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Microbiome analysis of saliva from oral squamous cell carcinoma (OSCC) patients and tobacco abusers with potential biomarkers for oral cancer screening

Bolaji Fatai Oyeyemi [a,b], Urvinder S. Kaur [c], Amit Paramraj [d], Chintamani [d], Ravi Tandon [c,***], Abhinav Kumar [d,e,**], Neel Sarovar Bhavesh [a,*]

[a] Transcription Regulation Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi, 110067, India
[b] Department of Science Technology, The Federal Polytechnic, P.M.B. 5351, Ado-Ekiti, Nigeria
[c] Laboratory of AIDS Research and Immunology, School of Biotechnology, Jawaharlal Nehru University, New Delhi, India
[d] Department of Surgery, Vardhman Mahavir Medical College (VMMC)-Safdarjung Hospital, New Delhi, 110058, India
[e] Division of Trauma Surgery and Critical Care, Department of Surgical Disciplines, Jai Prakash Narayan Apex Trauma Center, All India Institute of Medical Sciences (AIIMS), New Delhi, 110029, India

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer and accounts for about 95% of all head and neck cancers with high mortality, usually at a late stage. Dysbiosis in the oral microbiome can lead to chronic inflammatory responses and may predispose to the development and progression of OSCC. Tobacco abuse plays an essential role in oral microbiome dysregulation and OSCC pathogenesis. We used 16S rRNA gene amplicon next-generation sequencing to examine microbial signatures unique to saliva from OSCC patients, tobacco abusers (TA) and controls (n = 10 for each group) to elucidate oral microbiome changes associated with tobacco abuse and OSCC. Overall, the oral microbiome compositions of class Betaproteobacteria and Epsilonproteobacteria, order Neisseriales, Burkholderiales and Campylobacterales, family Burkholderiaceae and Campylobacteraceae and genera Campylobacter and Leptotrichia revealed significant differences among OSCC patients, TA and control. Our preliminary pilot study not only serves as a basis for future studies with large sample size but also gives an indication of microbiome-based potential non-invasive biomarkers for early screening and monitoring of oral carcinogenesis transition due to tobacco abuse.

1. Introduction

Humans are not unitary but 'superorganisms' made up of their own and microbial cells that reside on the epithelial barrier surfaces

* Corresponding author. Transcription Regulation Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi, 110067, India
** Corresponding author. Division of Trauma Surgery and Critical Care, Department of Surgical Disciplines, Jai Prakash Narayan Apex Trauma Center, All India Institute of Medical Sciences (AIIMS), New Delhi, 110029, India
*** Corresponding author. Laboratory of AIDS Research and Immunology, School of Biotechnology, Jawaharlal Nehru University, New Delhi, India
E-mail addresses: ravitandon@jnu.ac.in (R. Tandon), dr.abhinav@aiims.gov.in (A. Kumar), neelsb@icgeb.res.in (N.S. Bhavesh).

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of the human body [1]. There is still no consensus on the number of microorganisms present in humans. Studies by Turnbaugh, Ley, Hamady, Fraser-Liggett, Knight and Gordon [2] and Sender, Fuchs and Milo [3] reported that the ratio of microorganisms to human cells is in a ratio of 10:1 and 1:1, respectively. The Human microbiome has been annotated to determine the association between microbiota perturbation and health/disease [2,4]. Microorganisms residing in the human oral cavity are termed oral microbiome; they are one of the essential components of the human microbiome [5]. These microorganisms are crucial to host health by resisting pathogens, maintaining delicate homeostasis and their profound modulating influence on immune cell function [6,7]. The human oral cavity contains more than 2000 bacteria taxa, opportunistic pathogens. They are usually considered the second most diverse microbial community after the gut [5]. They are thus important for metagenomics biomarker discovery for oral health. Reports have shown that altered oral microbiota may be related to oral health [4,6], especially oral carcinogenesis [4,5,7–9]. This was recently supported as a significant perturbation in the abundance of Lactobacillus, Neisseria, Phyllobacterium, Campylobacter, Lautropia, and unclassified Neisseriaceae were direct correlates of oral cancer [6,7,10].

Oral hygiene and habits like tobacco (smoke and smokeless) abuse and alcohol consumption can disturb the delicate balance in the oral microbiome composition [11,12]. The dysbiotic oral microbiome can generate toxins (genotoxins, lipopolysaccharide, nucleoside diphosphate kinase, gingipains, cytolethal distending toxin), inflammation-induced DNA damage and potentially tumor-promoting metabolites (nitrosamines, acetaldehyde). External stimuli, immune subversion, bacterial invasion, pro-inflammatory and autoimmune reactions, and cyto-genotoxic damages might eventually damage epithelial cells, resulting in cancer [5]. Tobacco and alcohol abuse are thus essential risk factors for oral cancer [13]. Habitual tobacco (smoke and smokeless) and alcohol abusers need regular oral screening so that potential oral cancer in the form of epithelial precursor lesions can be detected early, as studies have shown the transformation from epithelial precursor lesion (leukoplakia, oral submucosal fibrosis, and others) to cancer over time [14]. Subsequently, the relationship between oral cancer-inducing factors and the resident microbiome should not be neglected.

Earlier studies on the role of oral microbiota on OSCC have focused on the premalignant lesion (oral fibroepithelial polyp (FEP)

Table 1
Demography, clinical and other details of all subjects.

Details		OSCC (n = 10)	TA (n = 10)	Control (n = 10)
Age*		55.30 (14.40)	34.30 (11.88)	23.60 (0.97)
T. abuse	Now	9 (90.0)	10 (100.0)	
	Past	1 (10.0)	–	
	Never	–	–	10 (100)
T. per day	1 packet	5 (50.0)	3 (30.0)	
	2-3 packets	5 (50.0)	7 (70.0)	
	4-10 packets	–	–	
	Non-smoker	–	–	10 (100)
Smokeless T	Now	5 (50.0)	9 (90.0)	–
	Past	5 (50.0)	–	–
	Never	–	1 (10.0)	10 (100)
Smokeless T per day	1 packet	6 (60.0)	7 (70.0)	–
	2-3 packets	3 (30.0)	1 (10.0)	–
	4-10 packets	1 (10.0)	1 (10.0)	–
	Non-smoker	–	1 (10.0)	10 (100)
Chew T.	Never	1 (10.0)	2 (20.0)	10 (100)
	Now	6 (60.0)	8 (80.0)	–
	Past	3 (30.0)	–	–
Chew T. per day	1 packet	4 (40.0)	1 (10.0)	–
	2-3 packets	–	1 (10.0)	–
	4-10 packets	5 (50.0)	4 (40.0)	–
	>10 packets	–	2 (20.0)	–
	Non-chewer	1 (10.0)	2 (20.0)	10 (100)
Alcohol abuse	Never	–	1 (10.0)	10 (100)
	Now	7 (70.0)	9 (90.0)	–
	Past	3 (30.0)	–	–
Alcohol abuse per week	1–5 units	2 (20.0)	–	–
	6–10 units	4 (40.0)	5 (50.0)	–
	11–20 units	4 (40.0)	1 (10.0)	–
	>20 units	–	3 (30.0)	–
	Non drinker	–	1 (10.0)	10 (100)
Cancer stage	Stage I	1 (10.0)	–	–
	Sage II	1 (10.0)	–	–
	Stage III	4 (40.0)	–	–
	Stage IV	4 (40.0)	–	–
	No cancer	–	10 (100)	10 (100)
Histological type	Squamous cell carcinoma	100 (100.0)	–	–
	No cancer	–	10 (100)	10 (100)

Age reported as mean (SD), Number (percentage), T means tobacco which include cigarettes, cigars, pipes, bidis, smokeless T are chewing tobacco, oral snuff/snus and other unburnt products, and Chew T. includes betel quid/pan/gutkha/pan masala containing areca nut (supari).

* The mean difference is significant at the 0.05 level.

tissues, leukoplakia and oral submucosal fibrosis), OSCC patients and healthy controls [8,10,15,16], while others have elucidated the effects of smoking habits on oral microbiota [11,12]. However, there is a dearth of information on the difference in oral microbiota among tobacco abusers, OSCC and healthy controls in a single report. In this study, we show the alteration in the oral microbiome due to OSCC and tobacco abuse in a single study to find a potent biomarker of the development of OSCC.

2. Materials and methods

2.1. Ethics statement

The Institute Ethical Committees (IEC) of Vardhman Mahavir Medical College (VMMC)-Safdarjung Hospital and ICGEB New Delhi reviewed and approved the study vide approval numbers IEC/SJH/VMMC/PROJECT/JULY-2016/611, ICGEB/IEC/2017/03 and ICGEB/IEC/2018/01, respectively. Written and signed informed consent were sought and obtained from all participants. Approval from the Institutional Bio-Safety Committee was obtained and the biosafety guidelines of the Department of Biotechnology, Government of India were strictly followed.

2.2. Patients and saliva collection

Unrelated participants were randomly and independently recruited at the Department of Surgery's outpatient division (OPD) at VMMC-Safdarjung Hospital, New Delhi. All participants received information describing the study and were recruited only after they gave informed written consent for enrollment. OSCC was diagnosed based on clinical and histopathological criteria as described by the American Joint Committee on Cancer (AJCC) procedure [17,18]. Both pathological biopsy and specialized consultant confirmed the diagnosis of OSCC. OSCC-free individuals were categorized as tobacco abusers if they reported any ''current'' or history of daily cigarette smoking. Cigarettes, cigars, pipes, bidis, chewing tobacco, oral snuff/snus, betel quid/pan/gutkha/pan masala containing areca nut (supari) and other unburnt products were categorized as tobacco. Details of the study participants are shown in Table 1. All subjects recruited for the study were male with a median age of 31 years. Standardized questionnaires were used to collect information on fundamental traits, including smoking and drinking habits, general health, and denture use. The inclusion criteria included no past history of head and neck cancer. Data on basic characteristics were recorded using a structured questionnaire, including smoking and alcohol use habits, general health status, and denture wearing. Healthy control participants had never consumed tobacco, either active or passive and were free from any disease. Cancer-free status was confirmed by clinical screening every month over three months to cover the window period. All subjects were free from diseases that may affect their participation and not on medication, chemotherapy or radiotherapy before sample collection. The sample size for this study is thirty divided into groups of 10 subjects each. We matched our participants for diet while recruiting them for the study.

2.3. DNA extraction and 16S rRNA gene sequencing

DNA was extracted from 1.5 mL of saliva samples. Precipitate from centrifuged (12,000×*g* for 15 min at 4 °C) saliva was used for DNA extraction (Qiagen DNeasy Blood & Tissue Kit). The extracted DNA was quantified by Nanodrop 2000 (Thermo Scientific).

Approximately 25 ng of DNA was used as a template to amplify 16S rRNA hypervariable region V3–V4. The reaction includes KAPA HiFi HotStart Ready Mix and 100 nM final concentration of modified 341F and 785R primers (F 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3′, R 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′) with partial Illumina sequencing adapters overhangs. The PCR involved an initial denaturation of 95 °C for 5 min followed by 30 cycles of 95 °C for 30s, 55 °C for 45s and 72 °C for 30s and a final extension at 72 °C for 7 min. Additional eight (8) cycles of PCR were performed using Illumina barcoded adapters to prepare the sequencing libraries. The amplicons were purified using Ampure beads to remove unused primers before sequencing. Amplicon sequencing was performed on the Illumina MiSeq platforms. Libraries of Metagenome were produced using a TransNGS® DNA Library Prep Kit for Illumina® and sequenced with standard sequencing oligos on the Illumina MiSeq platform with 301 × 2 read length.

2.4. 16S rRNA gene analysis

Quality controls of the Illumina reads were performed using FASTQCToolkit.v0.11.7 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) to estimate the base call errors. Downstream sequence analysis was done in the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) software suite [19], and reads from all the samples were merged and subjected to de-noising. The reads were subjected to Chimera Filtering using UCHIME. No sliding window was used. OTU picking was performed using the *pick_otus.py* command with the default UCLUST algorithm. The UCLUST algorithm uses the USEARCH algorithm to assign sequences to a cluster [20].

The most abundant read in each OTU was selected as the representative sequence; this step was performed using pick_rep_set. py. Assign_taxonomy.py was used for the classification of each of the representative sequences. The default UCLUST consensus taxonomy assigner and 2013 Greengenes ribosomal reference database (13_8 release) [http://greengenes.secondgenome.com/downloads. Consequently] were used to assign taxonomy with 97% identity. Abundance for each sample is subjected to Cumulative Sum Scaling (CSS) normalization. Rarefaction curves were calculated from OTU tables using alpha diversity and rank abundance scripts within the QIIME pipeline. Beta diversity was also calculated with QIIME scripts with default metrics like unweighted_unifrac, and

weighted_unifrac. OTUs were used to analyze alpha diversity indices, specifically abundance-based coverage estimators [ACE], Chao1, Fisher, Shannon and Simpson, as executed in MirobiomeAnalyst [21].

2.5. Statistical analysis

Microbial community-wide dissimilarity measures: α-diversity was calculated using Chao1 and Shannon indices, while β-diversity was calculated from unweighted and weighted UniFrac distance. At the same time, the calculation of principal coordinate analysis (PCoA) was performed using Bray-Curtis dissimilarity (microbiomeanalyst). Ward clustering algorithm was used to build heatmap and dendrogram based on euclidean Bray-Curtis Index distance measures respectively as executed in microbiomeanalyst [21].

Group-based (phylum, class, order, family, genus, and species) percentage abundance in the stack bar and interactive pie charts were also computed to understand variation in oral microbiota in the study group. These abundances were subjected to univariate statistical comparisons by the Mann-Whitney/Kruskal-Wallis test using both p and FDR values. The significant microbiome was also subjected to ANCOVA, where age was used as the covariate. Group-based non-parametric factorial Kruskal-Wallis sum-rank analysis was calculated to understand taxa with significant differential abundance, followed by Linear Discriminant Analysis (LDA) Effect Size (LEfSe) with p values and Log LDA score > 1.0 to show pairwise abundance for every taxa [21].

Next-Generation Sequencing (NGS) raw data from this study have been deposited in the NCBI database with Sequence Read

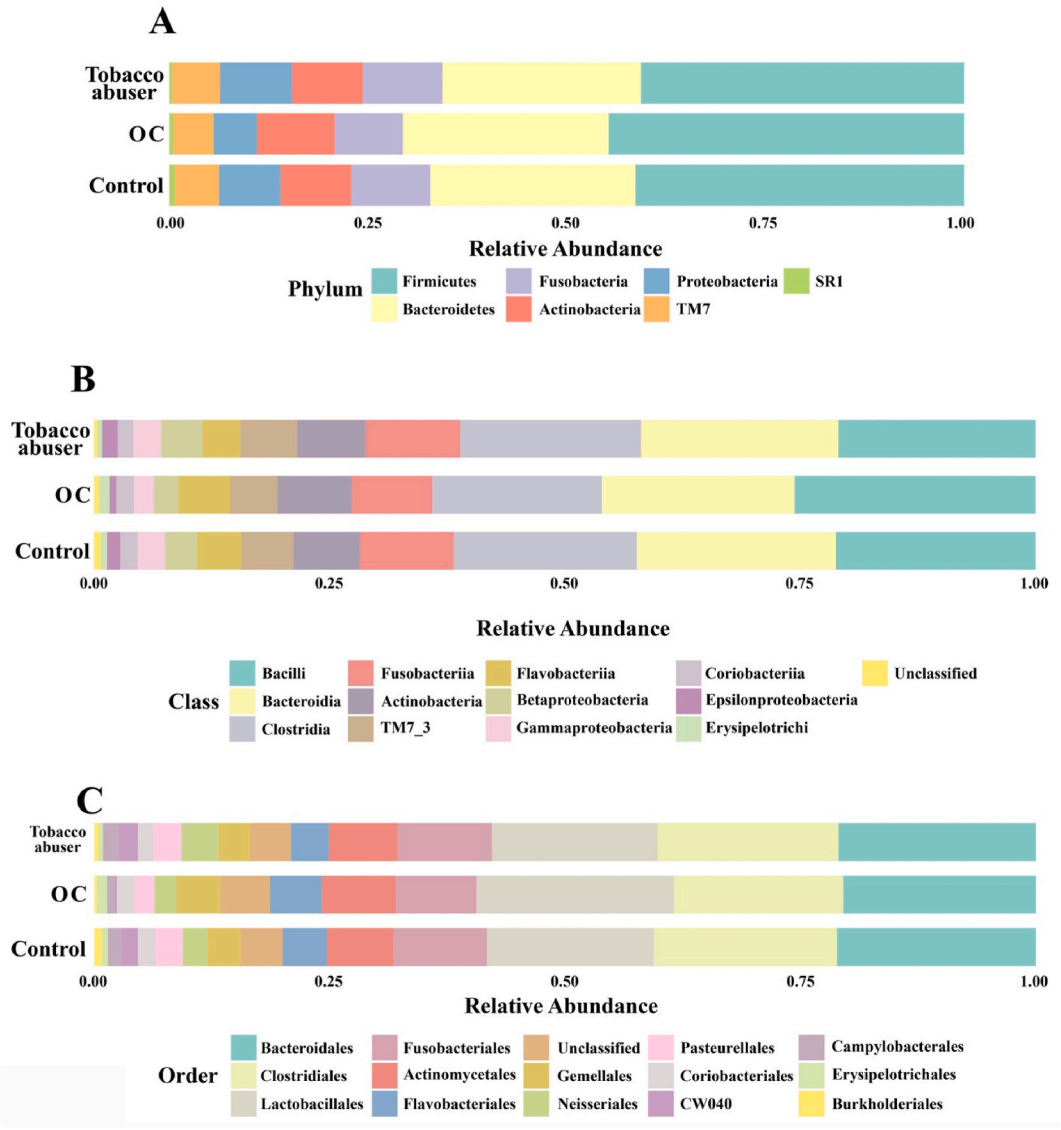

Fig. 1. Stacked bar plot showing mean relative abundance of most abundant **(A)** phyla, **(B)** class and **(C)** order from the saliva of healthy controls, tobacco abusers and OSCC.

Archive (SRA) accession number: PRJNA548462, available at https://www.ncbi.nlm.nih.gov/sra/PRJNA548462 and the Indian Nucleotide Data Archive (INDA) of the Indian Biological Data Centre (IBDC) with INDA Accession: INRP000100 and INSDC Bioproject Accession No.: PRJEB67576 available at https://ibdc.rcb.res.in/inda/home.

3. Results

3.1. Distinct pattern of oral microbiome among OSCC patients, tobacco abusers and controls

A total read count of 27,297 with 909 average counts per sample, 2028 and 119 maximum and minimum counts per sample, respectively, was reported, while group read statistics are shown in Supplementary Table 1. Both Principal Component Analysis and validated Partial Least Squares Discriminant Analysis did not reveal the difference in oral microbiota among the study group (Supplementary Fig. 1). All groups share a common microbial community as shown by non-significant alpha diversity values in the form of;

Fig. 2. Stacked bar plot showing mean relative abundance of the most abundant **(A)** family, **(B)** genus and **(C)** species from the saliva of healthy controls, tobacco abusers and OSCC.

ACE (p = 0.41416), Chao1 (p = 0.49926), Fisher (p = 0.40867), Shannon (p = 0.34995) and Simpson (p = 0.12782) (Supplementary Table 2 and Supplementary Fig. 2). To lend credence to this, a quantitative heatmap of varying phylum, class, order (Supplementary Fig. 3), family, genus, and species (Supplementary Fig. 4) fails to differentiate the study group.

The study group's most abundant phyla include Firmicutes and Bacteroidetes (Fig. 1A), while Bacilli, Fusobacteria and Clostridia are the most abundant class (Fig. 1B). As shown in Fig. 1C, Bacteroidales, Clostridiales, Lactobacillales and Fusobacteriales were the most abundant order. Group-based relative abundance in the form of stack bars of different Families, Genera and Species is presented in Fig. 2.

3.2. *Effect of OSCC and TA on oral microbiome*

The relative abundance of phyla (A–D), class (E–H) and order (I–L) from the saliva of the study group was presented in Fig. 3A–L. These varying abundant taxa were subjected to univariate analysis, and a significant reduction in class Betaproteobacteria and Epsilonproteobacteria in healthy controls when compared with OSCC and tobacco abusers (p = 0.020 and 0.034 respectively) was reported (Fig. 3E–F). Similarly, order CW040, Neisseriales, Burkholderiales and Campylobacterales were also downregulated in healthy controls with a corresponding increase in OSCC (except Neisseriales) (p = 0.001, 0.011, 0.022 and 0.034, respectively) (Fig. 3I–L). The abundance of CW040 and Campylobacterales are not significantly different in OSCC and tobacco abusers, as revealed by Dunn's multiple comparisons (Fig. 3I and L). There is no significant difference in the abundance of Burkholderiales in healthy controls and tobacco abusers, while order Neisseriale is significantly increased among tobacco abusers (p = 0.011).

Fig. 4A–L shows the comparative abundance of family, genus and species from the saliva of healthy controls, tobacco abusers and OSCC. As shown in Fig. 4A–D, the relative abundance of family F16 (p = 0.002), *Burkholderiaceae* (p = 0.022) and *Campylobacteraceae* (p = 0.034) were remarkably prevalent in the OSCC with corresponding reduction in healthy controls. *Neisseriaceae* is highly abundant among tobacco abusers, followed by OSCC and the least in healthy controls (p = 0.011). *Leptotrichiaceae* is significantly lower in OSCC

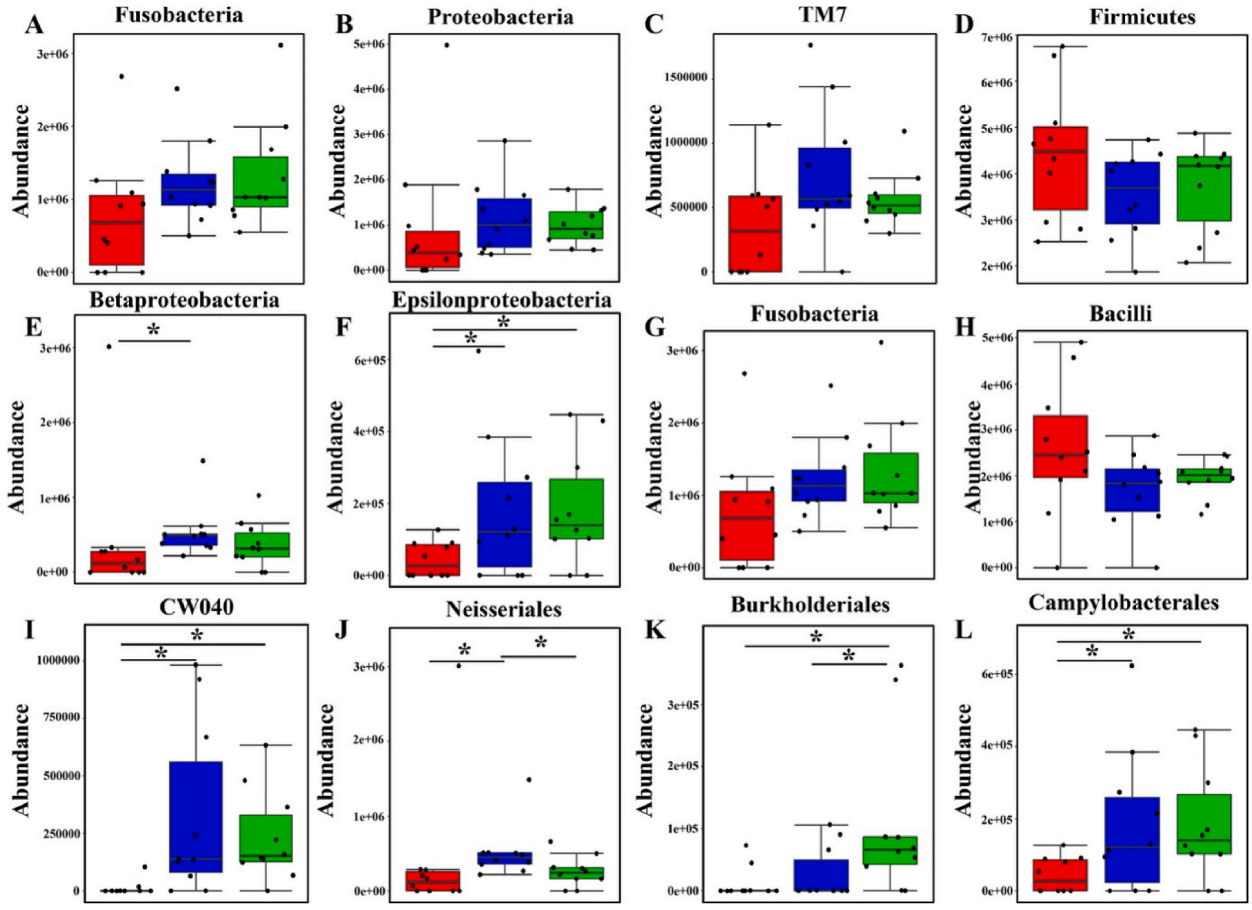

Fig. 3. Box and Whisker plots showing the comparison of relative abundance of phyla **(A–D)**, class **(E–H)** and order **(I–L)** from the saliva of healthy controls, tobacco abusers and OSCC. The horizontal line and deviation in the dot plot represent the median and interquartile range (IQR), respectively. Statistical differences in the abundance of oral microbiota at phylum, class and order levels were computed using the Kruskal-Wallis test with Dunn's multiple comparison. *p < 0.05 was considered to be significant.
Red, blue, and green colour are for OSCC, TA and control, respectively.

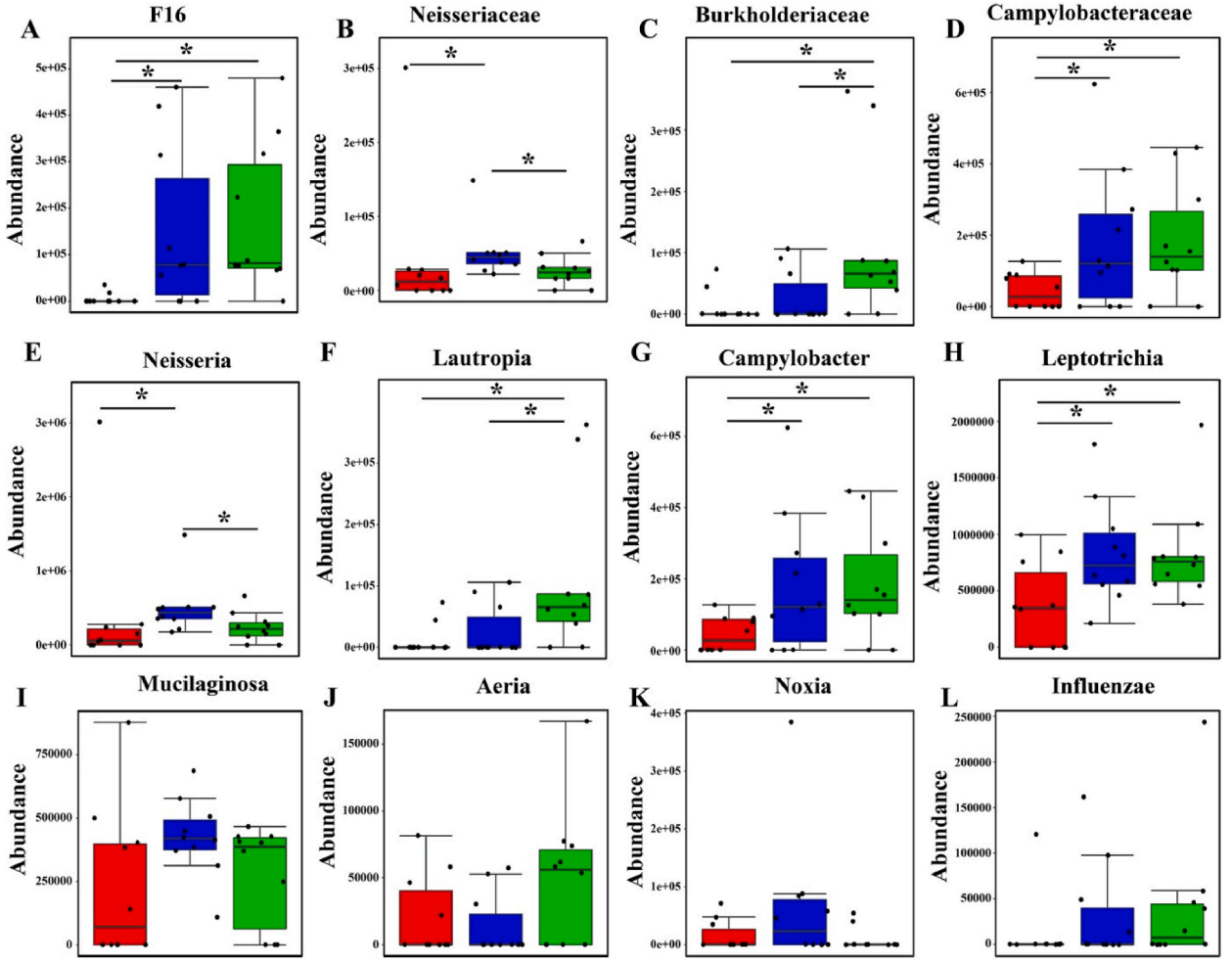

Fig. 4. Box and Whisker plots showing the comparison of relative abundance of **(A–D)** family, **(E–H)** genus and **(I–L)** species from the saliva of healthy controls, tobacco abusers and OSCC. The horizontal line and deviation in the dot plot represent the median and interquartile range (IQR), respectively. Statistical differences in the abundance of oral microbiota at family, genus and species levels were computed using the Kruskal-Wallis test with Dunn's multiple comparison. *$p < 0.05$ was considered to be significant.
Red, blue, and green colour are for OSCC, TA and control, respectively.

when compared with tobacco abusers and healthy controls ($p = 0.038$). The abundance is similar in healthy controls and tobacco abusers.

Both genera *Campylobacter* ($p = 0.034$) and *Leptotrichia* ($p = 0.038$) were significantly increased in OSCC, and tobacco abusers as compared to controls (Fig. 4G–H). There is also a higher abundance of *Lautropia* among OSCC patients with corresponding low values in the controls and tobacco abusers ($p = 0.022$) (Fig. 5B). A significantly higher abundance of *Neisseria* was reported among tobacco abusers than others ($p = 0.010$) (Fig. 4E). Non-significant increased abundance of phyla Fusobacteria and Proteobacteria were found in OSCC and tobacco abusers compared to healthy controls (Fig. 3A and B). A direct opposite was observed for phylum Firmicutes, though not significant as p is > 0.05 (Fig. 3D).

3.3. Microbial taxa associated with OSCC and TA

Differentially abundant phylum, class, order, family, genus and species in control and OC were analyzed with Linear Discriminant Analysis (LDA) Effect Size (LEfSe) as shown in Fig. 5A–F, respectively. These differences are also confirmed with pairwise linear discriminate analysis (LDA) effect size (LEfSe) using p-value < 0.05 and Log LDA score > 1.0. As shown in Fig. 5, Phylum Tenericutes (5A), Class Mollicutes (5B), Order Mycoplasmatales, Burkholderiales, Campylobacterales and CW040 (5C), Family *Mycoplasmataceae, Burkholderiacea, Campylobacteraceae, F16 and Leptotrichiaceae* (5D), Genus *Mycoplasma, Lautropia, Campylobacter and Leptotrichia* (5E) abundance are significantly different ($p < 0.05$) between healthy controls and OSCC. Supplementary Fig. 5 shows no significant difference in microbiota among controls and tobacco abusers. Differentially abundant phylum, class, order, family, genus and species in OC and tobacco abusers were analyzed with LEfSe as shown in Fig. 6A–F, respectively. Class Betaproteobacteria and

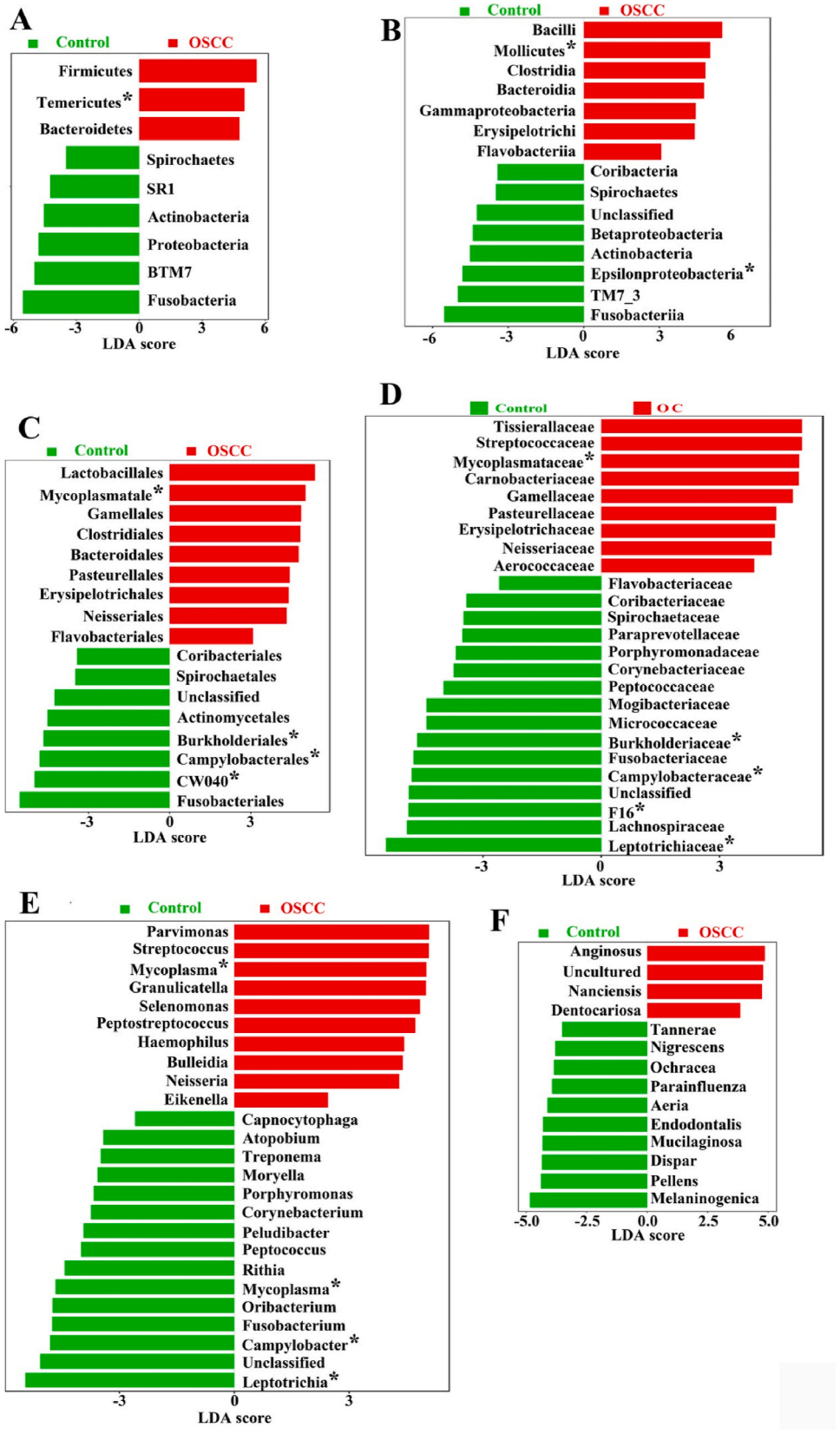

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Fig. 5. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) showing differentially abundant **(A)** phylum, **(B)** class, **(C)** order, **(D)** family, **(E)** genus and **(F)** species in controls and OC patients. *abundance significantly different at $p < 0.05$.

Epsilonproteobacteria (Fig. 6B), Order Neisseriale, Campylobacterales and CW040 (Fig. 6C), Family *Leptotrichiaceae, Neisseriaceae, F16 and Campylobacteraceae* (Fig. 6D)*, Genera Leptotrichia, Oribacterium, Neisseria* and *Campylobacter* (Fig. 6E) abundance are significantly different ($p < 0.05$) between OSCC and tobacco abusers.

4. Discussion

This study characterized differential oral microbiome profiles from saliva of OSCC, tobacco abusers and healthy control. To date, only a few studies have assessed the microbiome profiles of OSCC with respect to tobacco abusers and healthy individuals to identify diagnostic tools. Our results revealed a significantly perturbed oral microbiome in the study group. The characteristics bacteria of OSCC and tobacco abuser groups were identified and might be possible biological signatures for early detection of OSCC.

In 1994, the International Agency for Research on Cancer classified *H. pylori* as a carcinogen [22], this thus paved the way for scientists to understand the role of bacteria as a possible correlate of cancer development. Knowledge of the composition, diversity, function and relationship of oral microbiota with health and disease have significantly improved in recent time due to the state-of-the-art equipment recently developed. Proper balance in the collection of the oral microbial ecosystem is pertinent in maintaining integrity and health as dysbiotic oral microbiome has been implicated in both oral and systemic diseases [6,23,24]. Among three groups, three major taxa (which include, Firmicutes, Bacteroidetes and Fusobacteria) constituted the majority salivary microbiome, and this is in line with previous studies [4,6,25,26].

A plethora of studies have supported this claim, especially in oral health and malignant, tobacco abuse, cancer therapy and nutrition [4,26,27]. However, knowledge of the role of oral microbiota in the initiation and development of OSCC is still at its infancy, as there is currently no consensus among researchers on the dysbiotic nature of oral microbiota for diagnosis and screening of OSCC. This is because different studies have produced inconsistent results where they reported perturbation in varying types of oral microbiota in OSCC [8,10,15,28]. These studies have only focused on perturbation in oral microbiota due to OSCC and tobacco abuse separately [10,11,15]; this might be one of the cogs in the wheel for finding a reliable biomarker for OSCC. Studies have also not been able to understand if perturbed bacteria in OSCC are involved in the aetiology of cancer or just a consequence of it. It is thus imperative to study dysregulation in oral microbiota due to OSCC and tobacco abuse in a single study to find the elusive biomarker for OSCC as tobacco abuse has been implicated in the development of OSCC. This study thus analyzed differential microbiota due to both OSCC and tobacco abuse with respect to healthy controls to unravel differential microbiomes from tobacco abusers to OSCC.

Although the bacterial flora at the phylum level was not significantly different ($p > 0.05$), there is a reduction in the relative abundance of Firmicutes among tobacco abusers and OSCC with respect to controls; this is in line with an earlier report by Schmidt, Kuczynski, Bhattacharya, Huey, Corby, Queiroz, Nightingale, Kerr, DeLacure and Veeramachaneni [28] but contrary to a report by Hayes, Ahn, Fan, Peters, Ma, Yang, Agalliu, Burk, Ganly and Purdue [8] that reported higher abundance of Firmicutes among Head and Neck Squamous Cell Cancer (HNSCC) patients as compared with healthy controls. The elevated relative abundance of Fusobacteria in both OSCC and tobacco abusers was in line with the result from Refs. [9,28], where they reported an increase in their abundance in OSCC patients as compared to the control group. A recent study also presented elevated Fusobacteria in oral cancer patients but lower in tobacco abusers when compared with healthy control [26].

A higher abundance of class Betaproteobacteria in tobacco abusers with respect to controls and OSCC reported herein is not in agreement with data from Wu, Peters, Dominianni, Zhang, Pei, Yang, Ma, Purdue, Jacobs, Gapstur, Li, Alekseyenko, Hayes and Ahn [11] were they showed significantly lower levels of Betaproteobateria in current smokers with reference to healthy control. More so, Hayes, Ahn, Fan, Peters, Ma, Yang, Agalliu, Burk, Ganly and Purdue [8] also reported a significantly lower abundance of Betaproteobacteria in HNSCC patients as compared to healthy control.

Significantly increased abundance of genus *Leptotrichia* in both controls and tobacco abusers with respect to OSCC agrees with data from Pushalkar, Ji, Li, Estilo, Yegnanarayana, Singh, Li and Saxena [29] and Pushalkar, Mane, Ji, Li, Evans, Crasta, Morse, Meagher, Singh and Saxena [30] that reported abundant *Leptotrichia* in non-tumor library (controls) when compared with OSCC. But not in congruent with an earlier study by Sharma [31] that reported a significantly higher abundance of *Leptotrichia* in the saliva of oral cancer patients than in controls.

The result from this study was also not incongruent with an earlier study that reported depletion in the genera *Leptotrichia* among individuals who smoke *hookah* (smoking tobacco from a pipe with some sweet flavors) with respect to healthy controls [32]. Dysbiosis in oral commensal family *Leptotrichiaceae* and genus *Leptotrichia* reported in this study (downregulated in OSCC as compared with healthy controls, and tobacco abusers) might be due to oral cancer. This has been supported by a recent review suggesting their role in cancer [33].

Our result is in congruent with an earlier report that showed a reduced level of *Campylobacter* in Oral lichen planus (OLP) patients [34]. But the reduced abundance of *Campylobacter* in OSCC patients when compared with the control and tobacco abuser cohort reported in this study is not congruent with recent reports in India [4,25,35], other places [10,24], in breast and esophageal cancer [36, 37]. Consequently, perturbed Campylobacter might be due to poor oral hygiene as earlier study has supported the claim [36]. Both *Leptotrichia* and *Campylobacter* are not significantly different in controls and tobacco abusers, the perturbation in their abundance might thus be a sign for OSCC development.

Significant increase in genus *Neisseria* among tobacco abusers with respect to OSCC and controls reported here is not in agreement

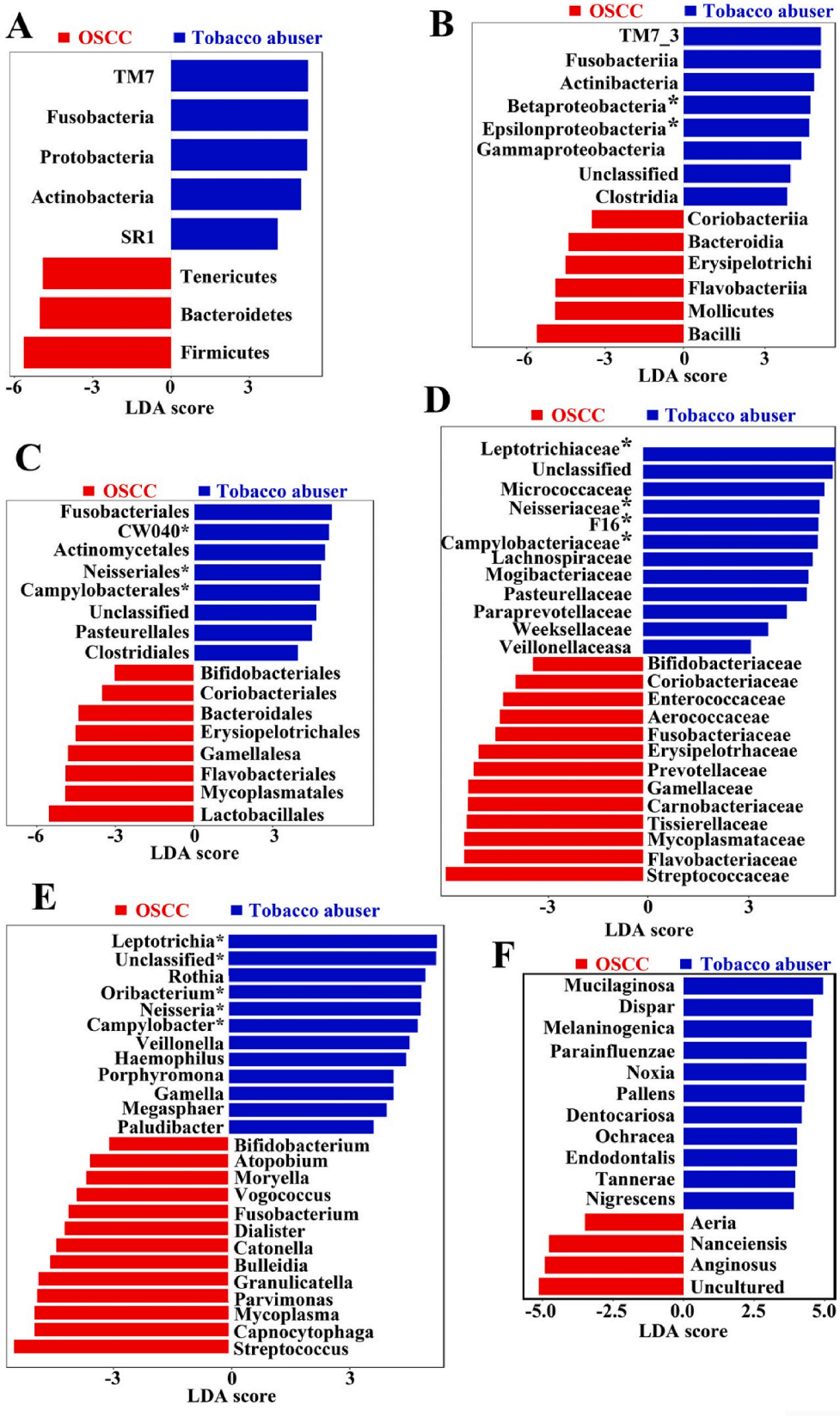

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Fig. 6. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) showing differentially abundant phylum **(A)**, class **(B)**, order **(C)**, family **(D)**, genus **(E)** and species **(F)** in OC and tobacco abusers. *abundance significantly different at $p < 0.05$.

with a recent study by Beghini, Renson, Zolnik, Geistlinger, Usyk, Moody, Thorpe, Dowd, Burk, Segata, Jones and Waldron [32] where they found them to be lower in current cigarette smokers compared to never smokers. Results from this study are also not incongruent with reports that showed reduced abundance of Neisseriales; *Neisseriaceae*; and *Neisseria* in HNSCC when compared with controls [7, 8].

The relative abundance of OTU annotated as *Lautropia* was found to be lower in OSCC and tobacco abusers than in controls is similar to a recent report by Beghini, Renson, Zolnik, Geistlinger, Usyk, Moody, Thorpe, Dowd, Burk, Segata, Jones and Waldron [32] where they reported a lower abundance of *Lautropia* in current cigarette smokers compared to never smokers.

Order Campylobacterales, family *Campylobacteraceae* and family *Campylobacter* were enriched in healthy controls, followed by tobacco abusers (not significant), and the least in OSCC reported in this study is not in tandem with a recent report in Shanghai, China that revealed a significantly higher abundance of *Campylobacter* in the cancer samples than controls [10]. Nonetheless, perturbation in these taxa may thus be an excellent molecular marker for OSCC. Several studies have suggested that these acid-utilizing bacteria in their natural habitat (oral cavity to the gastrointestinal tract) can induce acute inflammation [38].

Neisseria usually produces acid that lowers the oral cavity's pH, which culminates in increased enamel demineralisation; they are thus potentially complex organisms as they may be capable of both acid production and utilization. They also can utilize lactate, produce organic acid and at the same time metabolize glucose [39]. These show that *Neisseria* species are germane as they perform a complex role in oral cavity health. Dysbiosis in order Neisseriales, family *Neisseriaceae* and genus *Neisseria* reported as high abundance in tobacco abusers, slightly followed by OSCC and the least in controls (although no significant difference between OSCC and controls) reported here is a pointer to depreciating oral health due to tobacco abuse.

Finally, dysregulated microflora or selective growth of some bacteria might the important in carcinogenicity [40]. The biological role of this microbiome in the OSCC pathogenesis can be elucidated by bacterial simulation, pathogenesis, and production of potential carcinogens [41].

This study showed that Phylum Tenericutes, Class Mollicutes, Order Mycoplasmatales, Burkholderiales, Campylobacterales and CW040, Family Mycoplasmataceae, Burkholderiacea, Campylobacteraceae, F16 and Leptotrichiaceae, Genus Mycoplasma, Lautropia, Campylobacter and Leptotrichia abundance are significantly different ($p < 0.05$) between healthy controls and OSCC. However, there was no significant difference between tobacco abusers and healthy controls. While Class Betaproteobacteria and Epsilonproteobacteria, Order Neisseriale, Campylobacterales and CW040, Family Leptotrichiaceae, Neisseriaceae, F16 and Campylobacteraceae, Genera Leptotrichia, Oribacterium, Neisseria and Campylobacter abundance are significantly different ($p < 0.05$) between OSCC and tobacco abusers.

While a study with 30 samples can provide preliminary insights and generate hypotheses, it's essential to interpret the findings cautiously, recognizing the limitations of the small sample size. Further studies with larger samples would be necessary to validate and expand on any findings. However, the study's small sample size can be considered a limitation; combining oral cancer samples with tobacco abusers and healthy controls in a single study presents unique strengths. The study offers a more comprehensive perspective on the effects of tobacco and its potential role in mediating changes in the oral microbiome leading to cancer because it highlights potential microbial shifts that might be precursors to OSCC, emphasizing the risks of tobacco even before cancer develops. This study's novelty thus provides potential for groundbreaking findings, and its ability to pave the way for future, more extensive research in the area.

5. Conclusion

The study emphasizes the important part played by dysregulated microbial taxa in the development of oral squamous cell carcinoma (OSCC) and its link to smokeless tobacco use. By triggering protracted inflammatory reactions that may activate oncogenes and other tumor-promoting factors, abnormal alterations in the oral microbiota have been linked to several malignancies. Microbial imbalance, or dysbacteriosis, frequently precedes and follows the emergence of tumors. Therefore, maintaining a balanced microbial makeup (eubiosis) may be essential for stopping the development of cancer. This study offers a preliminary knowledge of how smokeless tobacco affects the oral microbiota and its possible contribution to the change from a healthy oral microbiome to one linked to inflammation. Potential indicators discovered in OSCC patients as well as tobacco users provide credence to the relationship between tobacco use and oral microbiome dysbiosis. However, as the study only used a small sample size and had a narrow geographic focus, more extensive metagenomic research is required. In areas like India and other South Asian nations, where OSCC incidence is high and is frequently linked to widespread tobacco product usage, comprehensive multi-omics analysis will improve our knowledge of tobacco's role in OSCC.

Disclosure of interest

The authors report no conflict of interest.

Data availability statement

The raw microbiome dataset (Raw sequence reads) from this study can be retrieved from the online repository at NCBI resources Bioproject: https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA548462.

BioProject ID/Accession number: PRJNA548462 and Submission ID: SUB5746919.

And Indian Nucleotide Data Archive (INDA) of Indian Biological Data Centre (IBDC) with INDA Accession: INRP000100 and INSDC Bioproject Accession No.: PRJEB67576. https://ibdc.rcb.res.in/inda/home.

CRediT authorship contribution statement

Bolaji Fatai Oyeyemi: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. **Urvinder S. Kaur:** Investigation, Methodology, Writing – original draft. **Amit Paramaraj:** Data curation, Investigation. **Chintamani:** Conceptualization, Methodology, Supervision, Writing – review & editing, Conceptualization, Methodology, Supervision, Writing – review & editing. **Ravi Tandon:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Abhinav Kumar:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Neel Sarovar Bhavesh:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21773.

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