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Molecular Detection of *Methylobacterium* Species in Oral Fibro-Epithelial Polyp Tissues of Male Patients and Their Risk Habits

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ABSTRACT

Objectives: This study aimed to assess the relative abundances of *Methylobacterium species* in oral fibro epithelial polyp (FEP) tissues of a cohort of male patients and evaluate associations with their life style risk habits.

Materials and Methods: The sample comprised 25 histologically confirmed oral FEP tissues of male patients selected from Oral & Maxillofacial Units across Sri Lanka. Amplification of nucleotides of extracted DNA from frozen tissues was performed using degenerate primers for the V1 to V3 region of the 16S r RNA gene and sequencing of the amplicon with Illumina's 2X 300–bp chemistry. Standard methodologies were used for sequencing, data processing and taxonomy assignment upto species level. Comparisons were made on percent average relative abundances of given *Methylobacterium* species by betel quid chewing, smoking and alcohol consumption habits using Fisher's exact test.

Results: The percent average relative abundances of *Methylobacterium sp. Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* of oral FEP tissues were 0.06%, 0.03% and 0.01% respectively. Significant differences were not evident in occurrence of given *Methylobacterium* species by betel quid chewing, smoking, and alcohol consumption habits categorized as never, past and current ($p>0.05$).

Conclusions: Our preliminary findings established and quantified *Methylobacterium sp. Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* in oral FEP tissues of male patients. Despite less than 0.1% of relative abundances of given species which is common to many taxa of the oral microbiome, those findings provide novel insights into molecular detection and diversity of oral *Methylobacterium*. Nevertheless, given the limitations of the present study, further research is warranted with large sample sizes to generate more conclusive evidence on implications of *Methylobacterium* species for oral health and possible associations with life-style risk habits.

Keywords: *Methylobacterium* species, Ubiquitous bacterium, Relative abundance. Betel quid chewing, Smoking, Alcohol consumption, Oral-fibro-epithelial polyps.

1. Introduction

The major entryway to the human body, the oral cavity, denotes one of the unique niches for ubiquitous microbes. Oral cavity demonstrates an exceptional microhabitat as the second most diverse residential microbiome in humans with a variety of metabolic and atmospheric requirements (1). The oral microbiome is dominated by the bacteriome ranging from 687-1072 species according to the human oral microbiome databases (2,3). There is an array of actions and reactions between the heterotrophic bacterial species solely depending on metabolites produced by the host

(4). Recent advances in genomic technologies, such as next generation sequencing (NGS), third generation sequencing (TGS) and bioinformatics enabled detailed exploration of oral microbiome along with functional and metabolic alterations in health and disease (5,6).

Methylotrophs are considered to be a group of heterotrophs among normal inhabitants and ubiquitous members of oral biofilm communities and human feet. Both the niches are rich in methylated sulphides that provide the energy source for this group of bacteria (7-11). They utilize carbon compounds containing one or more carbon atoms (5), mostly the methylated volatile compounds (methane, methanol, methanethiol, formaldehyde and dimethyl sulphide) containing sulfur as carbon and energy sources (7-11). Methanol dehydrogenase is an important enzyme implicated for methylotrophic metabolism which catalyzes oxidation of methanol to formaldehyde with subsequent metabolism to formate. Additionally, methylotrophs' catalase positivity is linked to a decrease in oral malodor. Therefore, it is reasonable to speculate that methylotrophic that metabolism may result in odor reduction in the mouth (7). In contrast, evidence also suggests that increased oral malodor is due to methylotrophic metabolism together with hydrogen sulphide and disulphide groups released from proteins (8).

The genus *Methylobacterium* accounts for mostly studied methylotrophic genera. It belongs to the family *Methylobacteriacea*, first described by Patt et al, in 1976 (12). It consisted of more than 20 species, including some previously grouped in the *Pseudomonas* genus (eg, *P. mesophilica*, *P. radiora*, and *P. rhodos*) subsequently amended by Green and Bousfield in 1983 (13). This was aimed at accommodating all pink pigmented facultative methylotrophic bacteria (often referred to as PPFM in the literature) (14) and to eliminate the original description of utilizing methane as a characteristic of this genus (15,16). With the advancements in 16S rRNA gene sequencing technologies the number of species assigned to the genus *Methylobacterium* was expanded to over 50 validly published species (17).

Methylobacterium species are abundant in the atmosphere, soil, dust, rhizophere, phyllosphere, plant and leaf surfaces (18,19) and aqueous environments including potable water supplies and hospital tap water (20). Furthermore, they have been isolated from water in dental and blood bank purification units (20-22). *Methylobacterium* species are commonly detected in environmental samples, but rarely found in clinical samples (23). Hence, the frequent presence and colonization of these strictly aerobic, fastidious, Gram-negative, and slow-growing bacilli in hospital environments and medical devices can lead to cross-contamination during endoscopy, catheterization and bronchoscopy procedures (23,24). The formation of biofilms and the resistance of those bacteria to disinfectants, high temperatures, and drying may be the reason for cross contamination (21). It has also been revealed that the *Methylobacterium* in biofilms could survive after contact with other cleaning agents, including 1% benzalkonium chloride for 24 hours (21). As a result, this opportunistic pathogen, despite its low virulence, can cause healthcare-associated infections in immunocompromised patients (23,24).

There is promising evidence on phenotypic and genotypic characterization of PPFM bacterium from upper molars of a male patient diagnosed with periodontitis and dental caries (11). Further, diverse methylotrophic bacteria were isolated from the tongue, and supra- and sub-gingival plaque in the mouths of volunteers and patients with periodontitis (7-10). Nevertheless, there is lack of in-depth investigations into the diversity and relative abundance of methylotrophic bacteria in the various niches of the oral cavity with their potential implications for oral health and overall health.

Fibro epithelial polyps (FEPs) denote common, benign intra oral lesions, manifesting as polypoid, pale, and firm swellings that are reactive and hyperplastic, attributed to chronic irritation (25,26). Hence, oral FEPs occurring on buccal mucosa and tongue among males with past or current life-style risk habits are not uncommon. Not only carcinogenic, but also inflammogenic materials are present in the masticatory substances used by these patients pertaining to betel quid chewing, smoking and alcohol consumption (6). Consequently, those habits induce oral mucosal trauma and chronic inflammation. The hyperplastic parakeratinized, stratified squamous epithelium with arcading pattern and mixed inflammatory cell infiltrate dominated by lymphocytes and plasma cells usually describes the anatomical pathology of oral FEPs (26). Therefore, it might be rational to argue that the oral FEP patients engaging in life-style risk habits, possessing

less optimal oral mucosal health status attributed to chronic inflammation induced by masticatory substances. Increasing evidence indicates oral microbiome as a key player in tumour development (6). Mechanisms such as chronic inflammation underpinned by microbial dysbiosis, mucosal barrier disruption, biofilm formation, and the production of genotoxic metabolites, are implicated for tumorigenesis by oral microbiome (6), Hence, exploration of microbiome in oral FEP tissue micro-environment among males engaging in life-style risk habits could offer novel insights into prevention and control of oral cancers.

As this study is focused on males with past and present life-style risk habits, reduction or enhancement of oral malodor could have important implications for their oral health. Further, it is rational to argue that methylotrophic metabolism could exert mixed outcomes with alcohol use. For example, methanol is found in spirits and traditionally brewed alcoholic beverages, which is considered to be toxic (27). However, to support or refute such speculations, a prerequisite would be exploring how relative abundances of *Methylobacterium* species are associated with life-style risk habits. Further, establishment and quantification of *Methylobacterium* species by relative abundance has not been reported previously. Hence, to address these gaps, this study is aimed at molecular detection and quantification of *Methylobacterium* species in oral FEP tissues of a cohort of Sri Lankan male patients, as well as to ascertain possible associations with their life style related risk habit status.

2. Materials and Methods

2.1 Study Design, Sample Size Calculation and Study Population

This was a sub-set (n=25) of a multi-center study that provided data for the assessment of microbiome profile of oral squamous cell carcinoma tissues compared to oral FEP tissues of male patients in Sri Lanka (3). This study was conducted in selected Oral & Maxillofacial Units in six provinces; namely, Western, Southern, Sabaragamuwa, North Western, Uva and Central (3). These numbers were based on a sample size calculation with a two-sided significance level of 0.05, a power of 90, a proportion of controls with exposure of 0.4, a proportion of cases with exposure of 0.6 and a ratio of sample size of 1 (28). The representative sub-sample for this study comprised 25 Sinhala males with histologically confirmed oral FEPs in buccal or tongue mucosa.

2.2 Eligibility Criteria

Males belonging to Sinhalese ethnicity with histologically confirmed FEPs arising from buccal mucosa or tongue, practicing/practiced at least one life style risk habit and those who were not on antibiotics for the past two months from the day of data collection comprised the inclusion criteria. In previous microbiome studies, patients who were not on antibiotics for past 1 month to 3 months were included to detect microbiome profiles (29). Thus, patients who were on antibiotics prior to 2 months of the day of data collection were included in this study, assuming that original flora was established after 2 months. This was assessed by recall and cross-checked with clinical records.

Exclusion criteria included ethnicities other than Sinhalese, those who did not practice any life risk habit, those who were on antibiotics within the past two months from the day of data collection and FEPs in sites other than buccal mucosa and tongue, as well as those not having histological confirmation.

2.3 Tissue Sampling

Deep tissue samples (~100 mg each) were dissected from each excisional biopsy of FEPs, avoiding contamination from the tumor surface. The samples were then stored at −80 °C (3,30).

2.3.1 DNA Extraction and 16S rRNA Sequencing

DNA was extracted using the Gentra Puregene Tissue kit (Qiagen) according to the manufacturer's protocol for solid tissue, with minor modifications. Those modifications included incubation in the lysis buffer performed overnight, an additional lysis step using 50 units of mutanolysin at 37 ^{0} C for 1.5 hours to digest cell wall of Gram positive bacteria prior to the addition of proteinase K. The V1 to V3 region of the 16S r RNA gene was amplified for sequencing using the degenerate primers 27FYM (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GWATTACCGCGGCKGCTG-3′). Library preparation, indexing, and sequencing were conducted at the Australian Centre for Eco-genomics (University of Queensland, Australia) using v3 2 × 300 bp chemistry on an Illumina MiSeq platform (3,30).

2.3.2 Sequencing, Data Processing and Taxonomy Assignment up to Species

Data was deposited at Sequence Read Archive under project no. PRJNA415963. Pre-processing of data, including primer trimming, merging, quality filtration, alignment, and chimera check, was performed with the exception of using a less stringent Q-score average cut-off for the sliding 50-nucleotide window (30 instead of 35). The high-quality nonchimeric merged reads were classified with the prioritized species-level taxonomy assignment algorithm as implemented by Al-Hebshi et al. (31-33). Each read was BLASTN searched against 4 databases of 16S r RNA gene reference sequences at alignment coverage and percentage identity ≥98% and then assigned species-level taxonomy of the hit with highest percentage identity and bit score, and belonging to the highest-priority reference set. Reads with no matches at the set cut-offs were subject to de novo operational taxonomic unit calling and assigned to the closest species (31-33).

2.4 Ethical Approval

Ethical approval for this study was granted by the Ethics Research Committee of the Faculty of Dental Sciences at the University of Peradeniya, Sri Lanka, and by the Griffith University Human Research Ethics Committee in Australia. Written informed consent was obtained from each participant.

2.5 Data Collection and Statistical Analysis

An interviewer administered, pre-tested questionnaire was used to collect data on socio-demographic characteristics and life-style related risk habits. Betel quid chewing, smoking and alcohol consumption were assessed as never, past, and current based on self-reports. Self-reports were partially complemented by objective measures, such as betel /tobacco stains and halitosis. Past behavior meant betel quid chewing, smoking and alcohol consumption given up a year or more before the time of data collection.

Data entry and analysis were carried out using IBM SPSS-26, (IBM Corp., Armonk, NY, USA) statistical software package. The percent average relative abundance of *Methylobacterium* species in oral FEP tissues was used to quantify the three *Methylobacterium* species. It was also used as the cut-off point to dichotomize the occurrence of selected *Methylobacterium* species in oral FEP tissues as < percent average relative abundance and ≥ percent average relative abundance. Occurrences were compared by betel quid chewing, smoking and alcohol consumption habits categorized as never, past and current by Fisher's exact test of statistical significance. $P<0.05$ was considered as statistically significant.

3. Results

3.1 Socio-demographic Profile of Oral FEP Patients and Their Distribution by OMF Unit Location

As shown in Table 1, oral FEP patients were males belonging to Sinhalese ethnicity. Most of them obtained secondary and above secondary educational attainment and are employed in diverse categories of occupations. Table 2 illustrates the distribution of oral FEP patients by their respective OMF Unit location, as this study was a multi-center study. Notably, the majority of patients (28.0%) were recruited from the Teaching Hospital Ratnapura, followed by the Teaching Hospital Kurunegala (20.0%).

Table 1: Socio-demographic profile of oral FEP patients

Variable	
Age mean ± SD in years	49.96±13.38
Age range	28-72 years
Gender	N %
Male	25 (100.0)
Ethnicity	
Sinhalese	25 (100.0)
Level of Education	N %
No Schooling	1 (4.0)
Primary Education	4 (16.0)
Secondary Education	10 (40.0)
Above Secondary Education	10 (40.0)
Occupation	N %
Farmer	8 (32.0)
Skilled/unskilled manual categories	7 (28.0)
Clerical/Professional	10 (40.0)

Table 2: Distribution of oral FEP patients by OMF unit location

OMF Unit location	N	%
Colombo National Dental Hospital (Teaching) Sri Lanka*	4	16.0
Base Hospital Panadura	1	4.0
District General Hospital Kalutara	3	12.0
District General Hospital Kegalle	3	12.0
Teaching Hospital Ratnapura	7	28.0
Teaching Hospital Badulla	1	4.0
Teaching Hospital Kurunegala	5	20.0
Teaching Hospital Kandy	1	4.0
Total	**25**	**100.0**

**Formerly Dental Institute.*

3.2 Occurrence of *Methylobacterium* Species in Oral FEP Tissues

As shown in Figure 1, Percent Average Relative Abundances of *Methylobacterium sp. Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* were 0.06, 0.03 and 0.01, respectively. The occurrence of *Methylobacterium sp. Oral Taxon C7* was 96.0% and 4.0% respectively, when dichotomized as < percent average relative and ≥ percent relative abundance. Similar occurrences were reported for *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* (Figure 2).

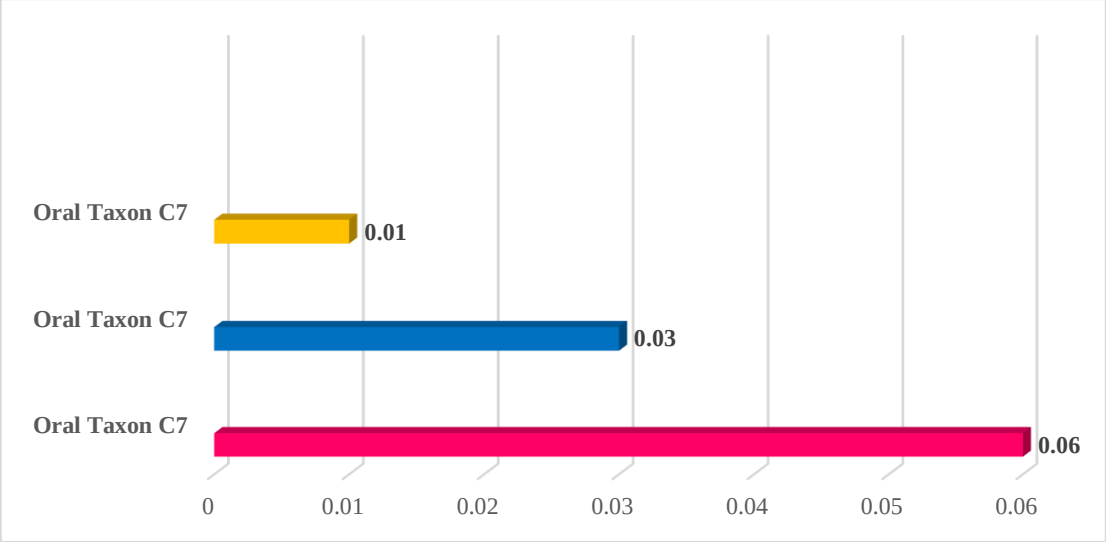

Figure 1: Percent average relative abundances of *Methylobacterium* species

3.3 Comparison of Occurrence of Methylobacterium Species by Life Style-related Risk Habits

Table 3 demonstrates occurrence of given *Methylobacterium* species in oral FEP tissues by betel quid chewing, smoking and alcohol consumption habits of males categorized as never, past and current. There were no statistically significant differences in the occurrence of *Methylobacterium sp. Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* by life style risk habits ($p > 0.05$).

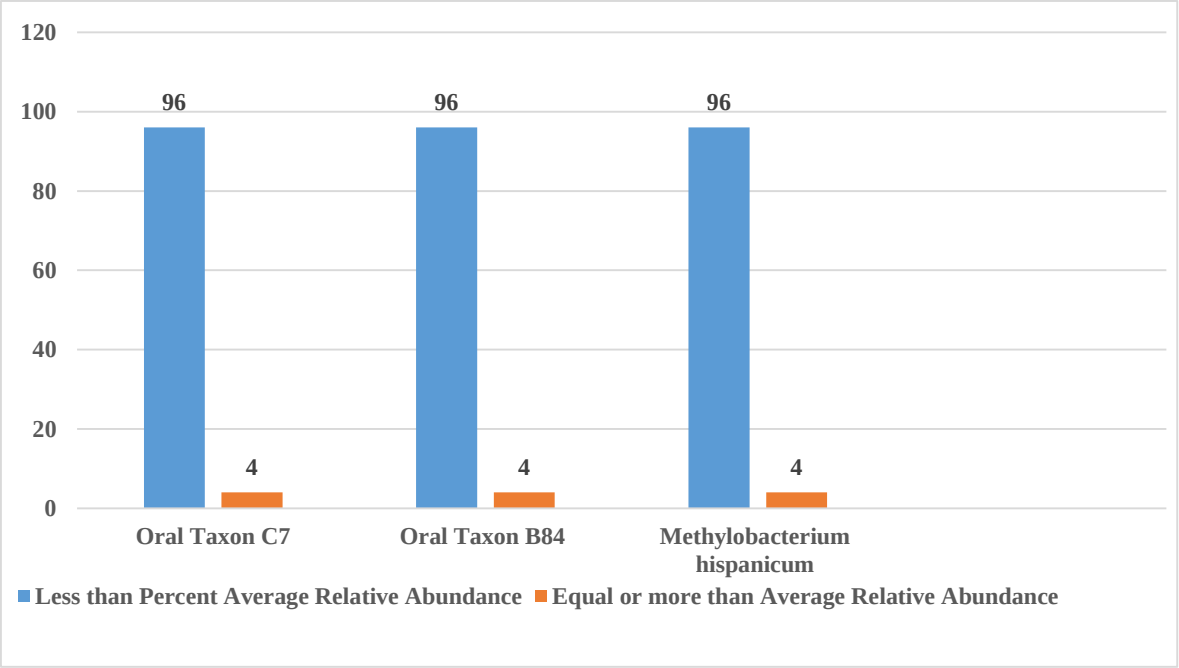

Figure 2: *Methylobacterium* species quantification by percent average relative abundance and assessment of their occurrence in oral FEP tissues of male patients

Table 3: Occurrence of *Methylobacterium sp. Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* by life style-related risk habits

Methylobacterium sp. Oral Taxon C7	**Risk Habit**	**<Percent Relative Abundance (<0.06) N (%)**	**≥Percent Relative Abundance (≥0.06) N (%)**	***p*-value***
	Betel quid chewing			0.08
	Never	4 (16.7)	0 (0.0)	
	Past	1 (4.1)	1 (100.0)	
	Current	19 (79.2)	0 (0.0)	
	Total	**24 (100.0)**	**1 (100.0)**	
	Smoking			0.56
	Never	7 (29.2)	1 (100.0)	
	Past	6 (25.0)	0 (0.0)	
	Current	1 (45.8)	0 (0.0)	
	Total	**24 (100.0)**	**1 (100.0)**	
	Alcohol consumption			1.00
	Never	8 (33.3)	0 (0.0)	
	Past	6 (25.0)	0 (0.0)	
	Current	10 (41.7)	1 (100.0)	
	Total	**24 (100.0)**	**1 (100.0)**	
Methylobacterium sp. Oral Taxon B84	Risk Habit	**<Percent Relative Abundance (<0.03) N (%)**	**≥Percent Relative Abundance (≥0.03) N (%)**	***p*-value**
	Betel quid chewing			1.00
	Never	4 (16.7)	0 (0.0)	
	Past	2 (8.3)	0 (0.0)	
	Current	18 (75.0)	1 (100.0)	
	Total	**24 (100.0)**	**1 (100.0)**	

	Smoking					1.00
	Never	8	(33.3)	0	(0.0)	
	Past	6	(25.0)	0	(0.0)	
	Current	10	(41.7)	1	(100.0)	
	Total	**24**	**(100.0)**	**1**	**(100.0)**	
	Alcohol consumption					
	Never	8	(33.3)	0	(0.0)	
	Past	6	(25.0)	0	(0.0)	
	Current	10	(41.7)	1	(100.0)	
	Total	**24**	**(100.0)**	**1**	**(100.0)**	
Methylobacterium hispanicum		**<Percent Relative Abundance (< 0.01)**		**≥Percent Relative Abundance (≥0.01)**		***p*-value**
	Betel quid chewing					1.00
	Never	4	(16.7)	0	(0.0)	
	Past	2	(8.3)	0	(0.0)	
	Current	18	(75.0)	1	(100.0)	
	Total	**24**	**(100.0)**	**1**	**(100.0)**	
	Smoking					1.00
	Never	8	(33.3)	0	(0.0)	
	Past	6	(25.0)	0	(0.0)	
	Current	10	(41.7)	1	(100.0)	
	Total	**24**	**(100.0)**	**1**	**(100.0)**	
	Alcohol consumption					0.56
	Never	7	(29.2)	1	(0.0)	
	Past	6	(25.0)	0	(0.0)	
	Current	11	(45.8)	0	(0.0)	
	Total	**24**	**(100.0)**	**1**	**(100.0)**	

*Fisher's exact test.

4. Discussion

Evidence supports isolation of diverse methylotrophic bacteria from the tongue, supra and sub-gingival plaque of healthy volunteers and patients with periodontitis (7-11). Oral methylotrophs are implicated for periodontitis; however, they are commonly detected among healthy volunteers as well (10,11). However, molecular detection with quantification of *Methylobacterum* species in the oral microbiome remains sparse. To the best of the authors' knowledge, this study is the first to detect any *Methylobacterium* species in oral fibro epithelial polyp tissues. Moreover, this becomes the first report of *Methylobacterium sp. Oral Taxon C7, Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* detected and quantified from the oral cavity. Thus, there are no corroborative studies to compare the results of the present study. *Methylobacterium hispanicum* has been previously detected in water samples (34), but not from the oral cavity.

Research on oral microbiome predominantly focused on molecular detection of species diversity and assessment of relative species abundance using 16S rRNA gene sequencing (35,36). Such NGS and recent TGS technologies have enabled generation of extensive data to identify and quantify microbial communities inhabiting different oral sites, such as tongue, buccal mucosa, palate, supra and sub-gingival plaque (6,35,36). However, there is notably limited research on assessment oral microbiome on benign oral mucosal lesions, such as FEPs (38). Our previous work on microbiome of oral FEP tissues revealed *Rothia mucilaginosa, Streptococcus mitis, Gamella haemolysans, Streptococcus sp. oral taxon 431, and Rothia dentocariosa* as the top five taxa among 810 bacterial species according to the percentage average relative abundance. *Rothia mucilaginosa* was elevated statistically significantly ($p< 0.05$) in oral FEP tissues (37). The genetic potential of xenobiotics and drug metabolism catalyzed by the P450 enzymes was observed for the first time as an attribute of bacteriome associated with oral FEP tissues dominated by *R. mucilaginosa* (37).

Microbial dysbiosis and pro-inflammatory bacteriome associated with oral squamous cell carcinoma micro tumor environment are well explored

(3,30,33,34). Recent research on microbiome of breast cancer tissues compared to non-cancerous tissues also demonstrated significant differences in microbiome profiles (39,40). Accordingly, breast carcinoma tissue microbiome clustered significantly differently from non-cancer tissues (p=0.03), largely driven by decreased relative abundance of *Methylobacterium* in cancer tissues (median 0.10 *vs.* 0.24, p=0.03) (38). This finding was contradicted by a systematic review and a meta-analysis that demonstrated significant elevation of *Methylobacterium radiotolerans* in gut and breast carcinoma tissues compared to healthy breast tissues (39). Therefore, emerging evidence indicates *Methylobacterium* as a potential biomarker of bacterial dysbiosis in breast carcinoma tissues (38,39). Importantly, *Methylobacterium radiotolerans* was detected in oral microbiome of 63 participants, especially from the tongue, gingival, and sub-gingival samples using DNA sequencing analysis of the 16S rRNA gene (7). Further, *Methylobacterium zatmanii* was detected in the oral cavity by a previous study (11). In contrast, our study did not detect *Methylobacterium radiotolerans* or *Methylobacterium zatmanii* in oral FEP tissues. This could be plausibly attributed to methodological difference in sample collection in those studies with regard to sample type and oral sites; i.e., swabs, plaque *vs*. FEP tissues.

The present study provides new insights into diversity of methylotrophs of oral microbiome by detecting and quantifying *Methylobacterium sp*. *Oral Taxon C7, Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* of oral FEP tissues. The percent average relative abundances of given species were 0.06%, 0.03% and 0.01%, respectively. Nevertheless, out of 25 samples, 24 (96%) contained < percent average relative abundances of given *Methylobacterium* species. Less than 0.1% of relative abundance seems common to many taxa of the oral microbiome (40). Further, relatively low abundant bacterial species have emerged as novel biomarkers in initiation of oral carcinogenesis (6). Therefore, low relative abundance could not be considered as a limitation for further research on *Methylobacterium* species in oral FEP tissues of male patients with life-style risk habits.

Betel quid chewing, smoking and alcohol consumption are among well-established etiological and risk factors for the burden of oral cancer (6.33). Betel quid, typically containing areca nut wrapped in a betel leaf accompanied by tobacco and slaked lime, denotes the key masticatory substance containing many carcinogenic chemical compounds implicated for the high burden of oral cancer in South and South East Asia (6,41). Chronic irritation due to masticatory substances could give rise to inflammation of the oral mucosa. Thus, life-style risk habits may cause benign mucosal lesions, such as oral FEPs, oral potentially malignant disorders (OPMD) and even oral cancers. Therefore, it might be rational to consider the oral FEP patients engaging in life-style risk habits, having less optimal oral mucosal health, oral malodor and elevated risk for OPMD and oral cancer.

Evidence suggests alterations in the relative abundances of different bacterial communities in the betel quid chewing and non-chewing population as well as smokers and alcohol users with betel quid chewing (41). However, it is not known how relative abundances of oral *Methylobacterium* species differ among betel quid chewers compared to non-chewers as well as smokers and alcohol users. Given the toxicity of methanol found in spirits and traditionally brewed alcoholic beverages (27), it is important to explore the impact of methylotrophic metabolism of methanol (42) among alcohol users. Surrounded by controversies, it is not clear whether methylotrophic metabolism reduces or increases oral malodour. Evidence supports opportunistic pathogenicity of *Methylobacterium* in immunocompromised patients (23,24) and their potential for acquiring antimicrobial resistance (10). As this bacterium is phylogentically closely related to certain species of *Pseudomonas*, acquiring multi drug resistance by horizontal gene transfer between related species becomes a possibility. Hence, further research is warranted to understand the pathogenic mechanisms of *Methylobacterium* species and their impact on oral health of betel quid chewers, smokers and alcohol users.

As this is a pilot study with a small sample size compounded by a low detection rate, it was not possible to find associations among percent relative abundances of *Methylobacterium* species and life-style risk habits. Low detection rate could be attributed to many reasons. For example, this study assessed and quantified given *Methylobacterium* species in oral FEP tissues, whilst supra and sub-gingival plaque and swabs of oral mucosal surfaces emerged as oral niches enriched in this group of bacteria (7-11). Habits were assessed

predominantly by self-reports partially complemented by objective measures, such as betel/tobacco stains and halitosis. Nevertheless, the possibility of social desirability response bias influencing self-reports of respondents could not be ruled out completely. This study assessed life-style risk habits as never, past and current. However, the possible associations of risk habits with *Methylobacterium* species could also be confounded by the duration of the habit, the amounts of betel quid chewed, smoked and alcohol consumed as well as the type of alcohol. Oral hygiene status and periodontal health status are among other confounding factors associated with risk habits as well as relative abundance of *Methylobacterium* species. However, they were not included in this analysis constrained by small sample size. Nevertheless, detection of *Methylobacterium* species in oral FEP tissues of otherwise healthy male patients with past and current practices of life-style risk habits necessitates further explorations. This will generate more conclusive evidence to suggest clinical implications. Given the emerging evidence on expanding diversity of Methylotrophic bacteria and their impact on halitosis, opportunistic pathogenicity and antimicrobial resistance, further research seems much warranted.

5. Conclusions

The present study detected and quantified three *Methylobacterium* species not reported from oral cavity previously. The percent average relative abundances of *Methylobacterium sp*. *Oral Taxon C7*, *Methylobacterium sp. Oral Taxon B84* and *Methylobacterium hispanicum* were 0.06%, 0.03% and 0.01%, respectively in oral FEP tissues of a cohort of Sri Lankan male patients. Despite less than 0.1% of relative abundances of given species, which is common to many taxa of the oral microbiome, those findings provide novel insights into this research arena of emerging importance. This study bridges the lack of information on molecular detection and quantification of *Methylobacterium* species in oral FEP tissues. Significant differences were not evident in occurrence of given *Methylobacterium* species by betel quid chewing, smoking, and alcohol consumption habits categorized as never, past and current ($p>0.05$) due to small sample size. This preliminary metagenomic study therefore, highlights the need for further studies with large sample sizes and rigorous methodologies. Future research should explore how life-style risk habits influence the relative abundances of *Methylobacterium* species and their implications for oral health.

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Conflict of Interests

No potential or actual conflict of interests was reported by the authors.

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