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Impact of Quorum Sensing Inhibitors on Population Dynamics of Oral Bacteria

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Abstract
Quorum sensing (QS) signaling system of bacteria play a crucial role in controlling their population densities and interactions with each other as well as with their environments. The human oral cavity harbors one of the most complex microbiota of which one third of the species are yet cultivated. Isolation and culturing of oral bacteria is critical to understand their impact on health and diseases. In this study, we investigated the effect of QS inhibitors on the cultivability of oral bacteria and their population density by disrupting their QS signals. Oral samples were collected from six healthy individuals and seeded on agar media in the presence of QS inhibitors. Based on colony morphology and Gram staining, 121 colonies grown only on the agar media containing QS inhibitors, but not on the control agar media without QS inhibitors, were picked and characterized using standard bacteriological methods. Of the 121 isolates, 26 strains were further characterized by 16S rRNA sequence analysis, and a phylogenetic tree was constructed. These 26 strains belonged to the phyla of *Actinobacteria*, *Firmicutes*, and *Proteobacteria*. The results of this study indicated that culturing oral bacteria in the presence of QS inhibitors may alter their population densities which in turn promote cultivation and identification of bacteria that may be suppressed in various environments including different parts of the human body.
Keywords: 16S ribosomal RNA, oral cavity, population density, quorum sensing
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1. Introduction

Quorum sensing (QS) is a phenomenon by which bacteria communicate with each other to regulate physiological and biochemical activities via small signaling molecules (Papenfort and Bassler, 2016). These signaling molecules regulate the expression of genes related to functions such as biofilm formation, swarming, toxin, exopolysaccharide, and pigment productions (Eberl et al. 1996; McClean et al. 1997; Ohtani et al. 2002; Marketon et al. 2003; Rice et al. 2005). The basis of QS phenomenon extends from a marine bacterium, *Vibrio fischeri* (Nealson et al. 1970), which produces bioluminescence using signaling molecules when it reaches high cell density (Eberhard et al. 1981). Several QS signaling molecules such as N-acyl homoserine lactones (AHLs), autoinducing peptides (AIPs), autoinducer–2 (AI–2), and cyclic dipeptides have been identified in bacteria (Lade et al. 2014).

In the oral cavity, which is comprised of teeth, the gingival sulcus, gingiva, tongue, cheek, lips, and hard and soft palates, there is a unique microenvironment and microbiome (Dewhirst et al. 2010). About 1.200 prokaryotic species of 15 different bacteria phyla exist in the oral cavity. Oral bacterial taxa mainly belong to *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochetes*, and *Fusobacteria* phyla (Dewhirst et al. 2010; Chen et al. 2010). The predominant species belong to the genera of *Streptococcus*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Porphyromonas* and *Fusobacterium* (Aas et al. 2005; Zaura et al. 2009).

To understand the effects of oral bacterial growth in host health and diseases, isolation and cultivation of oral bacteria are critically important. In the oral microbiome, about 57% species are known and culturable, 13% are unnamed yet culturable, and 30% are known yet unculturable (Chen et al. 2010). Many factors may affect cultivation, such as unknown culture conditions, absence of essential nutrients or the need to co-culture two or more microorganisms that cross-feed (Thompson et al. 2015). Quorum sensing signal molecules may play a crucial role in keeping bacterial populations at low levels, thus making isolation and cultivation of bacteria difficult (Diggle et al. 2007; Kuhner et al. 2011). In this study, we aimed to investigate the effect of QS inhibitors on cultivation of oral bacteria and on their population densities.

2. Materials and methods

2.1 Quorum sensing inhibitors

Quorum sensing inhibitors used in this study were selected from the published literature (Table 1). Five groups were randomly formed from 30 commercially available QS inhibitors to better manage the experiments and

increase the additive effect of the inhibitors. Stock solutions of QS inhibitors were prepared at the highest possible concentration per manufacturer's recommendations, and the garlic extract was prepared as described previously (Rasmussen et al. 2005). Final concentrations of QS inhibitors were determined according to the sub-MIC values described below.

Table 1. The QS inhibitors list

QS Inhibitors	Group	Stock Concentration (mg/mL)	Solvent	Final Concentration (µg/mL)
5-azacytidine	1	10	Distilled water	3
Acylase I		1	Potassium phosphate buffer	1
Apigenin		2	DMSO	2
Ellagic acid		40	DMSO	20
Phenothiazine		10	Ethanol	40
Vanillin		10	Distilled water	100
Caffeine	2	20	Distilled water	100
Cinnamaldehyde		10	Ethanol	10
D-aminoacylase		0.5	Potassium phosphate buffer	1
EGCG		2	DMSO	2
GABA		40	Distilled water	60
P-coumaric acid		30	Ethanol	25
Curcumin	3	10	DMSO	5
Kaempferol		5	Ethanol	3
Naringenin		5	DMSO	40
Salicylic acid		10	Ethanol	9
Tannic acid		100	Distilled water	10
Xylitol		50	Distilled water	500
Cyclohexanone	4	100	Distilled water	100
Hamamelitannin		2	DMSO	2
Indole		10	Methanol	117
P-benzoquinone		10	Distilled water	2
Quercetin		10	DMSO	25
S-adenosylhomocysteine		1	DMSO	1
2,4,5-tribromoimidazole	5	10	Methanol	4
4-NPO		25	Distilled water	5
Bergamottin		5	DMSO	2
Cycloleucine		20	Distilled water	100
E,E-Farnesol		10	Ethanol	15
Garlic extract		1000	Distilled water	2000

2.2 Media

Oral cavity samples were cultured with and without QS inhibitor groups on brain heart infusion (BHI) agar (Himedia, Mumbai, India), Reasoner's 2A (R2A) agar (Lab M, Lancashire, UK), and buffered peptone water (BPW) agar (1% peptone, 0.5% sodium chloride, 0.15% potassium dihydrogen phosphate, 0.35% disodium hydrogen phosphate, and 1.5% agar) (Merck, Darmstadt, Germany). Briefly, 20 mL of each of three base agars were poured into 90 mm petri plates and allowed to solidify. The top agar layers with 0.9% agar at 49°C containing QS inhibitors were poured over the solidified base agars. Agar media with QS inhibitor solvents were used as controls.

2.3 Subjects and sample collection

Six healthy volunteers, three female and three males, ages between 18–65 were included in the study. Volunteers were selected from non-smokers and non-alcohol users, who had not taken any antibiotics for the last 6 months. Samples were collected after obtaining informed consent from all the subjects. The study was approved by the Ethics Committee of Karadeniz Technical University (permission number: 2015/107). From each subject, saliva and microbiological swab samples from tongue, hard palate, buccal mucosa, keratinized gingiva, throat, and palatine tonsils, supragingival and subgingival plaques were collected. The samples were collected in the morning, 12 h after tooth brushing and 2 h after the last food and/or drink intake (Zaura et al. 2009; McInnes and Cutting 2010). Saliva samples were collected into sterile falcon tubes without induction. The swab samples were collected with sterile cotton swabs and sterile Gracey curettes from soft tissues and tooth surfaces and transferred to microcentrifuge tubes containing 1 mL reduced transfer fluid-glycerol (RTF-glycerol) buffer (Kiliç et al. 2001). The samples were vortexed for one minute to remove attached microorganisms into the solution, and the cells were pelleted by centrifugation at 10,000 rpm for 5 min with a microcentrifuge. Pellets were resuspended in 0.1 mL RTF-glycerol buffer and combined in a sterile test tube. The saliva samples were also centrifuged at 1,500 rpm for

5 min to separate coarse particles, and about 1 mL supernatant was transferred to the test tube containing swab samples of the same subjects.

2.4 Determination of MIC of QS inhibitors

Minimal inhibitory concentration (MIC) of each QS inhibitor was determined as described previously (Brackman et al. 2009) against oral bacteria present in the pooled saliva samples of three healthy individuals. Briefly, 100 µL of oral sample was mixed with SHI medium (Tian et al. 2010) containing one of the QS inhibitors in a 96-well plate (Greiner Bio-one, Frickenhausen, Germany). The plates were incubated for 24 h and presence of growth indicated by turbidity was measured at 600 nm using a microtiter plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The lowest concentration of a given QS inhibitor with antibacterial effect was determined as the MIC value. To prevent any possible anti-bacterial effects of QS inhibitors, the sub-MIC concentrations of QS inhibitors were used as final concentration in the bacteriological culture studies so that any effect on the bacterial growth or population density would be attributed to QS inhibition but not antibacterial activity.

2.5. Selection of QS inhibitor promoted microbial species

Each oral microbiological sample was divided into two portions; one portion was serially diluted and seeded directly on three different agar media containing QS inhibitor groups as well as on control media without QS inhibitors. The second portion of the sample was diluted 100-fold in 0.1% peptone water containing QS inhibitors for 24 h of pre-enrichment. The pre-enriched test and control samples were diluted and seeded as described above. The cultures were incubated up to four weeks and examined daily for bacterial growth. All agar cultures were incubated at 37°C under 5% CO2 atmosphere unless otherwise indicated. Colony morphologies were compared under a stereo microscope, and bacterial cells of these colonies were analyzed using Gram staining, catalase and oxidase tests, pigmentation and hemolysis on blood agar. Colonies appeared only on agar media containing QS inhibitors but not control media were picked and subcultured for further characterization again using standard bacteriological methods such as colony morphology, Gram staining, catalase, oxidase and hemolysis reactions.

2.6. 16S rRNA gene sequencing and phylogenetic analysis

Genomic DNAs of 26 stains that were grown only on media containing QS inhibitors were isolated as previously described (Chen and Kuo 1993) with some modifications. Briefly, 1 µg/mL lysozyme (AppliChem, Darmstadt, Germany) was added to the lysis solution and brief bead-beating using 1 mm diameter glass beads (Marienfeld, Lauda-Königshofen, Germany) was performed to facilitate the lysis of gram positive bacteria. The 16S rRNA genes were amplified using 27F (5′–AGAGTTTGGATCMTGGCTCAG–3′) and 1492R (5′–GGTTACCTTGTTACGACTT–3′) universal primers. PCR products were purified using the PureLinkTM Quick PCR Purification Kit (Invitrogen-Thermo Fisher, Carlsbad, CA, USA) according to the manufacturer's instructions and sequenced using BigDye Terminator Chemistry (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 capillary DNA sequencer (Applied Biosystems). One or more of the following internal primers: 27F, 517F (5′–CAGCMGCCGCGGTAA–3′), 534R (5′–GWATTACCGCGGCKGCTG–3′), 784F (5′–AGGATTAGATACCCT–3′), 1099F (5′–GYAACGAGCGCAACCC–3′) and 1492R were used for 16S rRNA gene sequence determination. The sequence results were compared with taxa present the Human Oral Microbiome Database (HOMD, http://www.homd.org) with the 16S rRNA RefSeq Version 15.1 data set. We used 98.5% similarity as a cutoff value for species differentiation (Chen et al. 2010). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987). Robustness of the phylogenetic tree was determined with 1.000 bootstrap replicates, using the Molecular Evolutionary Genetic Analysis (MEGA) 6.0 program package (http://www.megasoftware.net) as described elsewhere (Tamura et al. 2013). The 16S rRNA sequences were submitted to GenBank with accession numbers as shown in Table 3.

2.7. Statistical analysis

The data were analyzed using Mann-Whitney U tests using IBM-SPSS statistics version 23.0 (IBM, USA). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Bacterial cultures

The effects of QS inhibitors on the growth and population dynamics of oral bacteria were evaluated using 30 QS inhibitors in five combinations at sub–MIC concentrations (Table 1) on BHI, R2A and BPW agar media. The culture plates were evaluated daily for up to four weeks for growth. Visible colonies on each plate were inspected under a stereo microscope and picked using sterile toothpicks and replica-plated on the same agar media in the presence and absence of QS inhibitors. From direct and pre-enriched cultures derived from all subjects, 121 distinct colonies grown only on media containing QS inhibitors, but not on the control media without QS inhibitors, were selected for further analysis (Figure 1) (Table 2). Although culture plates were incubated for four weeks, most of

the colonies were picked within two weeks. Isolated colonies from each replica plate without QS inhibitors were streaked on BHI, R2A, BPW and blood agar plates for further characterization of colony morphologies, pigmentation, catalase and oxidase reactions as well as hemolysis. Each isolate was able to grow on each agar media after 24-48 h incubation. Most of the isolates were catalase and oxidase negative, gram positive cocci with alpha hemolysis. A single colony of each isolate was subcultured in BHI broth for DNA isolation and storage at -80°C. All 121 isolates were further compared to one another based on their morphological and biochemical features and 26 presumptively unique strains were selected for further identification using 16S rRNA sequence analysis.

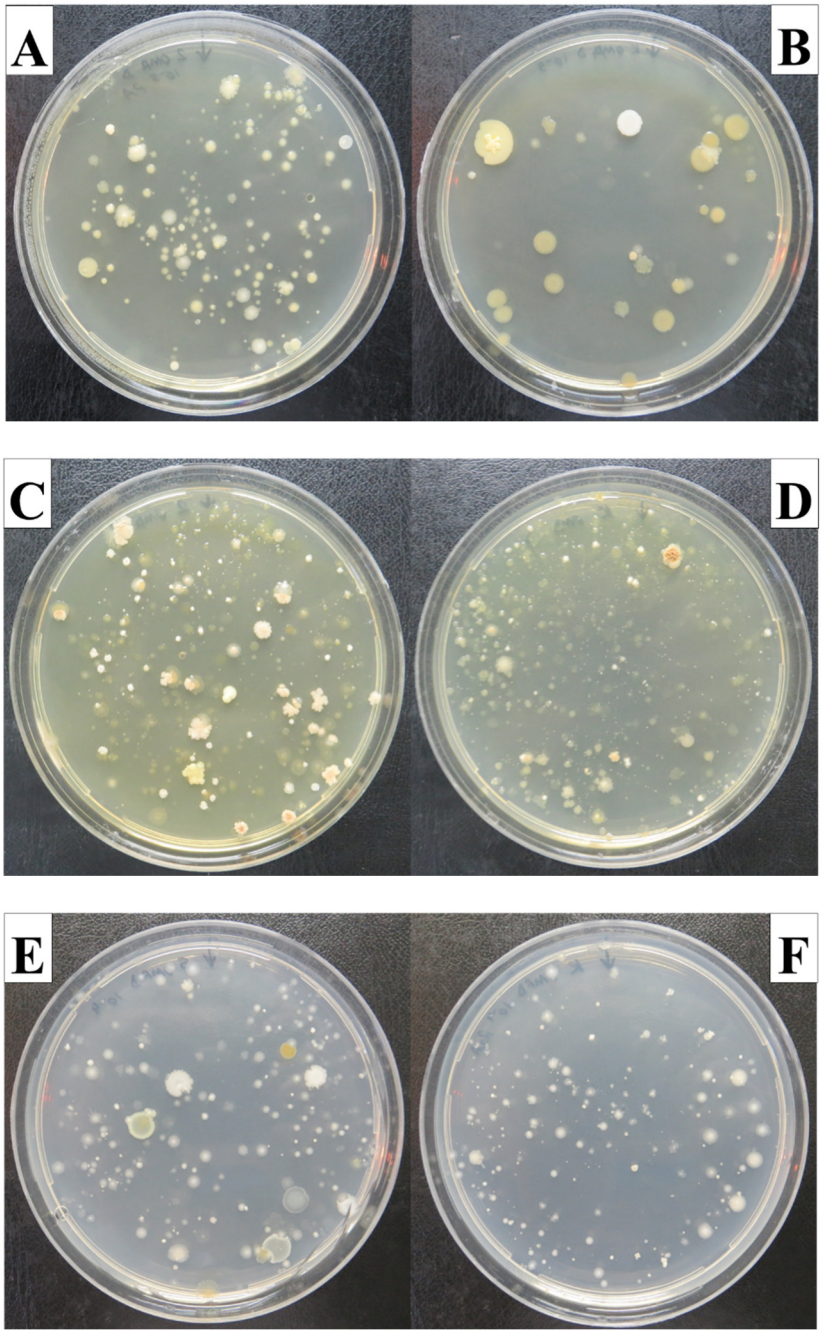

Figure 1. Examples of bacterial colony variations on agar media containing QS inhibitors. A and B, oral samples of subject 1 directly plated on BPW agar plates containing group 2 QS inhibitors (A) and control (B); C and D, oral samples of subject 4 directly plated on BPW agar plates containing group 2 QS inhibitors (C) and control (D); E and F, oral samples of subject 5 directly plated on R2A agar plates containing group 5 QS inhibitors (E) and control (F)

Table 2. Number of strains grown only on media containing QS inhibitors

Subject No	Direct cultures			Pre-enrichment cultures			Both of direct and pre-enrichment cultures			Total
	BHIA	R2A	BPWA	BHIA	R2A	BPWA	BHIA	R2A	BPWA	
1	2*/9	1*/4	2*/6	4	2	3	0	0	2	**5*/28**
2	1*/4	5	2*/6	1	2	0	1*/1	0	1	**4*/20**
3	3	2	2*/6	1	3	2	1*/1	1	1*/1	**4*/20**
4	1	3	2*/4	1*/2	0	1	2	0	0	**3*/13**
5	2	0	2*/3	0	1*/3	1*/1	1	3	1	**4*/14**
6	4	2*/8	4*/8	0	0	0	2	2	0	**6*/24**
Total	**3*/23**	**3*/22**	**14*/33**	**1*/8**	**1*/10**	**1*/7**	**2*/7**	**6**	**1*/5**	**26*/121**

* 16S rRNA sequences were determined

3.2. Effect of QS inhibitors on oral bacterial populations

The results of log Colony Forming Unit (CFU)/mL observed on each agar media with QS inhibitors and after pre-enrichment were compiled for six subjects are shown in Figures 2 and 3, respectively. Although there were no significant differences ($p > 0.05$) in the number of CFU between the QS inhibitor groups as compared to the controls (Figure 2), after pre-enrichment, the number of CFUs was reduced in Groups 1 and 3 compared to the controls regardless the media used ($p < 0.05$) (Figure 3). These results indicated that the number of colonies obtained on each agar media from oral samples of six subjects were not significantly different from each other regardless of direct seedings and pre-enrichments.

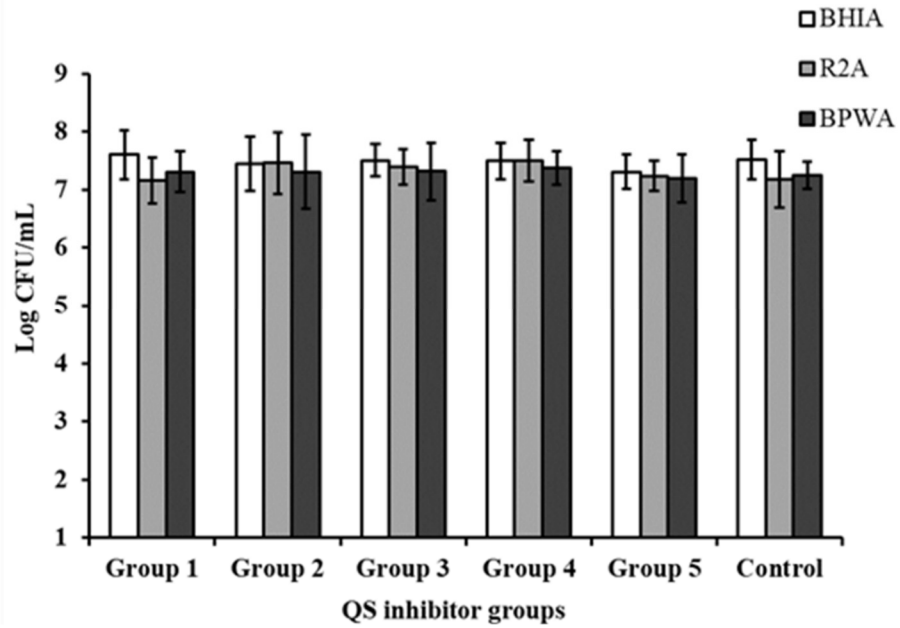

Figure 2. Average cell counts of oral samples of six individuals after direct seeding on agar media containing QS inhibitor groups 1 to 5 and control. Data are presented as the logarithm of mean CFU ± SD ($n = 6$, $p > 0.05$)

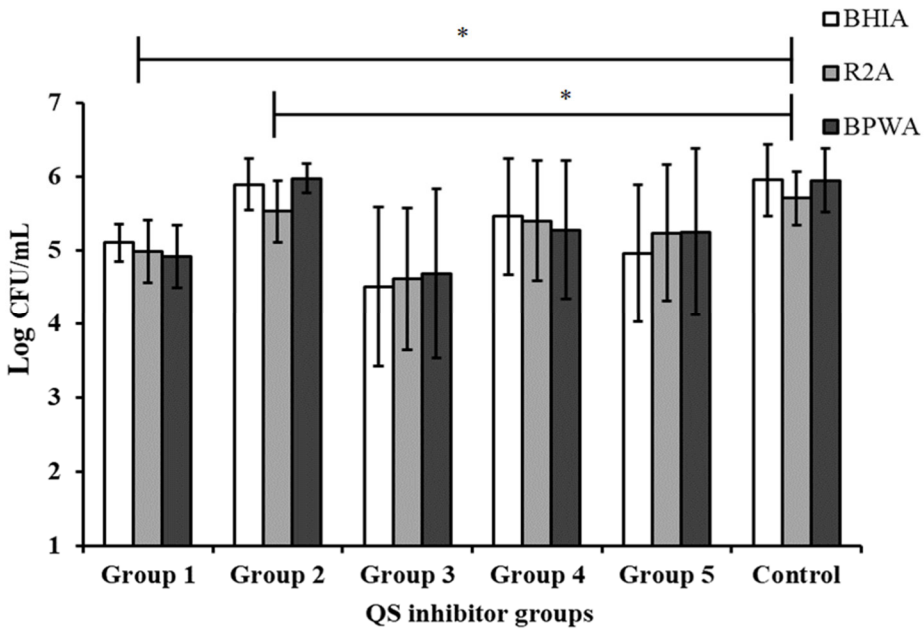

Figure 3. Average cell counts of oral samples of six individuals after pre-enrichment on agar media containing QS inhibitor groups 1 to 5 and control. Data are presented as the logarithm of mean CFU ± SD (n = 6, *p < 0.05)

3.3. 16S rRNA gene sequencing and phylogenetic analysis

Based on 16S rRNA sequence comparison with taxa present in the HOMD database, 25 of 26 strains showed homology with the known species, and one strain, TRO 148 showed 99.5% homology with a cultivable bacterium, *Streptococcus* sp. HMT–061 without any species designation. Sequence data 16S rRNA genes obtained from 26 strains ranged from 666 bp to up to the complete gene are presented in Table 3 with percent identity and mismatch bases. Twenty–six strains showed homology with 20 different bacterial taxa belonging to three major bacterial phyla (Table 3) (Figure 4), namely *Actinobacteria* (53.8%), *Firmicutes* (30.8%), and *Proteobacteria* (15.4%).

Table 3. Homology comparison of oral bacteria isolated from media containing QS inhibitors with Human Oral Microbiome Database (HOMD)

Strain	Accession Number	HOMD Taxon Name	Accession Number	Identities (%)	Mismatch (bp)
TRO 7	MG062894	*Neisseria bacilliformis*, HMT-013	EF509613	98.9	15/1393
TRO 18	MG062895	*Kocuria rhizophila*, HMT-197	NR_026452	99.9	2/1475
TRO 26	MG062896	*Neisseria flavescens*, HMT-610	EF511998	99.4	9/1404
TRO 30	MG062897	*Micrococcus luteus*, HMT-087	NR_075062	99.6	3/718
TRO 50	MG062900	*Streptococcus sanguinis*, HMT-758	AF003928	99.9	1/1400
TRO 55	MG062901	*Rothia aeria*, HMT-188	Y13025	99.9	1/1377
TRO 65	MG062902	*Streptococcus australis*, HMT-073	AF385525	99.3	11/1510
TRO 73	MG062903	*Corynebacterium durum*, HMT-595	Z97069	99.6	5/1326
TRO 75	MG062904	*Rothia aeria*, HMT-188,	Y13025	99.2	5/666
TRO 102	MG062905	*Streptococcus infantis* clade 431, HMT-431	AY278633	99.2	11/1389
TRO 114	MG062906	*Neisseria bacilliformis*, HMT-013	EF509613	98.5	21/1381
TRO 117	MG062907	*Streptococcus oralis* subsp. *tigurinus* clade 071, HMT-071	AY278631	99.4	9/1409
TRO 121	MG062908	*Rothia aeria*, HMT-188	Y13025	99.9	2/1375
TRO 148	MG029432	*Streptococcus* sp, HMT-061	AF432131	99.5	7/1510
TRO 155	MG062909	*Neisseria sicca*, HMT-764	AJ239292	99.8	3/1394
TRO 161	MG062910	*Corynebacterium matruchotii*, HMT-666,	AF543287	99.5	4/750
TRO 181	MG062911	*Rothia aeria*, HMT-188	Y13025	99.1	12/1383
TRO 182	MG062912	*Corynebacterium durum*, HMT-595	Z97069	98.5	20/1338
TRO 183	MG062913	*Streptococcus gordonii*, HMT-622	AF003931	99.0	13/1406
TRO 197	MG062914	*Streptococcus infantis* clade 431, HMT-431	AY278633	98.6	19/1398
TRO 213	MG062915	*Corynebacterium diphtheriae*, HMT-591	X82059	96.3	30/805
TRO 214	MG062916	*Rothia dentocariosa*, HMT-587	M59055	100	0/1393
TRO 215	MG062917	*Rothia aeria*, HMT-188	Y13025	99.3	9/1371
TRO 216	MG062918	*Actinomyces naeslundii*, HMT-176	AY008315	98.7	11/830
TRO 225	MG062919	*Janibacter indicus*, HMT-339	NR_134061	94.1	81/1379
TRO 229	MG062920	*Streptococcus infantis* clade 638, HMT-638	AB008315	99.0	14/1399

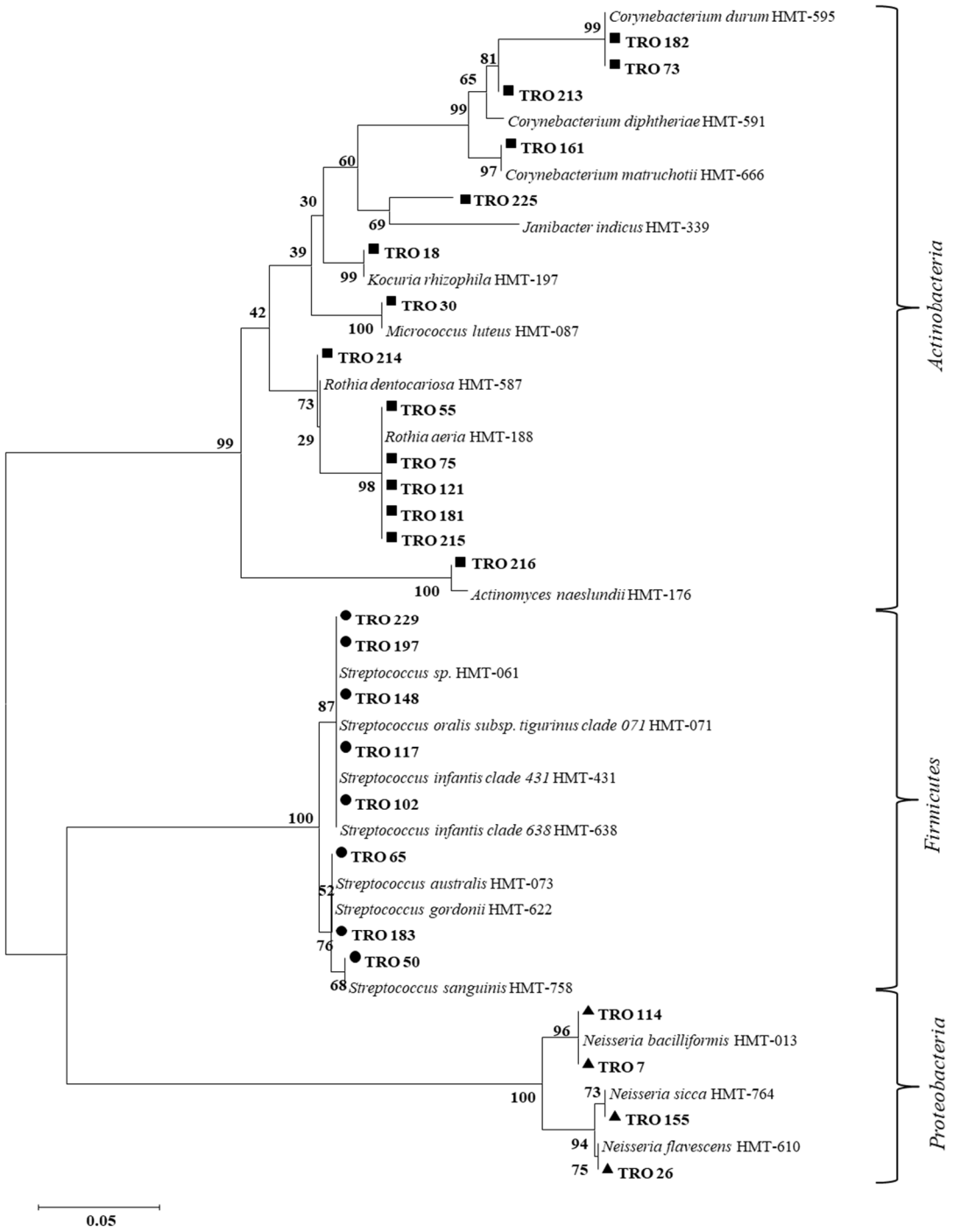

Figure 4. Phylogenetic tree of 26 strains isolated from agar media containing QS inhibitors. All strains belonged to either *Actinobacteria*, *Firmicutes* or *Proteobacteria* phyla. HMT, Human Microbial Taxon ID

4. Discussion

Many current bacteriological media are insufficient to grow and isolate all species in the complete oral microbiome (Tian et al. 2010). A variety of culture methods have been used to isolate previously uncultivatable or difficult-to-culture oral cavity bacteria, such as *Actinomyces* sp. HOT–172 (Human Oral Taxon), *Anaerolineae bacterium* HOT–439, *Lachnospiraceae* sp. HOT–500, and *Prevotella* sp. HOT–376 (Sizova et al. 2012; Thompson et al. 2015; Vartoukian et al. 2016). Another approach proposed by Kuhner and his colleagues (2011) involves the disruption of bacterial QS systems for culturing microorganisms. Certain bacteria regulate their growth and activities via QS systems in response to their population density and environment where nutrients are

homogeneously distributed. They do not need to compete for nutrients and prefer to disperse homogeneously rather than rapidly dividing and growth. Bacteria from such environments may not be cultured in vitro due to the population size control effect of QS systems. Since these dynamics may be present in the oral microbiome, we aimed to investigate the effect of QS inhibitors on microbial diversity and their population dynamics. To our knowledge, this is the first report showing the effects of QS inhibitors on culturing of oral bacteria.

The results of this study indicated that oral samples seeded directly on agar media in the presence of known QS inhibitors did not have any significant impact on the number of CFUs as compared to controls (Figure 1). Nevertheless, pre-enrichment of oral samples of six individuals in the presence of QS inhibitor Groups 1 and 3 resulted in significant reduction of total CFUs compared to the control. However, we observed slightly higher bacterial morphotypes grown on agar media containing QS inhibitors than on control media. Among the 121 strains, *Streptococcus* (Gram positive cocci chains, catalase-oxidase negative, and alpha hemolytic) was predominantly identified. This was an expected result given *Streptococcus* is dominant in the oral cavity (Dewhirst et al. 2010). Twenty-six of the 121 strains were identified by 16S rRNA sequencing. The results were compared with taxa found in the databases. Twenty–five isolates showed homology to known, named bacterial species and one of our isolates, TRO 148, shared 99.5% homology with the unnamed *Streptococcus* HMT-061. The status of *Streptococcus* sp. HMT–061 is indicated as "culture lost" in the HOMD. We were unable to confirm whether "culture lost" means the strain has been isolated previously and it cannot be found in stocks or it may not be revived from the stocks. *Streptococcus* sp. HMT–061 was first known as clone DN025 and found in the dorsal tongue of a halitosis patients via the use of culture–independent molecular methods (Kazor et al. 2003). We isolated TRO 148 from a healthy subject on BHIA media containing Group 4 QS inhibitors after pre-enrichment. Further characterization of TRO 148 is important to better understand its ecological and symbiotic role in the oral cavity since the cultivation of this strain is now possible.

In our work, it is noteworthy that there were five *Rothia aeria* HMT–188 isolates sequenced from the oral samples of four different subjects. Based on molecular analyses, the prevalence of *R. aeria* HMT–188 in the human oral cavity is 0.0602% presented in HOMD. *R. aeria* was first isolated in TGE medium (0.5% beef extract, 1% tryptone, 0.2% glucose, 0.1% triphenyl tetrazolium) from water and air specimens in the Russian Mir Space Station (Li et al. 2004). Oral *Rothia* species in saliva samples collected from 20 subjects were examined by culture method using selective medium which was developed by Tsuzukibashi and his colleagues (2017). In their study, *R. aeria* represented 0.8% of the total cultivable bacteria on general medium. This strain was frequently isolated from different subjects in our study. This suggested that *R. aeria* strains may be more easily and frequently isolated on agar media containing QS inhibitor groups, particularly Group 1 and Group 2.

According to the phylogenetic tree of our isolates, there were three main clusters. These clusters belonged to *Actinobacteria*, *Firmicutes*, and *Proteobacteria* phyla. Isolates were predominantly in *Actinobacteria* (53.8%), then *Firmicutes* (30.8%), and finally *Proteobacteria* (15.4%). Previous studies also showed that *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, and *Spirochetes* are the major phyla in the human oral cavity (Dewhirst et al. 2010; Chen et al. 2010).

There are several limitations in this study. First, the lack of anaerobic cultural condition may introduce bias toward aerobic and facultative species because strictly anaerobic species were not selected. Second, the selection method might allow a small number of colonies to randomly appear on the test but not the control agar media, producing a certain number of false–positive colonies. Third, among the 30 QS inhibitors used, there is a slight possibility that some chemicals might provide essential nutrients to certain oral bacterial species tested. If so, these bacteria species might grow up on the selective agar due to added nutrition, but not QS inhibition. However, this possibility is not high because at a higher concentration, these chemicals showed growth inhibition against oral bacteria in our MIC testing.

In summary, we have shown that culturing oral bacteria in the presence of QS inhibitors may alter their population densities and certain bacterial species may become frequently isolated. QS inhibitors may promote cultivation and identification of bacteria that may be suppressed in various environments, including different parts of the human body. In addition to the culture methods, metagenomic analysis may be required to better understand the impact of QS inhibitors on the oral bacterial population and the species diversity and relative abundance of the oral microbiome.

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Compliance with Ethical Standarts
The authors declare that they have no conflict of interest and any plagiarism issues.

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