

Detection, isolation, and identification methods
for *Scardovia wiggsiae* and its involvement
in adult dental caries

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Title: Detection, isolation, and identification methods for *Scardovia wiggsiae* and its involvement in adult dental caries

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Abstract

The present study was conducted to develop selective media for the isolation of *S. wiggsiae* and *S. wiggsiae*-specific PCR primers for its detection and identification, and also to investigate its role in the development of adult dental caries. *S. wiggsiae* was detected in the dental plaque samples of caries-free adults (DMFT=0), adults with low-moderate DMFT (DMFT<5), and adults with high DMFT (DMFT≥5) at 0.0002, 0.0012, and 0.1026%, respectively, of the total bacteria number. The detection, isolation, and identification methods for *S. wiggsiae* developed in the present study were very useful. Moreover, the proportion of *S. wiggsiae* in dental plaque samples was significantly higher in the HD group than in the CF and LMD groups ($P < 0.05$). The selective medium and PCR method used for the detection, isolation, and identification of *S. wiggsiae* may contribute to the diagnoses of not only severe ECC but also adult dental caries.

Keywords: *Scardovia wiggsiae*, dental caries, selective medium, oral cavity

1. Introduction

Dental caries begins as decalcified enamel or white spot lesions and progresses into dentin. Dental bacteria invade the pulp and root canal if untreated. Three risk factors have been identified for the onset of dental caries: cariogenic bacteria, such as mutans streptococci, susceptible hosts or teeth, and the frequent intake of dietary carbohydrates. The rate of progression of dental caries depends on the degrees of these risk factors ¹⁾. The progression of early childhood caries (ECC), dental caries of the primary dentition, also known as nursing (bottle) caries, is very rapid. ECC is a serious public health issue worldwide, with a global prevalence of untreated caries of 8.8% ²⁾. Severe ECC seriously damages the primary dentition, and is the main reason why infants visit dentistry hospitals ³⁾. A high prevalence of severe ECC of between 36 and 85% was previously reported for 3-year-olds in Taiwan, the Philippines, and Korea ⁴⁾. Moreover, severe ECC is a risk factor for dental caries in permanent teeth ⁵⁾, which makes successful treatment difficult because up to 50% of children develop new caries lesions after dental treatment ^{6, 7)}.

Streptococcus mutans is acidogenic and aciduric; therefore, it is considered to be the primary pathogen in ECC ⁸⁻¹¹⁾. Previous studies ^{12, 13)} suggested that bacteria other than *S. mutans* were cariogenic pathogens because *S. mutans* was detected in caries populations but not in all cases of ECC. Moreover, Tanner et al. ¹⁴⁾ compared the oral flora in children with severe ECC and caries-free children and found that the principal caries-associated bacterial species were *S. mutans* and a new *Bifidobacterium* species, *Scardovia wiggsiae*. Bifidobacteria are acidogenic, aciduric, Gram-positive, pleomorphic-branched, non-motile, non-spore forming, and non-filamentous rods that

consist of 7 genera (*Bifidobacterium*, *Aeriscardovia*, *Falcivibrio*, *Gardnerella*, *Parascardovia*, *Scardovia*, and *Alloiscardovia*) and approximately 40 species ¹⁵). Among *Bifidobacterium* species, the genus *Scardovia* comprises two species, i.e., *S. wiggisiae* and *S. inopinata* (<http://www.bacterio.net/scardovia.html>). The genus name “scardovia” was selected in honor of Lois Wiggs, a distinguished American microbiologist. The type strain of *S. wiggisiae* was isolated from the human oral cavity and an arm wound of an intravenous drug user. This organism has been implicated in the development of human dental root caries and other lesions in older adults ¹⁶). However, the relationship between this organism and adult dental caries remains unclear.

Therefore, to obtain a more detailed understanding of this relationship, the present study aimed to establish detection, isolation, and identification methods for *S. wiggisiae* using selective medium and a polymerase chain reaction (PCR) method.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All bacterial strains used in the present study are listed in Table 1 and 2. All strains were maintained by cultivation on BactTM Brain Heart Infusion (BHI, Becton, Dickinson and Co., Sparks, MD, USA) and 1.5% agar (BHI agar). Anaerobic bacteria, i.e., *S. wiggisiae* and *Fusobacterium nucleatum*, were cultured at 37°C for 48 h under anaerobic conditions with a gas pack system (AnaeroPack[®]; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *S. wiggisiae* isolates (NUM-Sw 8070, NUM-Sw 8071, NUM-Sw 8073, NUM-Sw 8076, and NUM-Sw 8077) were obtained with non-selective medium, i.e., BHI supplemented with 1% yeast extract (BHI-Y), from the human oral cavity in this study. Strains other

than anaerobic bacteria were cultured at 37°C overnight in an atmosphere of 5% CO₂ in a CO₂ incubator (MCO-18AIC; Sanyo Electric Co., Tokyo, Japan).

2.2. Development of a new selective medium

2.2.1. Evaluation of the base medium

Some representative base media were used to confirm whether the base medium was suitable as a selective medium with a previously described procedure ¹⁷⁾. The plates on which *S. wiggisiae* were inoculated were cultured at 37°C for 5 days under anaerobic conditions.

2.2.2. Susceptibility tests and recovery of S. wiggisiae and other representative oral bacteria on selective medium

Preliminary studies on antibiotic selection were performed with BHI-Y agar using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method with BHI broth was used for susceptibility testing ¹⁸⁾. The recoveries of the *S. wiggisiae* reference strain, *S. wiggisiae* isolates, and other representative oral bacteria on selective medium were assessed as previously described ¹⁷⁾. Anaerobic bacteria and other bacterial strains were pre-incubated in BHI-Y broth at 37°C overnight in an atmosphere of 5% CO₂ in a CO₂ incubator and under anaerobic conditions. The plates on which all bacterial strains were inoculated were cultured at 37°C for 6 days under anaerobic conditions.

2.3. Clinical samples

Seventy volunteers (age 20-62 years, male 32, female 38) participated in the present study. The numbers of decayed teeth, missing teeth because of caries, or filled teeth (DMF teeth) in all subjects were scored according to the criteria of the World Health Organization (WHO, 1997). Exclusion criteria were as follows: patients with systematic diseases and those taking immunosuppressive agents or antibiotics. Subjects were divided into three groups, i.e., 30 caries-free adults (CF; the number of DMF teeth=0), 20 adults with low-moderate DMF teeth (LMD; the number of DMF teeth <5), and 20 adults with high DMF teeth (HD; the number of DMF teeth ≥ 5).

Dental plaque samples on the buccal surfaces of the first permanent molars of all subjects were obtained using sterile micro-applicators (Benda[®] Micro; Centrix, Inc., Shelton, CT, USA) and placed in a sterile microcentrifuge tube containing 1.0 ml of Tris-HCl buffer (0.05 M, pH 7.2). Samples were dispersed, and 0.1 ml of each was diluted and inoculated on CDC and selective medium plates according to a previously described procedure¹⁷⁾. Bacterial DNA in the remaining 0.9 ml of each sample was extracted using a previously described method¹⁹⁾ that directly detects *S. wiggsiae* with a PCR analysis. CDC plates for total cultivable bacteria and selective medium plates were cultured at 37°C for 6 days under anaerobic conditions. After cultivation, CFU/ml in each sample was calculated. The present study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 18-17-012-1).

2.4. Development of the PCR method to detect and identify *S. wiggsiae* using designed primers

Detection directly from clinical samples and the identification of *S. wiggisiae*-like colonies on selective medium were confirmed by a PCR analysis using *S. wiggisiae*-specific primers designed in the present study (Table 3). *S. wiggisiae*-specific primers were designed as described previously ¹⁷⁾. The accession numbers of the 16S rRNA sequences of *S. wiggisiae* and genetically close species, i.e. *S. inopinata* and *Parascardovia denticolens*, used in the present study were AY278626, D89332, and D89331, respectively. The development of the PCR method to detect and identify *S. wiggisiae* using the primers designed was achieved as previously described ¹⁷⁾. MightyAmp DNA Polymerase Ver. 3 (Takara Bio Inc., Shiga, Japan) was used as the PCR enzyme. In the present study, PCR conditions consisted of an initial denaturation step at 98°C for 2 min, followed by amplification for 30 cycles at 98°C for 10 s and at 68°C for 1 min.

2.5. Statistical analysis

The proportions of *S. wiggisiae* to total bacteria numbers in dental plaque samples from the CF, LMD, and HD groups were assessed with a one-way analysis of variance (one-way ANOVA). ANOVA was followed by post-hoc Tukey's tests where appropriate to compensate for multiple comparisons. Values of $P < 0.05$ were considered to be significant. Statistical analysis was performed using SAS program package version 9.3 (SAS Institute, Cary, NC).

3. Results

3.1. Development of selective medium

3.1.1. Selection of the base medium

An optimum base medium to add into the selective medium for *S. wiggisiae* was examined. *S. wiggisiae* grew well on BHI-Y as well as on other media (data not shown). Based on its low cost and convenience, BHI-Y was ultimately selected as the base medium.

3.1.2. Susceptibility to antibiotics

S. wiggisiae was more resistant to ofloxacin than oral Gram-negative rods, such as *Fusobacterium*, *Leptotrichia*, *Aggregatibacter*, *Porphyromonas*, *Prevotella*, and *Tannerella* species. The minimal inhibitory concentration (MIC) of ofloxacin for *S. wiggisiae* was 50 µg/ml. Oral Gram-negative rods were sensitive to 13 µg/ml of ofloxacin. *S. wiggisiae* was more resistant to fosfomycin than some oral Gram-positive bacteria, such as *Streptococcus* species and *Rothia* species. The MIC of fosfomycin for *S. wiggisiae* was 50 µg/ml. *Streptococcus* species and *Rothia* species were sensitive to 13 µg/ml of fosfomycin. *S. wiggisiae* was more resistant to cinoxacin than oral Gram-negative cocci, such as *Neisseria* and *Veillonella* species. The MIC of cinoxacin for *S. wiggisiae* was 100 µg/ml. *Neisseria* and *Veillonella* species were sensitive to 40 µg/ml of cinoxacin. *S. wiggisiae* was more resistant to gentamicin than *Actinomyces* species. The MIC of gentamicin for *S. wiggisiae* was 25 µg/ml. *Actinomyces* species were sensitive to 7 µg/ml of cinoxacin. *S. wiggisiae* was more resistant to aztreonam than other *Scardovia* species and genetically close species, i.e., *S. inopinata* and *P. denticolens*. The MIC of aztreonam

for *S. wiggisiae* was 100 µg/ml. *S. inopinata* and *P. denticolens* were sensitive to 13 µg/ml of aztreonam.

3.1.3. Composition of the new selective medium

The new selective medium, designated *S. wiggisiae*-selective medium (SWSM), was composed of the following (per liter): 37 g of BHI, 10 g of yeast extract, 15 g of agar, 10 g of galactose, 17 mg of bromocresol purple, 13 mg of ofloxacin, 13 mg of fosfomycin, 40 mg of cinoxacin, 7 mg of gentamicin, 13 mg of aztreonam, and 5 mg of amphotericin B. Antibiotics, i.e., ofloxacin, fosfomycin, cinoxacin, gentamicin, aztreonam, and amphotericin B, were added after the base medium had been sterilized and cooled to 50°C.

3.2. PCR method for identifying *S. wiggisiae*

3.2.1. Primer design

The specific primer set covering the upstream region of the 16S rDNA sequence of *S. wiggisiae* was designed in the present study (Table 3). The amplicon size of *S. wiggisiae* was 552 bp.

3.2.2. Detection limit

A PCR method was used to identify the *S. wiggisiae*-amplified DNA fragment of the expected size for this organism (Fig. 1). The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 5-

10 CFU per PCR template (5.6 µl) for the *S. wiggisiae*-specific primer set with the DSM 22547 strain (data not shown).

3.2.3. Assay of *S. wiggisiae* and representative oral bacteria

The PCR method used to identify *S. wiggisiae* produced positive bands from the *S. wiggisiae* reference strain DSM 22547, *S. wiggisiae* clinical isolate NUM-Sw 8070, and a *S. wiggisiae*-positive dental plaque sample (Fig. 1). Some *Streptococci*, *Actinomyces*, *Neisseria*, *Corynebacterium*, *Rothia*, *Veillonella*, *Fusobacterium*, *Aggregatibacter*, and *Staphylococcus* species were used as representative oral bacteria in the PCR analysis with the designed primer set. No amplicons were produced from not only any of the representative oral bacteria or the *S. wiggisiae*-negative dental plaque sample (Fig. 1), but also genetically close species, i.e. *S. inopinata* and *P. denticolens* (data not shown).

3.3. Recovery of *S. wiggisiae* and inhibition of other representative oral bacteria on the selective medium

Table 1 shows the recovery of the *S. wiggisiae* reference strain DSM 22547 and isolates on SWSM relative to CDC agar. The growth recoveries of the *S. wiggisiae* reference strain and isolates on SWSM ranged between 92.9 and 99.1% (average 96.9%) that on CDC.

Table 2 also shows the inhibition of other representative oral bacteria on SWSM relative to CDC agar. The growth of other representative oral bacteria was markedly inhibited on the selective medium.

3.4. Clinical examination

The detection frequencies of *S. wiggsiae* in dental plaque samples from the CF (n=30), LMD (n=20), and HD (n=20) groups are shown in Table 4. The average DMFT of the CF, LMD, and HD groups were 0, 2.7 (range: 1-4), and 11.2 (range: 5-20), respectively. The numbers of *S. wiggsiae*-positive samples from the CF, LMD, and HD groups were 4 (13.3%), 2 (10.0%), and 20 (100%), respectively. Comparisons of the proportion of *S. wiggsiae* in dental plaque samples among the 3 groups are shown in Fig. 2. *S. wiggsiae* in the dental plaque samples of the CF, LMD, and HD groups was detected at 0.0002, 0.0012, and 0.1120%, respectively, of total bacteria. The proportion of *S. wiggsiae* in dental plaque samples was significantly higher in the HD group than in the CF and LMD groups ($P < 0.05$).

In the first isolation, *S. wiggsiae* colonies on SWSM commonly had a rough, dry, folded, and convex appearance. The colony color and average colony size of *S. wiggsiae* on SWSM were light yellow and 1.1 mm in diameter, respectively (Fig. 3).

4. Discussion

ECC, also known as nursing (bottle) caries, is one of the most common infectious diseases in childhood¹⁴⁾. Although one of the most common causes of ECC was previously considered to be *S. mutans*, a recent study more strongly implicated the involvement of *S. wiggsiae* in severe cases of ECC than *S. mutans*²⁰⁾. Dental biofilms play a role in the formation of ECC and are derived from various factors, such as oral bacteria on the dentin surface, saliva, and dietary carbohydrates²¹⁾. *S. wiggsiae* in addition to *S. mutans* and other acid-producing bacteria have also been associated with white spot

initial caries lesions and aggressive caries in adolescents ²²⁾.

S. wiggsiae was isolated from root caries in adults, thereby expanding the age range of patients with *S. wiggsiae*-associated dental caries lesions ¹⁶⁾. Metabolically, *S. wiggsiae* contributes to the acidification of dental biofilms and, thus, is regarded as a caries-associated bacterium ²¹⁾. Although caries-associated bacteria, such as *S. mutans* and lactobacilli, cause dental caries, other caries-associated bacteria, including *S. wiggsiae*, cause dental caries even in the absence of the former bacteria, suggesting that the cariogenic potential of the latter bacteria needs to be clarified ²³⁾. Kameda et al. ²⁴⁾ recently revealed that *S. wiggsiae* was highly acidogenic and aciduric, similar to *S. mutans*.

It currently remains unclear whether this organism is part of the normal oral flora. Moreover, it may contribute to the development of not only severe ECC, but also adult dental caries. Therefore, a suitable selective medium and reliable identification method are needed to assess the prevalence of *S. wiggsiae* in dental caries, including severe ECC.

In the present study, we designed species-specific primers to identify *S. wiggsiae* using a PCR method. These primers distinguished *S. wiggsiae* and did not react with representative oral bacteria. Moreover, the PCR method in the present study directly used bacterial cell suspensions with MightyAmp DNA Polymerase Ver. 3 and was completed within approximately 2 hours.

A useful selective medium for detecting and isolating *S. wiggsiae* may contribute to assessments of the distribution and role of this organism at various locations in humans; however, there is currently no selective medium that is suitable for this purpose. In the present study, *S. wiggsiae* strains were more resistant to ofloxacin, fosfomycin, cinoxacin, gentamicin, and aztreonam than other representative oral bacteria. The growth of oral

bacteria detected in the oral cavity was inhibited by the addition of 3 mg/L ofloxacin, 13 mg/L fosfomycin, 40 mg/L cinoxacin, 7 mg/L gentamicin, and 13 mg/L aztreonam to BHI-Y agar. Moreover, amphotericin B was added to the selective medium to inhibit oral fungal growth. All of the *S. wiggisiae* reference strains and isolates grew well on the new selective medium, designated SWSM, while the growth of other bacteria was markedly inhibited (Table 1, 2). Moreover, the addition of galactose and bromocresol purple to the selective medium facilitated the identification of *S. wiggisiae* by its characteristic colony morphology and color (Fig. 2). *S. wiggisiae* colonies on SWSM were light yellow in color due to acid production from galactose. *S. wiggisiae* was previously reported to be capable of producing acid from galactose ²⁵).

Dental caries experience is an ideal indicator for assessing the risk of dental caries; however, the ability to infer a risk before the disease manifests is desired. To date, there have been no studies on risk factors with high positive and negative predictive values. Oral pathogens are the direct cause of dental caries and disease progression; therefore, these organisms may be used as risk markers. According to the ecological dental plaque hypothesis, environmental acidification may increase the number of acid-tolerant bacteria, such as mutans streptococci, lactobacilli, *Actinomyces*, and *Bifidobacteria*, in tooth biofilms and shift the process towards the demineralization of tooth surfaces ²⁶). Among the pathogens associated with severe ECC, *S. wiggisiae* has potential as a microbial risk marker because its prevalence is associated with disease manifestation ²⁰). In the present study, the prevalence of *S. wiggisiae* was compared among 3 groups. The results obtained indicated that *S. wiggisiae* may not be a part of the normal oral flora because it was detected in all subjects in the HD group, but only in a minority of those in the CF and

LMD groups. Moreover, the proportion of *S. wiggsiae* in dental plaque samples was significantly higher in the HD group than in the CF and LMD groups ($P < 0.05$). Accordingly, monitoring *S. wiggsiae* levels may be useful as a clinical indicator for the diagnosis of adult dental caries and also as a microbial risk marker for the prevention of this disease.

We developed a selective medium, designated SWSM, to isolate *S. wiggsiae* in the oral cavity of humans. Since SWSM is highly selective for *S. wiggsiae*, it will be useful for assessing the distribution and role of this organism at various locations in humans. The selective medium and PCR method used for the detection, isolation, and identification of *S. wiggsiae* may contribute to the diagnoses of severe ECC as well as adult dental caries.

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Table1 Recovery of *S. wiggisiae* on CDC blood and SWSM

Strain	CDC blood	SWSM	Recovery, %
	CFU/ml, $\times 10^8$	CFU/ml, $\times 10^8$	
<i>Scardovia wiggisiae</i>			
DSM 22547	2.0±0.1 ^a	2.0±0.3	99.1
NUM-Sw 8070	2.1±0.3	2.1±0.2	96.9
NUM-Sw 8071	1.4±0.3	1.3±0.3	92.9
NUM-Sw 8073	1.1±0.2	1.1±0.3	98.0
NUM-Sw 8076	0.9±0.3	0.9±0.2	96.6
NUM-Sw 8077	0.7±0.2	0.7±0.3	98.1

^a Ave \pm SD

Table 2 Growth of other representative oral bacteria on CDC blood and SWSM

Strain		CDC blood	SWSM
		CFU/ml, $\times 10^8$	Recovery, %
<i>Streptococcus oralis</i>	ATCC 35037	3.0	0
<i>Streptococcus salivarius</i>	ATCC 10557	3.2	0
<i>Streptococcus anginosus</i>	ATCC 10557	4.4	0
<i>Streptococcus mutans</i>	ATCC 33397	0.6	0
<i>Actinomyces naeslundii</i>	NCTC 10449	0.5	0
<i>Actinomyces oris</i>	ATCC 12014	0.7	0
<i>Actinomyces israeli</i>	ATCC 27044	0.8	0
<i>Corynebacterium matruchotii</i>	ATCC 14266	0.8	0
<i>Corynebacterium durum</i>	ATCC 33449	1.3	0
<i>Rothia dentocariosa</i>	JCM 3067	0.7	0
<i>Rothia mucilaginosa</i>	JCM 10910	0.9	0
<i>Rothia aeria</i>	JCM 11412	1.2	0
<i>Neisseria sicca</i>	ATCC 29256	0.7	0
<i>Fusobacterium nucleatum</i>	ATCC 25586	0.8	0
<i>Staphylococcus epidermidis</i>	ATCC 14990	2.1	0
<i>Neisseria sicca</i>	ATCC 29256	0.7	0

Table 3 Locations and sequences of species-specific primers for the 16S rDNA of *S. wiggisiae*

Species	Primer	Sequence	Product size (bp)	Position	Accession number
<i>S. wiggisiae</i>	SWF SWR	ATGCCTGTGTGGTGTGGTG GGCATCATCCAGGTTATGT	552	428-446 979-961	AY278626

Table 4 Detection frequency of *S. wiggisiae* in each group

Group	Average DMFT (range)	No. of <i>S. wiggisiae</i> -positive samples (%)
CF (n=30)	0	4 (13.3)
LMD (n=20)	2.7 (1-4)	2 (10.0)
HD (n=20)	11.2 (5-20)	20 (100)

Figure legends

Fig. 1 PCR assay to detect *S. wiggsiae*

Primers are a mixture of SWF and SWR. Lanes: 1, *S. wiggsiae* DSM 22547; 2, *S. wiggsiae* NUM 8070; 3, *S. inopinata* JCM 12537; 4, *Parascardovia denticolens* JCM 12538; 5, *S. oralis* ATCC 10557; 6, *S. sanguinis* ATCC 10556; 7, *S. salivarius* JCM 5707; 8, *S. anginosus* ATCC 33397; 9, *S. mutans* NCTC 10449; 10, *A. naeslundii* ATCC 12104; 11, *A. odontolyticus* ATCC17929; 12, *A. israelii* ATCC 12102; 13, *R. dentocariosa* JCM 3067; 14, *Corynebacterium matruchotii* ATCC 14266; 15, *C. durum* ATCC 33449; 16, *Neisseria sicca* ATCC 29256; 17, *Veillonella parvula* JCM 12972; 18, *Fusobacterium nucleatum* ATCC 25586; 19, *Aggregatibacter actinomycetemcomitans* ATCC 33384; 20, *Staphylococcus epidermidis* ATCC 14990; 21, *S. wiggsiae*-positive dental plaque sample; 22, *S. wiggsiae*-negative dental plaque sample. M, molecular size marker (100-bp DNA ladder).

Fig. 2 Comparison of *S. wiggsiae* proportions in dental plaque among 3 groups.

Fig. 3 Appearance of *S. wiggsiae* colonies on SWSM. A: *S. wiggsiae* colonies on SWSM inoculated with a dental plaque sample. B: Stereomicroscope image of *S. wiggsiae* colonies on SWSM.

Fig.1

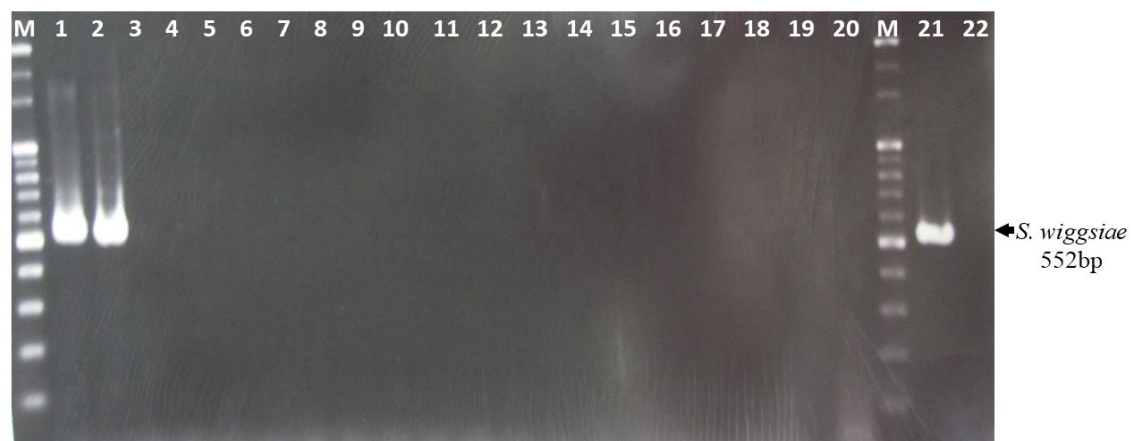


Fig.2

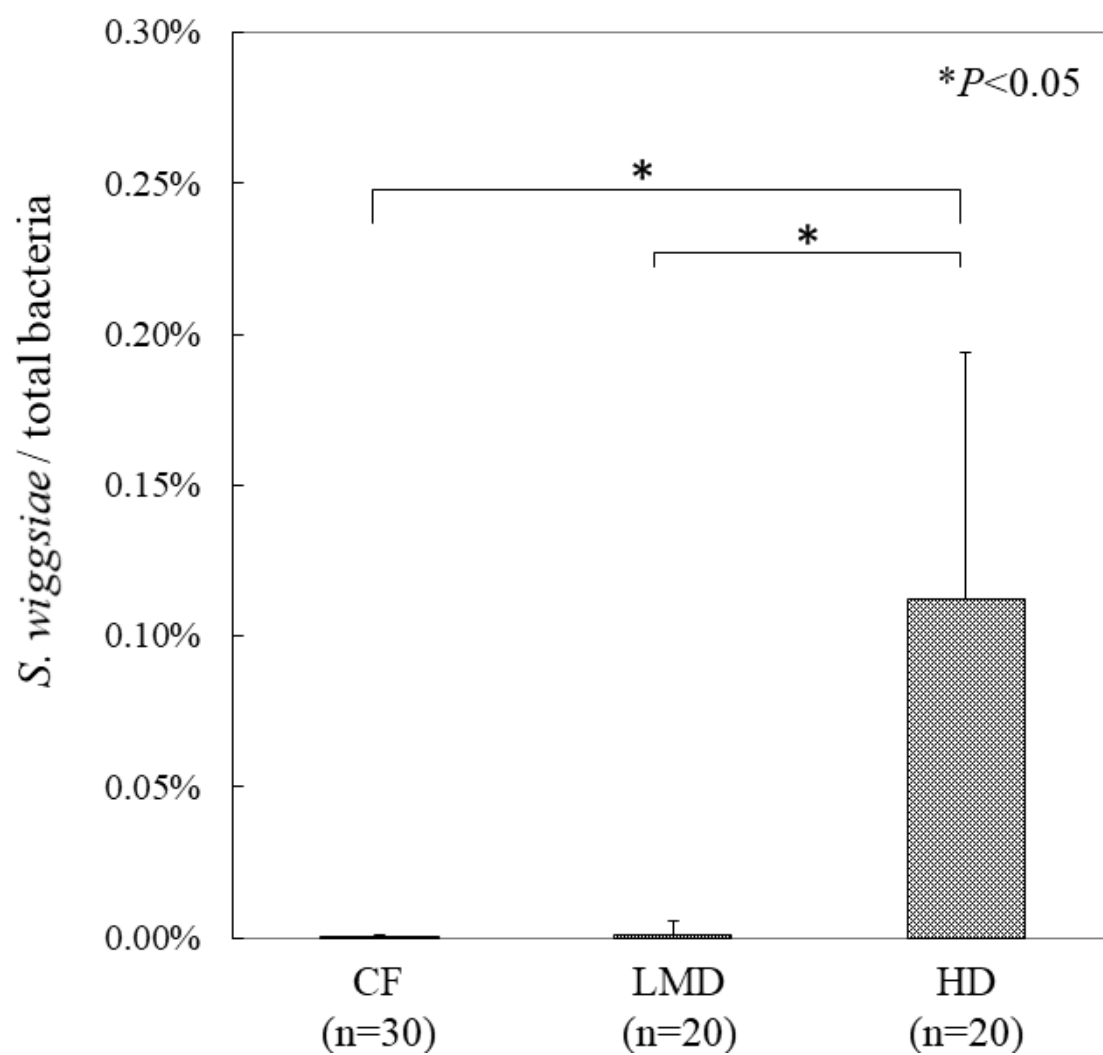


Fig.3

