previous studies using a continuous culture of mixed bacteria (3–5), although the pH values causing growth limitation seemed to be relatively lower than those from continuous culture studies. This discrepancy could be because of methodological differences between batch culture and continuous culture. The lower acid tolerance of bacteria in continuous culture can be ascribed to their growth under nutrient limitation with a concomitant lower capacity for adapting to the acid conditions. Continuous culture is generally accepted as the method of choice when evaluating bacterial growth at acidic pH. Overall, the present study, using a batch culture method, supports the previous finding that acid-tolerance of bacterial growth rate is an ecological determinant of microbial composition in supragingival plaque, as assumed in the 'ecological plaque hypothesis' (15, 16).

The acid-tolerance of bacterial growth rate indicates the bacterial ability to grow at different pH as described above, but this does not provide information regarding the acid-impairment of bacteria under acidic conditions. Acid-killing is known as one of the determinants of bacterial acid-impairment (1, 6, 9, 14). In the present study, the acidification to pH 4.0 gradually killed *A. naeslundii*, *S. sanguinis*, and *S. oralis*, while mutans streptococci and *Lactobacillus* survived the acidification. These results were similar to previous reports (21, 23), suggesting that *A. naeslundii*, *S. sanguinis*, and *S. oralis* are killed gradually at pH 4.0.

Were the cells that survived the acidification affected? Our previous study indicated that acidification at pH 4.0 for 1 h impaired the growth ability of *S. sanguinis* cells temporarily through a reversible inhibition of glycolytic enzymes, and that the impaired cells started to grow again at pH 7.0 with a longer lag phase (23). In the present study, a similar phenomenon was observed in *A. naeslundii*, *S. sanguinis*, and *S. oralis*. The cells that had survived during the acidification at pH 4.0 started to grow again, but the growth was apparently slower than the expected growth calculated from the number of surviving cells. These findings clearly indicate that physiological and transient acidification impairs the bacterial growth ability temporarily and that the growth ability recovers after the time required for repair.

Temporary bacterial impairment has not been considered as an ecological determinant in dental plaque because no clear influence, such as killing of bacteria, has been observed. However, it should be recognized that a usual method for counting live bacterial cells is to spread bacterial cells on agar plates and incubate them for 1–2 days for colony formation. Using this method it is difficult to detect a delay in bacterial growth. The combination of counting live cells on agar plates and monitoring bacterial growth in liquid media, as adopted in the present study, could enable us to observe temporary and reversible acid-impairment of bacterial growth if the bacteria are not acid-killed. The transient acidification occurs in dental plaque at least three times a day after each meal, probably having a significant influence on bacterial growth ability after pH recovery to neutral, and subsequently functioning as a driving force of ecological modification of dental plaque microflora.

In immature dental plaque, the pH falls to around 5, while the plaque pH becomes more acidic, to around 4, as the plaque matures (7). Transient acidification to around pH 5.5, as occurs in immature plaque, may increase bacterial acidogenicity and aciduranee through a series of bacterial acid-adaptation mechanisms. These include the induction of H^+-ATPase, stress proteins, and metabolic enzymes for alkaline production and the reinforcement of the bacterial membrane against H^+-penetration (1, 22, 25), probably along with a shift of energy usage from growth to maintenance metabolism (8, 13) and a resultant decrease in bacterial growth rate. However, a frequent and greater acidification to pH 4.0, as occurs in mature plaque, may weaken the competitiveness of *A. naeslundii*, *S. sanguinis*, and *S. oralis* through a reversible but repetitive impairment of growth ability. Furthermore, prolonged acidification around pH 4, as occurs in established caries lesions (11),

Table 1. Actual and expected lag times

Bacterial strains		Acidification time (h)		
		0.5	1	2
A. naeslundii ATCC 12104	Actual	1.87 (0.58)[1]	2.97 (1.04)	nc
	Expected	0.36 (0.09)	0.53 (0.07)	4.44 (0.95)
A. naeslundii WVU 627	Actual	2.26 (0.57)	3.52 (0.28)	nc
	Expected	1.14 (0.12)	1.98 (0.47)	20.2 (4.03)
S. oralis ATCC 10557	Actual	0.98 (0.57)	2.22 (0.69)	6.82 (4.11)
	Expected	0.39 (0.27)	0.63 (0.57)	4.41 (2.49)
S. sanguinis ATCC 10904	Actual	1.46 (0.30)	4.85 (1.51)	nc
	Expected	1.62 (0.59)	3.05 (1.13)	7.24 (4.06)
S. sanguinis ATCC 10556	Actual	0.82 (0.30)	2.65 (1.23)	7.19 (2.74)
	Expected	0.16 (0.10)	0.21 (0.19)	2.06 (1.94)
S. mutans NCTC 10449	Actual	0.13 (0.15)	0.34 (0.22)	0.33 (0.30)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
S. sobrinus 6715	Actual	0.14 (0.12)	0.00 (1.19)	0.00 (1.02)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
L. paracasei C12-4	Actual	0.33 (0.32)	0.28 (0.27)	0.00 (0.20)
	Expected	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

[1]The mean values with standard deviations (h) in parenthesis of three independent experiments are given.
nc, not calculated because of poor growth at 12 h after acidification.

may exclude these bacteria through acid-killing and leave more acidogenic and aciduric bacteria, such as mutans streptococci and *Lactobacillus*, as demonstrated by continuous culture studies (3–5, 17).

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Profiling of subgingival plaque biofilm microflora from periodontally healthy subjects and from subjects with periodontitis using quantitative real-time PCR

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Background and Objective: Qualitative and quantitative changes of the subgingival plaque biofilm microflora in periodontal pockets are thought to be associated with the development and progression of periodontitis. The aims of the present study were to quantify the proportions of nine periodontitis-associated bacterial species and four *Streptococcus* species in subgingival plaque, and to evaluate their relationship with periodontitis quantitatively.

Material and Methods: Subgingival plaque samples were obtained from 12 periodontally healthy subjects and from 28 patients with periodontitis. The amounts of total and target bacteria were measured by quantitative real-time PCR using universal and species-specific primers, respectively.

Results: The proportion of total obligate anaerobes was found to be higher in subjects with periodontitis than in periodontally healthy subjects ($p < 0.05$). Among obligate anaerobes, *Tannerella forsythia* ($2.04 \pm 5.27\%$, $p < 0.05$), *Porphyromonas gingivalis* ($0.54 \pm 1.41\%$) and *Eubacterium saphenum* ($0.30 \pm 0.96\%$) were detected at high proportions in subjects with periodontitis, but not in periodontally healthy subjects. By contrast, the proportion of total streptococci was lower in subjects with periodontitis ($p < 0.05$). Specifically, the proportion of *T. forsythia*, *P. gingivalis* or *E. saphenum* increased ($\geq 2.78\%$) and the proportion of *Streptococcus* species decreased to virtually undetectable levels, in subjects with periodontitis.

Conclusion: Obligate anaerobes, including *T. forthysia*, *P. gingivalis* and *E. saphenum*, were identified predominantly in microflora from subjects with periodontitis, whereas *Streptococcus* species were identified predominantly in microflora from predominantly healthy subjects, suggesting a change in the subgingival environment that resulted in conditions more suitable for the survival of obligate anaerobes. The proportion of these obligate anaerobes in the subgingival plaque of subjects with periodontitis appears to be associated with the status of human periodontitis.

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The human oral cavity is considered healthy when the oral microflora is composed of indigenous bacteria and is balanced properly (1). Numerous environmental changes in the oral microflora, including pH, anaerobiosis and nutrition, may lead to an accumulation of periodontitis-associated bacteria in the subgingival sulcus, resulting in the initiation of human periodontitis (2–6). Both qualitative and quantitative changes in subgingival plaque biofilms in periodontal pockets are thought to be highly associated with both the initiation and progression of periodontitis (6,7). The establishment of anaerobic bacterial culture and molecular biological methods has enabled researchers to isolate and detect various species of periodontitis-associated bacteria from subgingival plaque biofilms (8–11). It has been estimated that more than 600 species of bacteria are present in the human subgingival plaque biofilm (9–11). Of these, *Porphyromonas gingivalis* and *Mogibacterium timidum* are detected more frequently in the subgingival microflora of subjects with periodontitis than in the subgingival microflora of periodontally healthy subjects (8,9,11,12).

However, few studies have specifically investigated the proportions of periodontitis-associated bacteria as well as early colonizers on the teeth (namely *Streptococcus* species) in the subgingival plaque biofilm. The aims of the present study were to quantify the proportions of *P. gingivalis* and *M. timidum*, in addition to seven other periodontitis-associated species of bacteria (*Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eubacterium saphenum*, *Prevotella tannerae*, *Prevotella intermedia*, *Slackia exigua* and *Tannerella forsythia*) and four *Streptococcus* species (*Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus salivarius*) in the subgingival plaque of periodontitis-affected subjects and periodontally healthy subjects by quantitative real-time PCR, and to evaluate the relationship between periodontitis and the quantity of these bacteria.

Material and methods

Subjects and sample collection

Twenty-eight subjects with periodontitis (mean age 62 ± 9.9 years; range 41–80 years; mean numbers of residual teeth 24.5 ± 4.1) and 12 periodontally healthy subjects (mean age 26 ± 1.8 years; range 22–29 years; mean numbers of residual teeth 27.9 ± 1.5) were included in the present study. The subjects had not received periodontal treatment or antimicrobial therapy for at least 6 mo before sampling and were free of systemic diseases. Informed consent was obtained from each subject before the collection of samples. This study was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan. Probing depths were measured in all teeth, at six sites per tooth for each subject, and the teeth with the deepest probing depths were chosen as the target sites for sampling. The deepest probing depths were < 4 mm (range 2.0–3.0 mm; mean 2.4 ± 0.5 mm) in periodontally healthy subjects (H1–H12, $n = 12$) and ≥ 4 mm (range 4.0–10.0 mm; mean 6.7 ± 1.9 mm) in subjects with periodontitis (P1–P28, $n = 28$). The samples were then collected using sterile periodontal pocket probes. All samples were immediately suspended in 1 mL of sterile distilled water and stored at −20°C before extraction of genomic DNA.

Bacterial strains

Nine strains of anaerobic bacteria, including the microaerophilic bacterial strains *A. actinomycetemcomitans* JCM 8578, *C. rectus* JCM 6301, *E. saphenum* ATCC 49989, *M. timidum* ATCC 33093, *P. gingivalis* W83, *P. intermedia* JCM 12248, *P. tannerae* ATCC 51259, *S. exigua* JCM 11022 and *T. forsythia* JCM 10827, and three mitis groups of *Streptococcus*, including *S. gordonii* JCM 12995, *S. oralis* JCM 12997 and *S. sanguinis* JCM 5708, and the salivarius group of *Streptococcus*, *S. salivarius* JCM 5707, were cultured in an anaerobic glove box (Model AZ-Hard; Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2 at 37°C for 2–7 d. One loopful of a colony of each strain was then suspended in 1 mL of sterile distilled water and used for genomic DNA extraction.

DNA extraction

Genomic DNA was extracted using the GFX Genomic Blood DNA Purification kit (GE Health Care Bio-Science Corp., Piscataway, NJ, USA) and the InstaGene Matrix kit (Bio-Rad Laboratories, Richmond, CA, USA), in accordance with the manufacturer's instructions.

Quantification of total bacterial levels using quantitative real-time PCR

In order to quantify the total amount of bacteria in the samples, quantitative real-time PCR was undertaken using the 16S ribosomal RNA gene universal primers 357F and 907R (13,14) and iQ SYBR Green Supermix (Bio-Rad Laboratories), according to the manufacturer's instructions. The primer sequences were: 357F, 5′-CTC CTA CGG GAG GCA GCA G-3′; and 907R, 5′-CCG TCA ATT CMT TTR AGT TT-3′. Quantitative real-time PCR cycling conditions have been described previously (14) and were undertaken using an iCycler (Bio-Rad Laboratories) programmed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 55°C for primer annealing and 30 s at 72°C for extension. During the extension step, fluorescence emissions were monitored and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). Standard curves were analyzed by comparing the universal primer set against a serial dilution of *P. gingivalis* W83 genomic DNA.

Quantification of specific bacterial species by quantitative real-time PCR

In order to quantify populations of specific bacteria in the samples, quan-

titative real-time PCR was performed as described above, using bacterial species-specific primers (Table 1). Quantitative real-time PCR amplification protocols for each bacterium were as follows. (i) *C. rectus*, *E. saphenum*, *P. gingivalis*, *S. exigua*, *T. forsythia*, *S. gordonii*, *S. salivarius*, *S. sanguinis* and *S. oralis*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. (ii) *A. actinomycetemcomitans*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 83°C for 10 s. (iii) *M. timidum* and *P. tannerae*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 82°C for 10 s. (iv) *P. intermedia*: initial denaturation at 95°C for 3 min, followed by 40 PCR cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, with fluorescence emissions monitored at 81°C for 10 s.

Detection frequency of each bacterial species

Based on the results obtained from the quantitative real-time PCR, the detection frequency of each bacterial species in subgingival plaque was calculated (i.e. the prevalence, in per cent, of each bacterial species was determined).

Data analysis

Fisher's exact probability test and Tukey tests were used to determine statistical significance. A p-value of < 0.05 was considered statistically significant.

Results

The quantitative real-time PCR analysis results demonstrated that the total amounts of bacteria per probing depth in periodontitis subjects (4.10 ± 0.85 Log fg DNA/μL per probing depth mm) were similar to those in periodontally healthy subjects (3.86 ± 0.53 Log fg DNA/μL per probing depth mm) (Fig. 1). By contrast, the proportion of total obligate anaerobes (*T. forsythia*, *P. gingivalis*, *E. saphenum*, *M. timidum*, *P. intermedia*, *S. exigua* and *C. rectus*) in subgingival plaque biofilm was higher in the subjects with periodontitis than in the periodontally healthy subjects ($p < 0.05$, Fig. 2). *T. forsythia* (2.04 ± 5.27%, $p < 0.05$), *P. gingivalis* (0.54 ± 1.41%) and *E. saphenum* (0.30 ± 0.96%) were detected at high proportions in subjects with periodontitis (Fig. 3) and were not detected at all in periodontally healthy subjects, with the exception of *P. gingivalis*, which was detected in one sample (H12, Fig. 4).

The proportions of *S. exigua*, *C. rectus* and *P. intermedia* were slightly higher in subjects with periodontitis than in periodontally healthy subjects (Fig. 3). *M. timidum* was only detected in subjects with periodontitis, but at a relatively low proportion (0.04 ± 0.21%). *A. actinomycetemcomitans* and *P. tannerae* were not detected in either group of subjects in the present study.

Table 1. Target bacteria and their species-specific primers used in the present study

Species	Target genes	Sequence (5′ 3′)	Product size (bp)	References
Aggregatibacter actinomycetemcomitans	16S rRNA	CTC AGA GAT GGG TTT GTG CC AGA TTC ACT CCC CAT CGC TG	273	(15)
Campylobacter rectus	16S rRNA	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598	(16)
Eubacterium saphenum	16S rRNA	TCT ACT AAG CGC GGG GTG A ATA CCC GAT TAA GGG TAC	430	(17)
Mogibacterium timidum	16S rRNA	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524	(17)
Porphyromonas gingivalis	16S rRNA	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518	(15)
Prevotella intermedia	16S rRNA	TCC ACC GAT GAA TCT TTG GTC ATC CAA CCT TCC CTC CAC TC	98	(18)
Prevotella tannerae	16S rRNA	CTT AGC TTG CTA AGT ATG CCG CAG CTG ACT TAT ACT CCC G	550	(19)
Slackia exigua	16S rRNA	GCC AAG CGG CCT CGT CGA AG GCC GGC TTT AAG GGA TTC GCT CG	697	(17)
Tannerella forsythia	16S rRNA	AGC GAT GGT AGC AAT ACC TGT C TTC GCC GGG TTA TCC CTC	88	(18)
Streptococcus gordonii	*gtf*G	CTA TGC GGA TGA TGC TAA TCA AGT G GGA GTC GCT ATA ATC TTG TCA GAA A	440	(20)
Streptococcus oralis	*gtf*R	TCC CGG TCA GCA AAC TCC AGC C GCA ACC TTT GGA TTT GCA AC	374	(20)
Streptococcus salivarius	*gtf*K	GTG TTG CCA CAT CTT CAC TCG CTT CGG CGT TGA TGT GCT TGA AAG GGC ACC ATT	544	(20)
Streptococcus sanguinis	*gtf*P	GGA TAG TGG CTC AGG GCA GCC AGT T GAA CAG TTG CTG GAC TTG CTT GTC	313	(20)

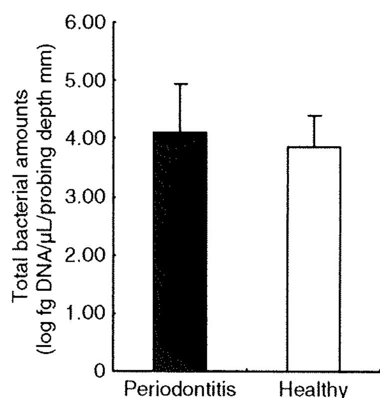

Fig. 1. Comparison of total bacterial amounts per probing depth between subgingival plaque biofilm obtained from subjects with periodontitis and that from periodontally healthy subjects.

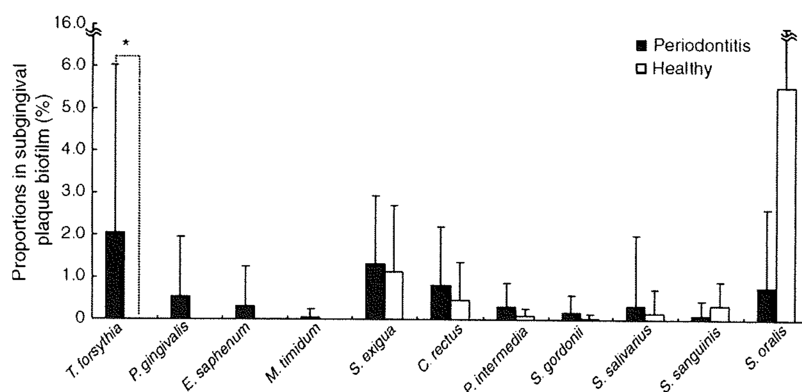

Fig. 3. Proportions of *Tannerella forsythia*, *Porphyromonas gingivalis*, *Eubacterium saphenum*, *Mogibacterium timidum*, *Slackia exigua*, *Campylobacter rectus*, *Prevotella intermedia*, *Streptococcus salivarius*, *Streptococcus gordonii*, *Streptococcus sanguinis* and *Streptococcus oralis* in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects. *$p < 0.05$.

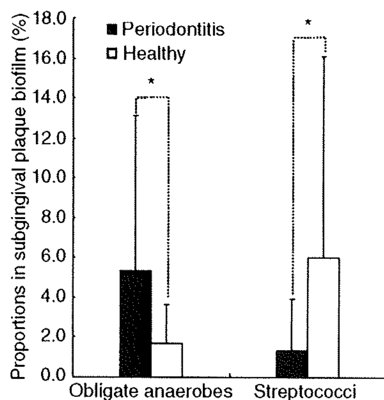

Fig. 2. Proportions of total obligate anaerobes and total streptococci in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects. *$p < 0.05$.

The proportion of total streptococci was significantly higher in periodontally healthy subjects than in periodontitis subjects ($p < 0.05$, Fig. 2). In particular, the proportions of *S. oralis* (5.53 ± 9.81%) and *S. sanguinis* (0.33 ± 0.57%) were higher in periodontally healthy subjects than in periodontitis subjects (Fig. 3), while the proportions of *S. salivarius* (0.32 ± 1.68%) and *S. gordonii* (0.16 ± 0.42%) were slightly higher in periodontitis subjects than in periodontally healthy subjects (Fig. 3).

The detection frequencies of *T. forsythia*, *P. gingivalis*, *E. saphenum*, *M. timidum*, *S. exigua*, *C. rectus*, *P. intermedia* and *S. gordonii* were higher in subjects with periodontitis than in periodontally healthy subjects (Fig. 5).

The proportions of target bacteria for each sample and its probing depth are shown in Fig. 4. Among obligate anaerobes, *T. forsythia*, *P. gingivalis* and *E. saphenum* were found in 43% of subjects with periodontitis (samples P17–P28), while *Streptococcus* species were detected in small amounts (≤ 8.91%) in 25% of subjects with periodontitis (samples P17–P22, P26). However, *Streptococcus* species disappeared as any of *T. forsythia*, *P. gingivalis* or *E. saphenum* increased (2.78–27.04%) in subjects with periodontitis (samples P23–P25, P27 and P28).

In the subjects with periodontitis, there was no apparent relationship between probing depths and total numbers of bacterial species present or between probing depths and the proportions of the above-mentioned three species (*T. forsythia*, *P. gingivalis* and *E. saphenum*) (Fig. 4).

Discussion

In the present study, the total bacterial amounts per probing depth in subjects with periodontitis were similar to those in periodontally healthy subjects. However, the predominant bacterial species identified in subgingival plaque biofilm microflora from subjects with periodontitis differed from those identified in periodontally healthy subjects. The proportions of obligate anaerobes, in particular *T. forsythia*, *P. gingivalis* and *E. saphenum*, were higher in subgingival plaque biofilm microflora from subjects with periodontitis. However, the proportions of *Streptococcus* species, especially *S. oralis* and *S. sanguinis*, were higher in subgingival plaque biofilm microflora from periodontally healthy subjects, indicating that *Streptococcus* species are one of the major components of periodontally healthy subgingival plaque biofilm. Furthermore, the findings of the present study led to an understanding of total subgingival plaque biofilm and its significant difference in health and disease.

Focusing attention on the proportion of *T. forsythia*, *P. gingivalis* and *E. saphenum* that are frequently isolated and detected in human periodontal pockets (8,9,11,21,22), periodontitis subjects were divided into three groups; these were termed the no-detection group (samples P1–P16), the low-level group (0.01–1.38%, samples P17–P22) and the high-level group (2.78–27.04%, samples P23–P28). In order to investigate the relationship between the three obligate anaerobes (*T. forsythia*, *P. gingivalis* and *E. saphenum*) and *Streptococcus*, the proportions of the three obligate

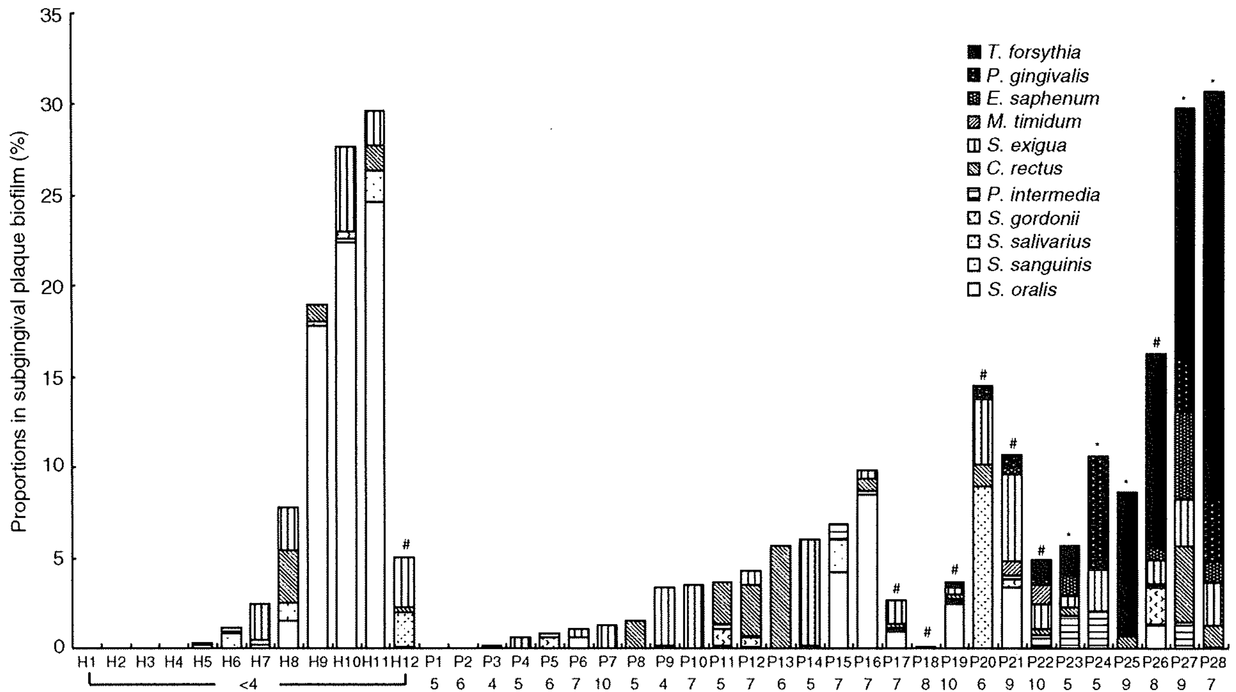

Fig. 4. Proportions of bacteria in the subgingival plaque biofilm of each periodontally healthy subject and each subject with periodontitis. *Any of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Eubacterium saphenum* was detected. #Streptococci and any of *P. gingivalis*, *T. forsythia* and *E. saphenum* were detected. Numerals (< 4 and 4 10) below each sample number indicate each probing depth (mm) of periodontally healthy subjects (H1 H12) and periodontitis subjects (P1 P28), respectively.

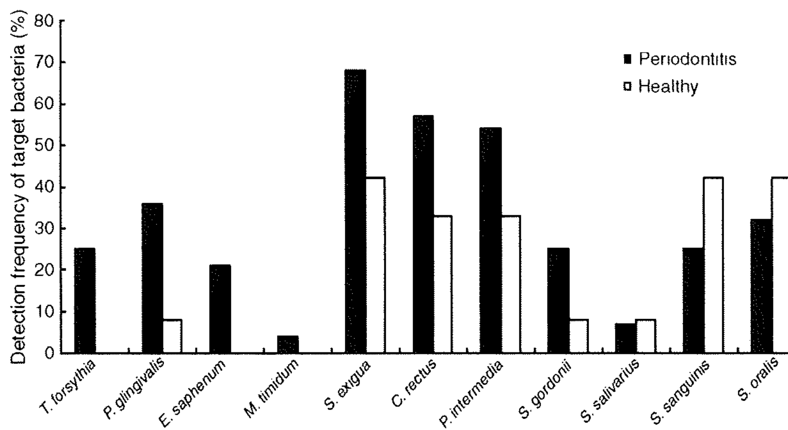

Fig. 5. Detection frequencies of target bacteria in subgingival plaque biofilm obtained from subjects with periodontitis and in periodontally healthy subjects.

anaerobes for each sample are shown in sequence in Fig. 4. *Streptococcus* species were specifically detected in the no-detection group and in the low-level group, but not in the high-level group, with the exception of sample P26 in the present study. These results suggest that environmental conditions, including oxygen concentration, nutritional supply and pH levels, are diverse among periodontal pockets and that the high-level group have an environment with low oxygen, nitrogenous-compound supply and stable neutral pH, which is suitable for the growth of periodontitis-associated bacteria (6,7).

The proportions of periodontitis-associated bacteria, such as *T. forsythia* and *P. gingivalis*, in subgingival plaque biofilm from subjects with periodontitis were relatively lower in the present study than in previous studies (18,23,24). This may be ascribed to the fact that the instruments used for sample collection in the present study (periodontal pocket probes), were different from those used in previous studies (Gracey curettes or paper points). Although it has been reported that the subgingival biofilm-forming bacteria detected were different when the sampling technique was different (25–27), the tendency to find higher proportions of periodontitis-associated bacteria in subgingival plaque biofilm from subjects with periodontitis was similar between the present study and previous studies. In addition, to our knowledge, this is the first study that specifically investigated, using real-time PCR, the proportions of early colonizers (such as *Streptococcus* species) in subgingival plaque biofilm isolated from the teeth of subjects with periodontitis.

Because we were unable to find periodontally healthy subjects in the elderly, the mean age of the perio-

dontally healthy subjects was 26 years in the present study. However, it has been reported that the subgingival microflora of periodontally healthy subjects does not change markedly with age (28,29), and thus the mean age of the periodontally healthy subjects may not influence greatly the results of the present study.

The present study demonstrated that bacterial species frequently detected in the subgingival plaque biofilm of subjects with periodontitis did not always represent a major proportion of the microflora (Figs 3 and 5). For example, the detection frequencies of *S. exigua*, *C. rectus* and *P. intermedia* were higher than those of *T. forsythia*, while the proportion of *T. forsythia* was higher than that of these three species. These results indicated the possibility that *T. forsythia* becomes predominant once it colonizes, although this bacterium cannot colonize efficiently, and suggest that the quantitative analysis is more reliable for evaluating bacterial status in the microflora.

In the present study, periodontal pocket depths were not found to correlate with either the total bacterial amounts or the proportions of individual bacteria in the microflora (Fig. 4). For instance, sample P7, obtained from a 10-mm periodontal pocket depth, did not contain *P. gingivalis*, *T. forsythia* or *E. saphenum*, while samples P23 and P24, obtained from a 5-mm periodontal pocket depth, contained large amounts of these bacteria. These results suggest that the depth of the periodontal pocket does not directly reflect the amount of bacteria found in the periodontal microflora.

In conclusion, facultative anaerobes, including *Streptococcus* species, were found to be the predominant bacterial species in subgingival plaque biofilm microflora from periodontally healthy subjects, whereas obligate anaerobes, including *T. forsythia*, *P. gingivalis* and *E. saphenum*, were the predominant bacterial species in subgingival plaque biofilm microflora from patients with periodontitis. The microbial diversity found in the present study suggests that environmental changes occur in the subgingival area, providing a more suitable environment for the survival of obligate anaerobes and thus the subsequent progression of periodontitis (6,7), and that the proportions of these obligate anaerobes in subgingival plaque from subjects with periodontitis are associated with the status of human periodontitis disease (21). The findings of the present study support the hypothesis that quantification of bacteria in subgingival plaque biofilm microflora is an appropriate tool for the diagnosis and prognosis of periodontitis (21). In addition to a large-scale study on the relationship between the periodontitis status and periodontitis-associated bacteria, a comparative and quantitative study on the changes observed in the oral microflora during the healing process, from periodontitis to healthy status, is required in order to verify the hypothesis.

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