〔Original〕

The wire method for generating experimental biofilms formed by oral *Streptococcus* and *Veillonella* species

Izumi MASHIMA & Futoshi NAKAZAWA

Department of Oral Microbiology, School of Dentistry, Health Sciences University of Hokkaido

Abstract

Complex interactions between microorganisms result in the formation of microbial biofilms as dental plaque and tongue surface debris, leading to caries, periodontal disease, and oral malodor. It is important to know the microbial composition of oral biofilms for the diagnosis and rational treatment of these diseases. In human oral cavities, *Streptococcus* species, especially *S. gordonii, S. mutans*, and *S. salivarius*, are suggested to be the initial colonizers of dental biofilms. Oral *Veillonella* spp., including *V. atypica*, *V. dispar*, *V. denticarosi*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis*, are also suggested to be early colonizers of dental biofilms. The formation of biofilms in *in vitro* experiments has been previously studied using 96−well microtiter plate−based biofilm assays or a flow cell system. However, limitations of the technical sensitivities or the high cost of these methods is prohibitive to their routine use. To circumvent these limitations, "the wire method" was used in this study. A cobalt−chrome alloyed wire was used as the scaffold for biofilm formation and quantitative real−time PCR was applied to quantify the number of bacterial cells in the biofilms. Moreover, the above−mentioned *Streptococcus* and *Veillonella* spp. were used as initial and early colonizers for developing biofilms on wires. The amounts of biofilm formed by *S. gordonii* increased in the presence of *Veillonella* spp. The amount of biofilm formed by *S. mutans* was less in the presence of *Veillonella* spp. than in the control, which contained *S. mutans* alone. The biofilm formation increased in co−cultures of *S. salivarius* and *V. dispar*, and *S. salivarius* and *V. denticariosi*. In the present study, we demonstrated that the wire method is appropriate for generating experimental biofilms, especially at early stages, such as the biofilms formed by *Streptococcus* and *Veillonella* spp. Furthermore, our data showed that the amount of biofilm formed by *Streptococcus* spp. depends on the *Veillonella* spp. that is used as the partner species.

Key words：Biofilm, *Streptococcus*, *Veillonella*, The wire method, Quantitative real time PCR

Introduction

The human oral cavity contains more than 19,000 microbial species (Keijser et al., 2008). These microorganisms interact with each other and with their host tissues (Kroes et al., 1999 ; Paster BJ et al., 2001). These complex interactions result in the formation of microbial biofilms as dental plaque and tongue surface debris, leading to caries, periodontal disease, and oral malodor. Biofilms are surface−adherent populations of microorganisms consisting of cells, water, and extra−cellular matrix material (Costerton et al., 1995 ; Sutherland, 2001). It is now known that organisms living in biofilms behave very differently from those living in a free−floating or planktonic state (Bhardwaj et al., 2009). The ability of bacterial cells to adhere to a privileged site to build protective structures and to communicate through chemical signals enables them to counteract the host's natural defenses (Bhardwaj et al., 2009). Recent studies have suggested that several oral bacteria are opportunistic pathogens that cause systemic diseases and adverse health outcomes, such as bacterial endocarditis

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(Douglas et al., 1993 ; Berbari et al., 1997 ; Tao & Herzberg, 1999), aspiration pneumonia (Scannapieco, 1999), pre−term low birth weight of infants born to mothers with periodontal disease (Offenbacher et al., 1998), and cardiovascular diseases (Genco et al., 2002). Consequently, it is important to know the microbial composition of oral biofilms for the diagnosis and rational treatment of these diseases.

Streptococcus mutans, the principal pathogen in dental caries, co−exists with over 500 other species of bacteria (Kroes et al., 1999 ; Moore & Moore, 2000 ; Paster et al., 2001) in an interactive community known as the dental biofilm, making it an organism of particular interest in the study of microbial interactions in biofilms. Furthermore, it has been suggested that many *Streptococcus* spp., especially *S. gordonii*, *S. mutans*, and *S. salivarius*, are initial colonizers of dental biofilms (Aas et al., 2005 ; Diaz et al., 2006 ; Saravanan & Kolenbrander, 2009 ; Saravanan & Kolenbrander, 2010).

The genus *Veillonella* consists of small, strictly anaerobic, gram−negative cocci that lack flagella, spores, and capsules. Members of this genus have been isolated from the oral cavity and intestinal tract of humans and from other animals that obtain energy by the utilization of short−chain organic acids (Delwiche et al., 1985 ; Sutter, 1984). The genus *Veillonella* is currently subdivided into 12 species : *V. atypica*, *V. caviae*, *V. criceti*, *V. denticariosi*, *V. dispar*, *V. magna*, *V. montpellierensis*, *V. parvula*, *V. ratti*, *V. rodentium*, *V. rogosae*, and *V. tobetsuensis* (Arif et al., 2008 ; Byun et al., 2007 ; Jumas et al., 2004 ; Kolenbrander & Moore, 1992 ; Mareike & David, 2008 ; Mashima et al., 2012 ; Mays et al., 1982 ; Rogosa, 1984). Of these species, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* have been isolated from the human oral cavity (Arif et al., 2008 ; Byun et al., Kolenbrander & Moore, 1992 ; Mareike & David, 2008 ; Mashima et al., 2012 ; Mays et al., 1982 ; Rogosa, 1984). Moreover, it has been suggested that oral *Veillonella* spp. are early colonizers of dental biofilms (Saravanan & Kolenbrander, 2009 ; Saravanan & Kolenbrander, 2010).

In the past, 96−well microtiter plate−based biofilm assays (Christensen et al., 1985) have been most widely used in many *in vitro* biofilm experiments. However, almost all 96−well microtiter plates are made of polystyrene. The dissimilarities between polystyrene and the materials found in dental plaque make it difficult to extrapolate the results of such biofilm assays to the natural behavior of bacterial biofilms in the oral cavity. Moreover, the force of gravity is involved with a large part of forming biofilms in this biofilm assays. It is unnatural circumstance for bacterial biofilms. In addition, to quantify the biofilm adhering to the wells in the microtiter plates, planktonic cells are removed using a generalized washing procedure. This procedure is likely to introduce a large margin of experimental error because it is possible to accidentally remove the biofilm formed on the well with such washing procedures. Furthermore, it is difficult to accurately estimate the total number of individual bacterial cells in biofilms in this plate−based assay method.

A flow cell system (Foster & Kolenbrander, 2004) has also been frequently used for *in vitro* biofilm experiments. The force of dynamic flow is involved with a large part of forming biofilm in this method. It is also unnatural circumstance for bacterial biofilms. Then, the flow cells are typically made of a high−density polyethylene block with a glass coverslip. The flow cell system is expensive to establish and difficult to maintain. Moreover, the bacterial composition in the biofilm is determined using fluorescence in situ hybridization (FISH), with which the mixed−species communities in the flow cells can be examined without disruption of the growing biofilm. The analyses of biofilms by using the flow cell system and FISH assays are expensive and tedious, making this method unsuitable for routine use in laboratory settings. There is a need for a low−cost assay for studying biofilm formation that can circumvent some of the limitations of the assays in current use.

In this study, a wire that is commonly used in orthodontic treatment was applied as the scaffold for biofilm formation. Bacteria use only their force to form biofilms in this wire method. We used a protocol for generating experimental biofilms on the wire by using *Streptococcus* and *Veillonella* spp. Moreover, we also quantified a number of individual bacterial cells in the experimental biofilms by using quantitative real−time PCR (q−PCR).

The aim of this study was to verify the practical application of the wire method for biofilm formation and to quantitatively analyze the biofilm formed by *Strepto-*

coccus and *Veillonella* spp., which were used as initial and early colonizers of oral biofilms.

Materials and methods

Bacterial strains and culture conditions

S. gordonii ATCC 10558^T, *S. mutans* Ingbritt, and *S. salivarius* ATCC 9222 were cultured on TY agar (TY ; Bacto™ Tryptic Soy Broth and Bacto™ Yeast Extract) in anaerobic conditions (N_2 : CO_2 : H_2, 80 : 10 : 10) at 37°C for 3 days. *V. atypica* ATCC 17744^T, *V. dispar* ATCC 17748^T, *V. denticariosi* JCM 15641^T, *V. rogosae* JCM 15642^T, *V. parvula* ATCC 10790^T, and *V. tobetsuensis* ATCC BAA−2400^T were cultured on BHI blood agar (BHI ; Bacto™ Brain Heart Infusion) supplemented with 5% defibrinated sheep blood and 2% sodium lactate in anaerobic conditions at 37°C for 5 days.

Wire preparation

Cobalt−Chrome alloyed wires of 0.9 mm diameter and 15 cm length were used in this study. The wire was made fine chase by filing. After fixing a rubber plug and flexible plastic tubing (TYGON® Saint−Gobain) to the wire ends, the wire apparatus was sterilized in an autoclave.

Biofilm formation on the wire

Two milliliters of a suspension of each *Streptococcus* spp., whose turbidity had been determined by measuring the absorption at OD_{660}, was inoculated into 18 mL BHI broth containing 10% sucrose and 2% sodium lactate in a test tube. A wire treated with artificial saliva (Saliveht® TEIJIN) was inserted in the test tube as a base for biofilm formation. After incubation in anaerobic conditions at 37°C for 3 days, the wire with the *Streptococcus* biofilm was transferred into suspensions of individual *Veillonella* cultures, whose turbidity had also been determined by measuring the absorption at OD_{660}, in test tubes containing the same media. The co−cultures were incubated in anaerobic conditions at 37°C for 4 days to generate mixed biofilms with *Streptococcus* and *Veillonella* spp. The media in the test tubes were replaced with fresh media every day. Biofilms containing *S. gordonii*, *S. mutans*, and *S. salivarius* alone were generated on the wires under the same conditions for 7 days, as controls.

DNA Extraction

All of the biofilm on the wire was removed into a sterilized tube, and DNA was extracted from the biofilms by using a modified alkaline lysis protocol (Chalmers et al., 2008 ; Hoshino et al., 2005 ; Periasamy et al., 2009). Briefly, the biofilm was immersed in 1400 μL of sterilized water, 5600 μL of 0.05 M sodium hydroxide was added, and the mixtures were incubated at 60°C for 60 min, after which 64.4 μL of 1 M Tris−HCl (pH 7.0) was added to neutralize the pH. This extract was used as the template DNA for quantitative real−time PCR (q−PCR) analyses (Chalmers et al., 2008 ; Hoshino et al., 2005 ; Periasamy et al., 2009). Bacterial genomic DNA for standard curves was extracted from 3−day−old pure cultures of the 3 *Streptococcus* spp. and 5−day−old pure cultures of the 6 *Veillonella* spp. with the InstaGene Matrix Kit (Bio−Rad) according to the manufacturer's instructions. Genomic DNA was stored at −20°C until further analysis.

Preparation of q−PCR standards and quantification of species in biofilms

For construction of standard curves, 10−fold serial dilutions (from 300 to 1.0×10^8) of target species genomic DNA preparations were analyzed by q−PCR by using previously described primers (Yoshida et al., 2003 ; Hoshino et al., 2004 ; Rinttilä et al., 2004). Primers specific for *S. gordonii* were designed using the *gtfG* gene as the species−specific reference gene. The sequence of the forward *gtfG* primer was 5'−CTATGCG GATGATGCTAATCAAGTG−3', and the sequence of the reverse *gtfG* primer was 5'−GGAGTCGCTA-TAATCTTGTCAGAAA−3' (Hoshino et al., 2004). Primers specific for *S. salivarius* were designed using the *gtfK* gene. The sequence of the forward *gtfK* primer was 5'−CTGTTGCCACATCTTCACTCGCTTCGG−3', and the sequence of reverse *gtfK* primer was 5'−CGTT GATGTGCTTGAAAGGGCACCATT−3' (Hoshino et al., 2004). Primers specific for *S. mutans* were designed using the *gtfB* gene. The sequence of the forward *gtfB* primer, Smut3368−F, was 5'−GCCTACAGCTCAGA-GATGCTATTCT−3', and the sequence of the reverse *gtfB* primer, Smut3481−R, was 5'−GCCATACAC-CACTCATGAATTGA−3' (Yoshida et al., 2003). Primers specific for the *Veillonella* spp.−*V. atypica*, *V. dis-*

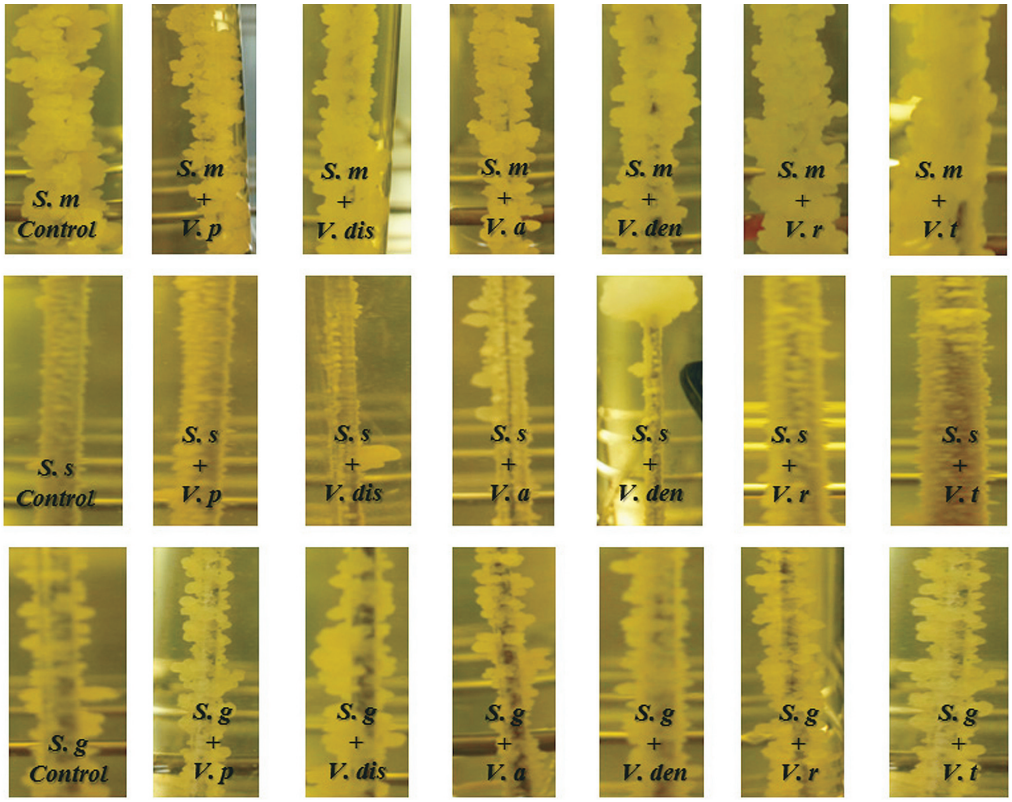

Fig.1, The biofilm formation with *Streptococcus species* and *Veillonella* species on the wire.
S. m (*S. mutans*), *S. s* (*S. salivarius*), *S. g* (*S. gordonii*), *V. p* (*V. parvula*), *V. dis* (*V. dispar*), *V. a* (*V. atypica*), *V. den* (*V. denticariosi*), *V. r* (*V. rogosae*), *V. t* (*V. tobetsuensis*).

par, *V. denticariosi*, *V. rogosae*, *V. parvula*, and *V. tobetsuensis*－were designed using the 16S rDNA gene, which is conserved among these *Veillonella* spp. The sequence of the 16S rDNA forward primer was 5'－A(C/T)CAACCTGCCCTTCAGA－3', and the sequence of 16 S rDNA reverse primer was 5'－CGTCCCGATTAACAG AGCTT－3' (Rinttilä et al., 2004). Quantification of *S. gordonii*, *S. salivarius*, *S. mutans*, *V. atypica*, *V. dispar*, *V. denticariosi*, *V. parvula*, *V. rogosae*, and *V. tobetsuensis* cells in biofilms was performed with q－PCR, using the SYBR green dye to detect the *gtfB*, *gtfK*, *gtfG*, and 16S rDNA gene amplicons. Each reaction (final reaction volume, 50 μL) contained 4 μL of template, 16 μL of sterilized water, 25 μL of KAPA SYBR® FAST qPCR MasterMix Universal (KAPA BIOSYSTEMS), 2 μL each of the forward and reverse primers at 400 nM, and 1.0 μL of ROX Low. q－PCR was performed in a 7500 Real Time PCR System (Applied Biosystems) by using the following thermocycles recommended for the Fast SYBR green Master mix (KAPA BIOSYSTEMS)： 95°C for 30 s, 40 cycles of 5 s at 95°C, and 34 s at 60°C. Dissociation curves were generated by incubating the reaction products at 95°C for 15 s and 60°C for 1 min, and incrementally increasing the temperature to 95°C. Fluorescence data were collected at the end of the 60°C primer－annealing step for 40 amplification cycles and throughout the dissociation curve analysis. The analysis of the melting curves with all the primer sets showed a single sharp peak. The standard curves were determined in triplicate to ascertain the accuracy and consistency of the q－PCR assay.

Data Analysis

Kruskal Wallis H－test post hoc Mann－Whitney U－test with Bonferroni correction was used to determine statistical significance. A p－value of < 0.05, < 0.01, and < 0.001 were considered statistically significant.

Results

The formation of biofilms formed on the wires after 7 days of incubation of the single or mixed cultures is shown in Fig. 1.

Ten－fold serial dilutions of genomic DNA extracted from *S. mutans* (Fig. 2) and *V. parvula* (Fig. 3) demonstrated a linear relationship when the copy number per milliliter was compared with Ct values generated from

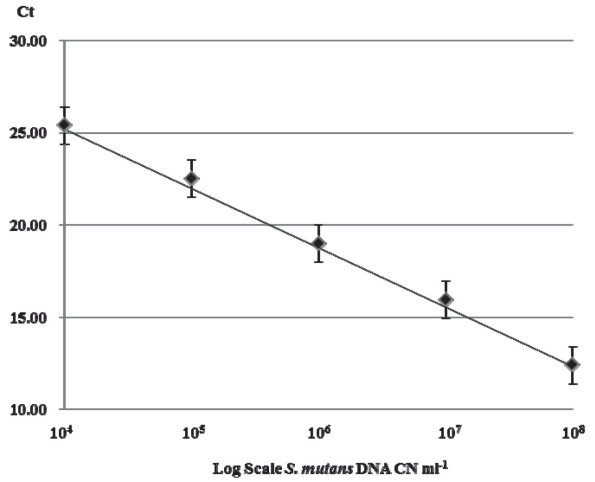

Fig.2, The standard curve of *Streptococcus* mutans.
Quantitative PCR (qPCR) of standardized *S. mutans* DNA. Ct values are plotted against DNA copy number (copy number ml^{-1}). 10−fold dilutions of DNA extracted from *S. mutans* cells (10^8 cells ml^{-1}) were used as the copy number standard. The Ct values were determined from the diluted copy number (n=3) for each dilution. Bar means standard error. CN, *S. mutans* copy number.

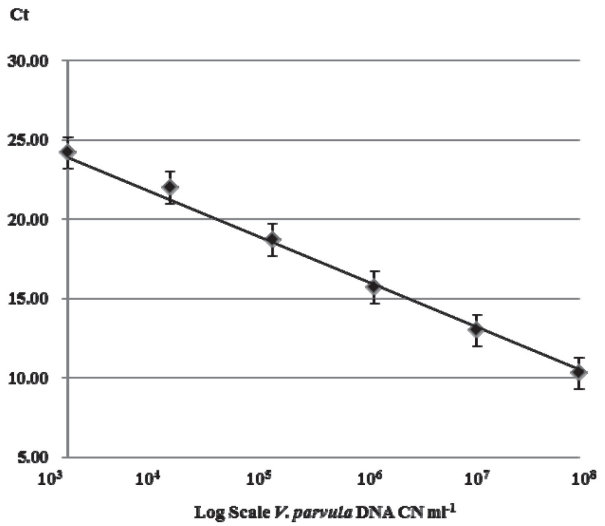

Fig.3, The standard curve of *Veillonella parvula*.
Quantitative PCR (qPCR) of standardized *V. parvula* DNA. Ct values are plotted against DNA copy number (copy number ml^{-1}). 10−fold dilutions of DNA extracted from *V. parvula* cells (10^8 cells ml^{-1}) were used as the copy number standard. The Ct values were determined from the diluted copy number (n=3) for each dilution. Bar means standard error. CN, *V. parvula* copy number.

the q−PCR analyses. Similarly, standard curves for *S. gordonii*, *S. salivarius*, *V. atypica*, *V. dispar*, *V. denticariosi*, *V. rogosae*, and *V. tobetsuensis* were also obtained (data not shown).

The amounts of biofilm formed by *S. mutans* and the 6 *Veillonella* spp., including a control, as determined from the results of the q−PCR analyses, are shown in Fig. 4. The amount of biofilm formed by *S. mutans* in

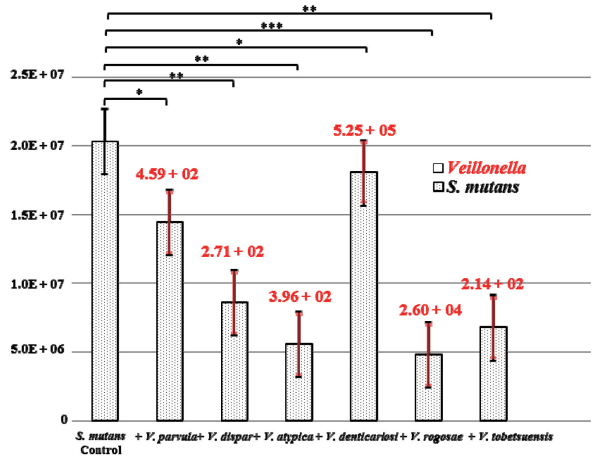

Fig.4, The amount of biofilm formation with *S. mutans* and 6 oral *Veillonella* species.
* P < 0.05, ** P < 0.01, ***P < 0.001
The red number in figure shows the average number of *Veillonella* species.
Bar means standard error.

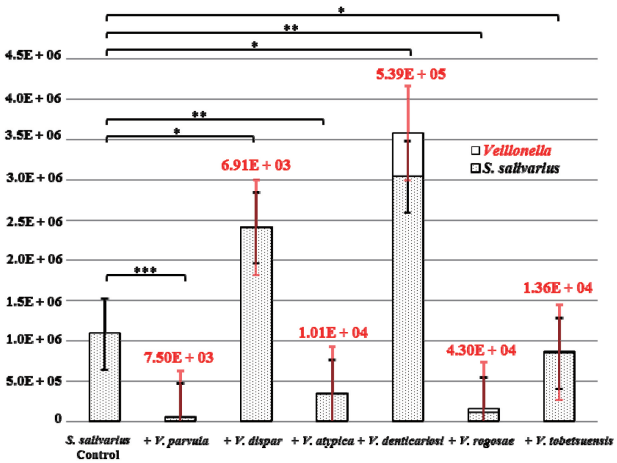

Fig.5, The amount of biofilm formation with *S. salivarius* and 6 oral *Veillonella* species.
* P < 0.05, ** P < 0.01, ***P < 0.001
The red number in figure shows the average number of *Veillonella* species.
Bar means standard error.

the presence of the 6 *Veillonella* spp. were less than that formed in the control, which contained *S. mutans* alone. On the other hand, in the case of *S. salivarius*, the presence of *V. dispar* and *V. denticariosi* increased the amount of biofilm formed on the wire (Fig. 5). Furthermore, the amount of biofilm formed by *S. gordonii* in the presence of the 6 *Veillonella* spp. was greater than that in the control, especially in the presence of *V. rogosae* (Fig. 6).

When the ratio of *Veillonella* to *Streptococcus* in the biofilm was analyzed, *S. mutans* constituted a large portion of the biofilms, accounting for more than 99% of the bacteria (data not shown). On the other hand, in

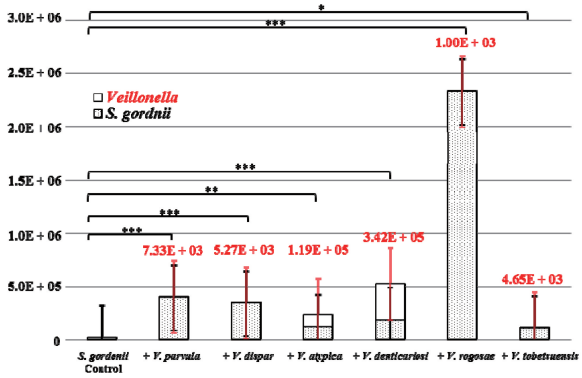

Fig.6, The amount of biofilm formation with *S. gordonii* and 6 oral *Veillonella* species.
* P < 0.05, ** P < 0.01, ***P < 0.001
The red number in figure shows the average number of *Veillonella* species.
Bar means standard error.

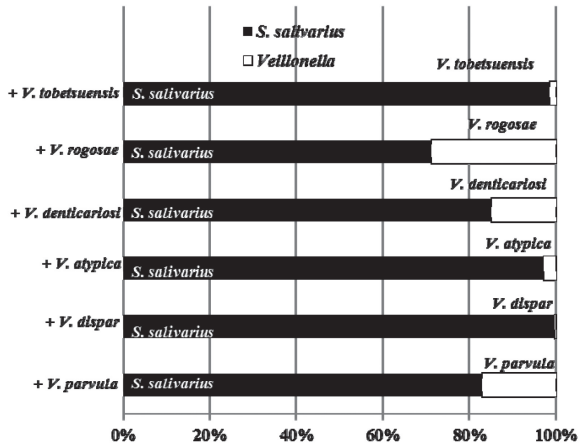

Fig.7, The ratio of 6 oral *Veillonella* species to *S. salivarius*.

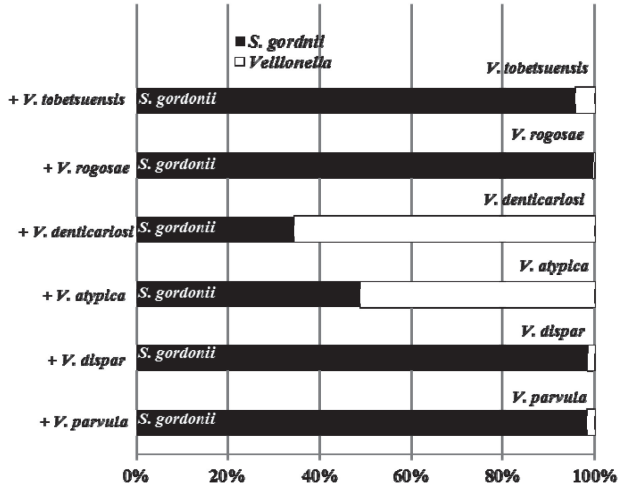

Fig.8, The ratio of 6 oral *Veillonella* species to *S. gordonii*.

case of the pairs of *S. salivarius* and *V. rogosae*, *S. salivarius* and *V. parvula*, *S. gordonii* and *V. denticariosi*, and *S. gordonii* and *V. atypica*, *Veillonella* spp. constituted 30%, 20%, 60%, and 50% of the respective biofilms (Figs. 7 and 8).

Discussion

From experiments in this time, it is suggested that this wire method has physical limits of the amount of biofilm formation to form biofilms at all stage. For this reason, in the present study, we demonstrated that the wire method is appropriate for generating experimental biofilms, especially biofilms at the early stages, such as those formed by *Streptococcus* and *Veillonella* spp. Our results indicate that this method may be useful for generating biofilms and quantifying the number of bacterial cells in the biofilm by using q−PCR. The wire method described here does not share the technical sensitivities or the high cost of other biofilm assays, such as the 96−well microtiter plate−based biofilm assays (Christensen et al., 1985) or the flow cell system (Foster & Kolenbrander, 2004), making it more suitable for routine use. Also, in the case of wire method, biofilms on the wire are formed by only bacterial properties.

The amount of biofilm formation with *Streptococcus* and the bacterial ratio in the biofilm depends on the particular *Veillonella* spp. used as the partner species. Our results suggest that *Veillonella* spp. plays important roles in the formation of the oral biofilm at the early stages. The data also suggest that *Veillonella* spp. assist the growth of *S. gordonii* and inhibit the growth of *S. mutans* on the wires in the biofilms formed after 7 days. Furthermore, the data suggest that oral *Veillonella* spp. have the ability to inhibit the caries activities caused by *S. mutans* because they inhibit their growth in biofilms. This fact may provide support for the findings of Mikx et al. (1972).

In this study, “the wire method” was used as a useful technique to generate biofilms for *in vitro* experiments. This method could be useful for investigating the relationship between biofilm formation and oral infections such as caries, periodontal diseases, and also systemic infectious diseases caused by oral biofilms. Moreover, this is the first report on the use of the wire method for generating early−stage biofilms from oral bacteria, using *Streptococcus* and *Veillonella* spp.

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眞島　いづみ

北海道医療大学大学院歯学研究科微生物学専攻博士課程第２学年

平成13年３月　富士見丘高等学校　卒業
平成16年４月　北海道医療大学歯学部歯学科　入学
平成22年３月　北海道医療大学歯学部歯学科　卒業
平成23年４月　北海道医療大学大学院歯学研究科　入学
平成24年現在　北海道医療大学大学院歯学研究科　在籍中