Baseline gut microbiota composition is associated with oral mucositis and tumour recurrence in patients with head and neck cancer: a pilot study

Ghanyah Al-Qadami (ghanyah.al-qadami@adelaide.edu.au)
University of Adelaide
Joanne Bowen
University of Adelaide
Ysabella Van Sebille
University of South Australia
Kate Secombe
University of Adelaide
Mohsen Dorraki
University of Adelaide
Johan Verjans
South Australian Health and Medical Research Institute
Hannah Wardill
University of Adelaide
Hien Le
Royal Adelaide Hospital

Research Article

Keywords: Radiotherapy, Head and neck cancer, Oral mucositis, Gut microbiome

Posted Date: August 3rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1895894/v1

Abstract

Purpose

Mounting evidence suggests the gut microbiome influences radiotherapy efficacy and toxicity by modulating immune signalling. However, its contribution to radiotherapy outcomes in head and neck cancer (HNC) is yet to be investigated. This study, therefore, aimed to uncover associations between an individual's pre-therapy gut microbiota and i) severity of radiotherapy-induced oral mucositis (OM), and ii) recurrence risk in patients with HNC.

Methods

In this prospective pilot study, 20 patients with HNC scheduled to receive radiotherapy or chemoradiotherapy were recruited. Stool samples were collected before treatment and microbial composition was analysed using 16S rRNA gene sequencing. OM severity was assessed using the NCI-CTCAE scoring system. Patients were also followed for 12 months of treatment completion to assess tumour recurrence.

Results

Overall, 80% of the patients were male with a median age of 65.5 years. 53% experienced mild/moderate OM while 47% developed severe OM. Further, 18% experienced tumour relapse within 1 year of treatment completion. A pre-treatment microbiota enriched of *Eubacterium*, *Victivallis*, and *Ruminococcus* was associated with severe OM. Conversely, a higher relative abundance of immunomodulatory microbes *Faecalibacterium*, *Prevotella*, and *Phascolarctobacterium* was associated with a lower risk of tumour recurrence.

Conclusion

Our results indicate that a patient's gut microbiota composition at the start of treatment is linked to OM severity and recurrence risk. We now seek to validate these findings to determine their ability to predict treatment outcomes in HNC, with the goal of using this data to inform second-generation microbial therapeutics to optimise treatment outcomes for patients with HNC.

Introduction

Head and neck cancer (HNC) is the sixth most common type of cancer with ~ 930,000 new cases and over 460,000 deaths reported worldwide annually [1]. Given the relative ease by which these tumours can be accessed, radiotherapy is commonly used to treat both early- and advanced-stage HNC [2] with both

curative and palliative intent [3]. While largely effective, one of the major challenges in HNC radiotherapy is the heterogeneity in tumour response, recurrence rate, and severity of impactful toxicities.

Oral mucositis (OM), inflammation of the oral/oropharyngeal mucosa, is a common, dose-limiting toxicity in patients treated with radiotherapy for HNC [4]. Curiously, the incidence and severity of OM vary between patients, even in highly homogeneous cohorts [5]. Unfortunately, it remains unclear what drives this variation in OM risk, with traditional risk factors related to patient demographics, disease/treatment variables, and specific genetic variants unable to sensitively identify high-risk patients [6]. The same challenge is faced for radiotherapy efficacy, with the cause of treatment failure and disease recurrence in some patients still largely unexplained [7, 8]. This lack of understanding severely impacts clinical decision-making, patient monitoring, and the provision of optimal supportive care.

Both radiotherapy-induced toxicities and anti-tumour responses are known to be influenced by host immune responses, which are either exaggerated to drive mucosal toxicity or impaired, thus failing to optimally clear residual tumour load [9]. This knowledge has directed attention to how the gut microbiota may contribute to individual treatment responses, with the gut microbiota a profound regulator of immune tone and immunogenic cell death [10]. Due to its immunomodulatory capacity and its impact on pathways/mechanisms critical to cancer treatment efficacy, such as drug metabolism and cell death and repair, the gut microbiota is emerging as a major driver of treatment outcomes in chemotherapy and immunotherapy, with distinct microbial phenotypes predicting the efficacy and toxicity of these therapies [11].

In the context of radiotherapy, the data is limited. However, accumulating evidence strongly suggests that the gut microbiota may also augment both the efficacy and toxicity of radiotherapy [12, 13]. Of note, olfactory signatures reflecting the structure of the gut microbiota community have been associated with gastrointestinal mucositis severity in patients undergoing pelvic radiotherapy [14]. Additionally, recent evidence in preclinical models demonstrates that the gut microbiota can modulate the radiotherapy-induced anti-tumour immune responses and hence impacting its anti-tumour activity [15, 16]. Together, these data indicate that the gut microbiota may similarly control radiotherapy outcomes as in chemotherapy and immunotherapy.

While not directly investigated in HNC, microbiota-dependent modulation of radiotherapy outcomes is supported by anecdotal data. For example, the use of antibiotics is associated with earlier progression and lower survival among patients with locally advanced HNC treated with chemoradiotherapy [17]. Similarly, the use of probiotics has shown promising results in reducing the severity of OM among patients with HNC [18]. Despite this, the association between the pre-therapy gut microbiota and treatment outcomes in HNC has yet to be investigated. This study, therefore, aimed to explore the association between the pre-treatment gut microbiota, OM severity, and tumour recurrence in an HNC cohort.

Materials And Methods

Ethical approval

This study was approved by the Royal Adelaide Hospital Human Research Ethics Committee (HREC/17/RAH/533 (R20171131)) and was conducted according to the Declaration of Helsinki. The study protocol was sufficiently discussed with participants and informed consent was obtained from each participant before enrolling in the study.

Patients and biospecimen collection

Patients were recruited from the Radiation Oncology Department at the Royal Adelaide Hospital between October 2018 and December 2019. Adult patients diagnosed with HNC and scheduled to receive radiotherapy alone or combined therapies were eligible and underwent screening. Patients were excluded if they had a medical history of chronic gastrointestinal disorders or intestinal symptoms (unrelated to cancer/treatment) or had previous colonic surgery. Pre-treatment stool samples were collected by patients in DNA/RNA Shield Faecal Collection Tubes (Zymo Research, USA) and stored at -80 °C until processing.

Clinical data collection

Patients were provided with an induction survey to collect demographic information and behavioural/lifestyle factors (*see supplementary materials*). Clinical data for tumours and treatment characteristics were obtained from medical case notes held at the Royal Adelaide Hospital. OM was scored using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v5.0 [19], which grades OM as Grade 1(G1): asymptomatic or mild symptoms; intervention not indicated, Grade 2 (G2): moderate pain or ulcer not interfering with oral intake; modified diet indicated; Grade 3 (G3): severe pain; interfering with oral intake; Grade 4 (G4): life-threatening consequences; urgent intervention indicated; Grade 5 (G5): death. Patients were also followed to assess tumour recurrence within 12 months of treatment completion.

DNA extraction and 16S rRNA sequencing

To extract genomic DNA, 2 mL of the sample were first transferred to a sterile microcentrifuge tube and centrifuged at 16,000*x* g for 20 min at 4 °C. The supernatant was then separated and kept in a tube (not discarded) while the pellet was used for DNA extraction. DNA extraction was performed using Qiagen DNeasy PowerLyzer PowerSoil kit (Qiagen, Germany) as per manufacturer instructions with few modifications. First, Powderbead and C1 solutions were added to the pellet and mixed by brief vortexing. To lyse bacteria cells, the pellet mixture was heated at 65°C for 10 min. Then, the mixture was added into the PowerBead tube and homogenised using QIAGEN Tissuelyser LT (Qiagen, Germany) at 50 oscillation/sec for 6 min. The remaining steps were performed as indicated in the kit protocol. The retained supernatant was added back along with the C4 solution during the MB Spin column loading step. To increase the purity of extracted DNA, samples were precipitated using ethanol and sodium chloride, resuspended in nuclease-free water, and stored at -20°C.

DNA concentration was quantified using Qubit 2.0 Fluorometer (Life Technologies, Australia). Samples were sent to the South Australian Genomics Centre for 16S rRNA gene sequencing, performed via Illumina Miseq (San Diego, USA) using primers targeting the hypervariable V3-V4 region:

Forward:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The bioinformatics analysis was performed using Qiagen CLC Genomics Workbench 21.0.3. Briefly, trimmed and filtered pair-end reads were mapped back to the operational taxonomic units (OTUs) using the Greengenes 97% similarity reference database (v13.8, 2013). The alpha and beta diversity were assessed by the Shannon diversity index and principal coordinates analysis (PCoA) (generalised UniFrac distances) respectively. PERMANOVA analysis was used to measure the significance of beta diversity between groups. Linear discriminant analysis effect size (LEfSe) analysis was conducted using Galaxy online tool using default settings (http://huttenhower.sph.harvard.edu/galaxy/) [20].

Statistical analysis

The statistical analysis was performed using GraphPad prism 9. For quantitative data, unpaired T-test, Mann–Whitney, ANOVA, or Kruskal-Wallis tests were used depending on the Gaussian distribution of the dataset. Fisher's exact test was used to analyse categorical datasets. Correlation analyses were calculated by Pearson correlation coefficients in Python 3.9.6. A p-value of <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 20 patients were recruited in this study. Patient characteristics are summarised in Table S1. Briefly, 80% of the patients were males with a median age of 65.5 years. Among patients, 75% were either smokers or ex-smokers and 85% reported drinking less than 10 drinks per week. Tumours were located either in the oral cavity (20%), oropharynx (25%), nasal cavity (10%), salivary glands (30%), or HN skin (15%). Half of the patients had early-stage disease (I/II) and the remaining had late-stage disease (III/IV). All patients completed the planned radiotherapy course except for one who discontinued treatment after completing two fractions and hence they were excluded from treatment-related factors analysis. Patients were treated with either radiotherapy alone (31.6%), postoperative radiotherapy (47.4%), chemoradiotherapy (15.8%), or postoperative chemoradiotherapy (5.3%). Overall, patients received an average of 58.62 ± 8.78 Gy cumulative dose in 2.53 ± 1.21 fraction over 5.53 ± 1.46 weeks with 79% treated for curative intent.

Among 19 patients who completed treatment, two received palliative treatment (36 Gy; 6 Gy/F) over 2 weeks. Due to the low exposure, they were excluded from treatment outcomes analyses. Among 17 patients included, 17.7%, 35.3%, 29.4%, and 17.7% experienced G1, G2, G3, and G4 OM respectively. Further, three patients (17.6%) developed recurrence within 12 months post-treatment completion.

Characterisation of HNC patients' gut microbiota

First, we characterised the gut microbiota of all 20 patients. At the genus level, patients' gut microbiota was predominantly composed of *Bacteroides* (39.9%), unclassified *Ruminococcaceae* (7.4%), *Faecalibacterium* (6.8%), *Parabacteroides* (5.6%), and unclassified *Lachnospiraceae* (4.8%) (Fig. 1A). The average number of positive OTUs was 603.9 [229 - 864 range] and the average Shannon index value was 3.2 [1.3 - 4.1 range] (Fig. S1A & S2A).

Sex was the only factor associated with a significant difference in the microbial diversity and richness between patients. Female patients had significantly lower OTUs richness (p= 0.0007) and alpha diversity (p= 0.0289). Moreover, the gut microbiota of male and female patients clustered in distinctive patterns as shown by PCoA (p= 0.0052) (Fig. 1B-D). Further, five genera, mainly *Prevotella* and *Phascolarctobacterium,* were enriched in males while unclassified Lactobacillales and *P-75-a5* were increased in females (Fig. 1E).

Although there was no significant difference in the microbial richness and diversity based on other factors (Fig. S1B-M & S2C-N), specific genera were found to be enriched in specific subgroups. For instance, *Faecalibacterium*, *Paraprevotella*, and *Ruminococcus-2* were enriched in <50, 55-65, and >65 age groups respectively (Fig. 1F). Further, patients with cutaneous tumours had an increased abundance of unclassified RF32 while *SMB35* was increased among patients with salivary gland tumours (Fig. 1G). *Phascolarctobacterium* was increased in early-stage disease while *Enterococcus* was enriched in the advanced disease group (Fig. 1H). Moreover, *Phascolarctobacterium* was enriched in patients with HPV+ tumours (Fig. 1I). The unclassified *Enterobacteriaceae* was enriched in patients treated with radiotherapy alone while *Faecalibacterium* and *Phascolarctobacterium* were increased in those treated with postoperative radiotherapy and chemoradiotherapy respectively (Fig. 1J). Differential compositional changes based on other patient-related factors were also observed (Fig. S3A-H).

Risk factors associated with OM

Three patients were excluded from OM severity analysis (discontinued treatment/received low radiation doses). Patients were divided into either mild/moderate OM (G1-2) or severe OM (G3-4). In this cohort, there was no significant impact of all factors, except treatment type, on OM severity. Expectedly, 75% of patients with tumours in the oral cavity or oropharynx developed severe OM compared to only 22% of patients with tumours in other sites, but the difference was not statistically significant. However, those treated with chemoradiotherapy had significantly more severe OM (100%) compared to those who received radiotherapy without chemotherapy (30.8%) (p= 0.029) (Table 1).

Table 1: Risk factors associated with OM severity

	G1-2 (n=9)	G3-4 (n=8)	*P value*
Age (Year; mean ± SD)	67.89 ± 10.83	62.13 ± 9.73	0.269
BMI (Mean ± SD)	28.62 ± 5.95	25.54 ± 3.28	0.241
Sex, n (%)			
Male	6 (46.1)	7 (53.9)	0.577
Female	3 (75.0)	1 (25.0)	
Tobacco smoking, n (%)			
Non-smoker	2 (50.0)	2 (50.0)	>0.999
Ex-smoker/ Smoker	7 (53.9)	6 (46.1)	
Alcohol (# drinks/week), n (%)			
≤10	8 (57.1)	6 (42.9)	0.577
>10	1 (33.3)	2 (66.7)	
Antibiotics (B/D radiotherapy), n (%)			
Yes	4 (50.0)	4 (50.0)	>0.999
No	5 (55.6)	4 (44.4)	
Tumour site, n (%)			
Within the oral cavity (Oral cavity/ Oropharynx)	2 (25.0)	6 (75.0)	0.057
Outside the oral cavity (parotid gland/ nasal cavity/ HN skin)	7 (77.8)	2 (22.2)	
Treatment type, n (%)			
Radiotherapy	9 (69.2)	4 (30.8)	0.029*
Chemoradiotherapy	0	4 (100)	
Cumulative dose (Gy; mean ± SD)	59.89 ± 4.26	62.84 ± 4.47	0.184
Treatment period (Week; mean ± SD)	5.78 ± 0.67	6.13 ± 1.13	0.445
*B/D, Before or during radiotherapy; Unpaired T-test; Fisher's exact test; * p< 0.05*			

Gut microbiota traits associated with OM

Characterising the gut microbiota based on OM severity, the most abundant genera in the G1-2 OM group were *Bacteroides* (40%), *Parabacteroides* (7.8%), *Faecalibacterium* (6.9%), *unclassified Ruminococcaceae* (6.8%), and unclassified *Clostridiales* (4.7%) compared to *Bacteroides* (41.9%), *Faecalibacterium* (7.9%), unclassified *Ruminococcaceae* (7.2%), *Prevotella* (5.5%) and unclassified *Lachnospiraceae* (4.2%) in G3-4 OM group (Fig. 2A) (Table S2). Although there was no significant difference in the OTUs richness, alpha, and beta diversity between groups (Fig. S4A-C), *Eubacterium*, *Victivallis*, *Ruminococcus*, *Oxalobacter*, unclassified *Victivallaceae,* and unclassified *desulfovibrionaceae* were significantly increased in patients with G3-4 OM while unclassified RF32, *Alistipes,* and unclassified ML615J-28 were increased in those with G1-2 OM (Fig. 2B) (Table S4-S5).

Among the six genera enriched in the G3-4 OM group, the relative abundance of *Eubacterium* (p= 0.019), *Victivallis* (p= 0.016), and *Ruminococcus* (p= 0.027) was significantly higher in G3-4 compared to G1-2 OM group (Fig. 2C-E). *Eubacterium* and *Ruminococcus* genera were most abundant in patients with G3 OM while *Victivallis* was most abundant among patients with G4 OM (Fig. 2F-H). In contrast, the relative abundance of unclassified RF32 genus (p= 0.032) was significantly higher among patients with G1-2 OM and was most abundant among patients with G2 OM (Fig. 2I-J). Correlation analysis showed a significant positive correlation between the relative abundance of *Victivallis* and OM severity grade (r= 0.67, p= 0.003) (Fig. 3).

Risk factors associated with tumour recurrence

Among 17 patients included in tumour recurrence analysis, 14 patients did not develop tumour recurrence while 3 patients had recurrence within 12 months of treatment completion. Overall, there was no significant association between any of the patients and treatment-related factors and tumour recurrence (Table 2). Those who developed recurrence had tumours in the oropharynx, nasal cavity, or salivary gland. One of them had early-stage disease and two had advanced-stage disease. All of these patients received similar treatment; however, 2 out of these three patients had treatment breaks or delays.

Table 2: Patient and treatment-related factors associated with tumour recurrence

	No REC (n= 14)	REC (n= 3)	*P value*
Age (Year; mean ± SD)	63.57 ± 10.65	72.67 ± 5.69	0.178
Sex, n (%)			
Male	12 (92.3)	1 (7.7)	0.121
Female	2 (50)	2 (50)	
BMI (Mean ± SD)	28.09 ± 4.88	21.56 ± 1.12	0.088
Smoking, n (%)			
Non-smoker	3 (75.0)	1 (25.0)	>0.999
Ex-smoker/Smoker	11 (84.6)	2 (15.4)	
Alcohol (# drinks/week), n (%)			
≤10	11 (78.6)	3 (21.4)	>0.999
>10	3 (100)	0	
Antibiotics (B/D radiotherapy), n (%)			
Yes	8 (100)	0	0.206
No	6 (66.7)	3 (33.3)	
Tumour site, n (%)			
Oral cavity	3 (100)	0	-
Oropharynx	4 (80)	1 (20)	
Nasal cavity	1 (50)	1 (50)	
Salivary gland	4 (80)	1 (20)	
HN skin	2 (100)	0	
Tumour stage, n (%)			

Early stage (I/ II)	8 (88.9)	1 (11.1)	0.577
Advanced disease (III/ IV)	6 (75)	2 (25)	
HPV+, n (%)	4 (100)	0	-
Treatment type, n (%)			
Radiotherapy	10 (76.9)	3 (23.1)	0.541
Chemoradiotherapy	4 (100)	0	
Cumulative dose (Gy; mean ± SD)	61.41 ± 3.39	60.67 ± 9.24	0.665
Dose/Fraction (Gy/F)	2.11 ± 0.23	2.13 ± 0.12	0.337
Treatment period (Week; mean ± SD)	6.00 ± 0.78	5.67 ± 1.51	0.941
Treatment intent, n (%)			
Curative	13 (86.7)	2 (13.3)	0.331
Palliative	1 (50)	1 (50)	
Treatment gaps/breaks, n (%)			
Yes	2 (50)	2 (50)	0.121
No	12 (92.3)	1 (7.7)	
REC: recurrence; B/D, Before or during; Unpaired T-test; Fisher's exact test			

Gut microbiota traits associated with tumour recurrence

Characterising the gut microbiota based on tumour recurrence, the most abundant genera among patients with no recurrence (no REC) were *Bacteroides* (39%), *Faecalibacterium* (8.9%), *unclassified Ruminococcaceae* (7.2%), *Parabacteroides* (5.9%), and *Prevotella* (4.9%) compared to *Bacteroides* (50%), *unclassified Clostridiales* (6.4%), unclassified *Ruminococcaceae* (6.0%), *Parabacteroides* (5.7%), and *Blautia* (4.5%) in recurrence (REC) group (Fig. 4A) (Table S3). Generally, there was no significant difference in the number of OTUs, alpha, and beta diversity between groups (Fig. S5A-B & Fig. 4B). However, *Faecalibacterium*, *Prevotella,* and *Phascolarctobacterium* were enriched in patients with no recurrence, and *Adlercreutzia*, *Pseudoramibacter_Eubacterium*, *Desulfitobacter*, *Eggerthella*,

Megasphaera, and *p-75-a5* were increased in patients with recurrence (Fig. 4C) (Table S4-S5). The relative abundance of *Faecalibacterium* (p= 0.029), *Prevotella (*p= 0.031), and *Phascolarctobacterium* (p= 0.019) was significantly higher in patients with no recurrence (Fig. 4D-F). Further, patients who did not develop recurrence also had a significantly higher *Prevotella to Bacteroides* (P/B) ratio (p= 0.047) (Fig. 4G). Conversely, the relative abundance of *Adlercreutzia* (p= 0.006) and *Eggerthella* (p= 0.006) genera was significantly higher in patients with recurrence (Fig. 4H-I). There was no significant difference between recurrence and no recurrence groups in the relative abundance of other genera (Fig. S5C-F).

Discussion

Despite the recent technological advances in radiotherapy, variability in radiotherapy outcomes in terms of efficacy and toxicity remains a key challenge. Here, we build on the growing consensus that an individual's unique, pre-treatment gut microbiota is associated with radiotherapy responses, identifying enrichment and reduction in key taxa linked with distinct treatment outcomes.

Although there was no difference between patients with mild/moderate or severe OM in both the microbial richness and diversity, six bacterial genera were enriched in patients with severe OM. Among these microbes, *Eubacterium (E. biforme* species*)*, *Victivallis*, and *Ruminococcus* genera were the most significantly increased. *Eubacterium,* a genus of gram-positive anaerobic bacteria belongs to the *Erysipelotrichaceae* family with *Eubacterium biforme (E. biforme)* classified as main species within this genus [21]*. Eubacterium* has been recently reclassified as *Holdemanella* and *E. biforme* as *Holdemanella biformis (H. biformis)* [22]*.* We refer to them here as *Eubacterium* and *E. biforme* based on the reference database used for the analysis. Both beneficial and detrimental effects of this bacterium have been reported. It has been reported that *E. biforme* can produce C18-3OH, a free long-chain fatty acid with potential anti-inflammatory properties, which in turn reduces colitis severity in mice [23]. Conversely, other studies have reported that an increase in *Eubacterium* is associated with severe cystic fibrosis [24], nonalcoholic steatohepatitis [25], irritable bowel syndrome [26], and HIV infection [27]. In vitro incubation of peripheral blood mononuclear cells from HIV positive and negative subjects with *E. biforme* bacterial lysates was associated with a higher tumour necrosis factor α (TNF-α) to interleukin 10 ratio as compared to incubating cells with lipopolysaccharides or three other bacterial species, suggesting a pro-inflammatory property of this species [27]. Another genus that showed a strong correlation with OM severity is *Victivallis. Victivallis,* a genus of gram-negative anaerobic bacteria, belongs to the *Victivallaceae* family. It is the only genus in the *Victivallaceae* family and includes one well-characterised species, *Victivallis vadensis* [28]. Currently, little is known about the function and impact of this on the human gastrointestinal tract; however, an increase in the abundance of the *Victivallaceae* family or its genus and species has been linked to inflammatory conditions including colorectal cancer [29], Hashimoto's thyroiditis [30], and cerebral ischemic stroke [31]. Although this genus is present in a low abundance, the detection rate (OTUs>0) was 62.5% of patients with severe OM compared to only 11.1% of those with mild/moderate OM. This suggests that *Victivallis* may contribute to OM severity despite its low abundance and warrants further investigation. *Ruminococcus,* a genus of strictly anaerobic gram-

positive cocci of the *Lachnospiraceae family [32],* was also increased in patients with severe OM. It comprises five species including *R. gnavus* and *R. torques* [33]. Both species are mucolytic and have been linked to the pathogenesis of chronic inflammatory conditions including inflammatory bowel disease [34]. *R. gnavus* can also secrete a pro-inflammatory polysaccharide inducing the production of TNF-α through the toll-like receptor 4-dependent pathway; hence, contributing to Crohn's disease pathogenesis [35]. This suggests that these mucolytic and pro-inflammatory species potentially contribute to OM pathogenesis through degradation of mucus layer and activating systemic inflammation. Together, present results *suggests that these three genera could contribute to OM severity, potentially due to their* pro-inflammatory properties. Further studies are needed to validate this association and to determine the mechanism by which these microbes may influence OM pathogenesis.

Among the compositional changes observed is the increased abundance of Unclassified RF32 in patients with mild/moderate OM. Since this genus was also increased in patients with HN skin tumours, and all developed mild/moderate OM, we believe that this genus is associated with tumour site rather than OM severity. Although the LEFSe analysis revealed the *Alistipes*, and unclassified ML615J-28 were also enriched in patients with mild/moderate OM, the comparison of relative abundance did not yield a significant difference between groups. Overall, this study did not identify any bacterial taxa to be specifically associated with mild/moderate OM.

In terms of tumour recurrence, there was no difference in microbial richness and diversity between patients. Interestingly, patients who did not develop recurrence had a significantly higher abundance of *Faecalibacterium, Prevotella,* and *Phascolarctobacterium*. Additional analysis at the species level identified that *Faecalibacterium Prausnitzii* (*F. prausnitzii*) *and Prevotella Copri* (*P. copri*) were enriched in patients with no recurrence. Generally, these three genera comprise gram-negative bacteria and have been linked to better immunotherapy outcomes in patients with melanoma and non-small cell lung cancer [36-38]. For instance, in patients with melanoma treated with anti-PD-1 immunotherapy, responders had an increased abundance of *Faecalibacterium* and *Phascolarctobacterium,* with *Faecalibacterium* associated with prolonged progression-free survival [36,38]. Furthermore, an increase in *P. copri* was associated with a preferred response in a cohort of patients with non-small cell lung cancer treated with anti-PD-1 immunotherapy [37]. We also noticed that those who did not develop recurrence had a significantly higher P/B ratio, which is an enterotype associated with a favourable response to anti-PD-1/PD-L1 immunotherapy in patients with gastrointestinal cancers [39]. Current evidence suggests that these microbes modulate immunotherapy anti-tumour response through enhancing $CD8^+$ T cell expansion and function [37,36]. This could be similar in the context of radiotherapy as anti-tumour immune response also plays a central role in radiotherapy-induced tumour control [13]. In a preclinical study, targeting gram-positive bacteria with vancomycin improved radiotherapy anti-tumour activity by enhancing tumour-associated antigen presentation to $CD8^+$ T cells [15]. Conversely*, Adlercreutzia* and *Eggerthella (E. Lenta),* both belonging to *the Eggerthellaceae* family, were increased in those who developed recurrence. Previous studies have reported that these genera are enriched in non-responders treated with

immunotherapy for metastatic melanoma [38]. Together, the current results suggest that certain gut microbes are positively or negatively associated with the risk of recurrence in HNC patients.

Overall, this is the first study to characterise the association between gut microbiota and radiotherapy outcomes in patients with HNC. It demonstrates that specific gut microbes are associated with OM severity and risk of tumour recurrence and, as such, supports that the gut microbiota could be exploited to predict radiotherapy outcomes. Another strength of the study is that it assessed microbial signatures associated with both efficacy and toxicity of radiotherapy, which is a critical approach to achieving optimal outcomes for cancer treatments [40]. However, the study is not without limitations. We recognise the small sample size of our cohort and the presence of different confounding factors at baseline and, therefore, emphasise our results must be interpreted with caution. The small sample size may result in biases in the association between OM severity, tumour recurrence, and patients and treatment-related risk factors as well as the microbial signature. Moreover, baseline confounding factors including heterogeneity in tumour primary sites and type of treatment received could impact OM severity and recurrence risk analysis. Therefore, future studies should validate these findings in a larger cohort with minimal variation in the baseline factors.

Conclusion

Our study demonstrates that a gut microbiota enriched of *Eubacterium*, *Victivallis*, and *Ruminococcus* is associated with severe OM. Additionally, enrichment for *Faecalibacterium*, *Prevotella*, and *Phascolarctobacterium* confers lower recurrence risk. These pilot data, therefore, reinforce the emerging hypothesis that an individual's unique microbiota can be used to predict treatment outcomes and be used to direct the provision of proactive supportive care. Moving forward, these data should be used to identify candidate microbes suitable for second-generation probiotics aimed at pre-conditioning the microbiota to optimise treatment outcomes.

Declarations

Funding: This study was supported by Royal Adelaide Hospital Clinical Project Grant (CI Wardill)

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

Author Contributions: GA, JB, YVS, KS, and HL made substantial contributions to study conceptualisation and design (GA, JB, and HL), patients recruitment and samples collection (GA and HL), DNA extraction, and microbial data analysis (GA and KS), drafting (GA) and revising the manuscript (GA, JB, YVS, KS, and HL). HW is PI for the study, securing relevant ethical approvals and seed funding as well as contributing to data analysis and revising the manuscript. MD and JV contributed to the data analysis and interpretation and reviewing of the manuscript.

Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Royal Adelaide Hospital Human Research Ethics Committee

(HREC/17/RAH/533 (R20171131)).

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Acknowledgements: We would like to sincerely thank the nurses and radiation oncologists at the Department of Radiation Oncology (Royal Adelaide Hospital) for their assistance during the recruitment process.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 71 (3):209–249. doi:https://doi.org/10.3322/caac.21660
2. Yeh SA (2010) Radiotherapy for Head and Neck Cancer. Semin Plast Surg 24 (2):127–136. doi:10.1055/s-0030-1255330
3. Gooi Z, Fakhry C, Goldenberg D, Richmon J, Kiess AP (2016) AHNS Series: Do you know your guidelines?Principles of radiation therapy for head and neck cancer: A review of the National Comprehensive Cancer Network guidelines. Head Neck 38 (7):987–992. doi:10.1002/hed.24448
4. Maria OM, Eliopoulos N, Muanza T (2017) Radiation-induced Oral Mucositis. Frontiers in oncology 7
5. Bachour PC, Sonis ST (2018) Predicting mucositis risk associated with cytotoxic cancer treatment regimens: rationale, complexity, and challenges. Curr Opin Support Palliat Care 12 (2):198–210. doi:10.1097/spc.0000000000000339
6. Wardill HR, Sonis ST, Blijlevens NMA, Van Sebille YZA, Ciorba MA, Loeffen EAH, Cheng KKF, Bossi P, Porcello L, Castillo DA, Elad S, Bowen JM, On behalf of The Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral O (2020) Prediction of mucositis risk secondary to cancer therapy: a systematic review of current evidence and call to action. Supportive Care in Cancer 28 (11):5059–5073. doi:10.1007/s00520-020-05579-7
7. Begg AC, Stewart FA, Vens C (2011) Strategies to improve radiotherapy with targeted drugs. Nature Reviews Cancer 11 (4):239–253
8. Orth M, Lauber K, Niyazi M, Friedl AA, Li M, Maihöfer C, Schüttrumpf L, Ernst A, Niemöller OM, Belka C (2014) Current concepts in clinical radiation oncology. Radiation and environmental biophysics 53 (1):1–29. doi:10.1007/s00411-013-0497-2
9. Cytlak UM, Dyer DP, Honeychurch J, Williams KJ, Travis MA, Illidge TM (2021) Immunomodulation by radiotherapy in tumour control and normal tissue toxicity. Nature Reviews Immunology. doi:10.1038/s41577-021-00568-1
10. Roy S, Trinchieri G (2017) Microbiota: a key orchestrator of cancer therapy. Nature Reviews Cancer
11. Alexander JL, Wilson ID, Teare J, Marchesi JR, Nicholson JK, Kinross JM (2017) Gut microbiota modulation of chemotherapy efficacy and toxicity. Nature Reviews Gastroenterology and Hepatology

14 (6):356–365

12. Al-Qadami G, Van Sebille Y, Le H, Bowen J (2019) Gut microbiota: implications for radiotherapy response and radiotherapy-induced mucositis. Expert Review of Gastroenterology & Hepatology:1–12. doi:10.1080/17474124.2019.1595586
13. Tonneau M, Elkrief A, Pasquier D, Socorro TPD, Chamaillard M, Bahig H, Routy B (2020) The role of the gut microbiome on radiation therapy efficacy and gastrointestinal complications: A systematic review. Radiother Oncol. doi:10.1016/j.radonc.2020.10.033
14. Covington JA, Wedlake L, Andreyev J, Ouaret N, Thomas MG, Nwokolo CU, Bardhan KD, Arasaradnam RP (2012) The detection of patients at risk of gastrointestinal toxicity during pelvic radiotherapy by electronic nose and FAIMS: a pilot study. Sensors (Basel) 12 (10):13002–13018. doi:10.3390/s121013002
15. Uribe-Herranz M, Rafail S, Beghi S, Gil-de-Gómez L, Verginadis I, Bittinger K, Pustylnikov S, Pierini S, Perales-Linares R, Blair IA, Mesaros CA, Snyder NW, Bushman F, Koumenis C, Facciabene A (2020) Gut microbiota modulate dendritic cell antigen presentation and radiotherapy-induced antitumor immune response. J Clin Invest 130 (1):466–479. doi:10.1172/jci124332
16. Yang K, Hou Y, Zhang Y, Liang H, Sharma A, Zheng W, Wang L, Torres R, Tatebe K, Chmura SJ, Pitroda SP, Gilbert JA, Fu Y-X, Weichselbaum RR (2021) Suppression of local type I interferon by gut microbiota–derived butyrate impairs antitumor effects of ionizing radiation. Journal of Experimental Medicine 218 (3). doi:10.1084/jem.20201915
17. Nenclares P, Bhide SA, Sandoval-Insausti H, Pialat P, Gunn L, Melcher A, Newbold K, Nutting CM, Harrington KJ (2020) Impact of antibiotic use during curative treatment of locally advanced head and neck cancers with chemotherapy and radiotherapy. Eur J Cancer 131:9–15. doi:10.1016/j.ejca.2020.02.047
18. Shu Z, Li P, Yu B, Huang S, Chen Y (2020) The effectiveness of probiotics in prevention and treatment of cancer therapy-induced oral mucositis: A systematic review and meta-analysis. Oral Oncol 102:104559. doi:10.1016/j.oraloncology.2019.104559
19. Trotti A, Colevas AD, Setser A, Basch E (2007) Patient-reported outcomes and the evolution of adverse event reporting in oncology. J Clin Oncol 25 (32):5121–5127. doi:10.1200/jco.2007.12.4784
20. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation. Genome Biol 12 (6):R60. doi:10.1186/gb-2011-12-6-r60
21. Wade WG (2006) The Genus Eubacterium and Related Genera. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The Prokaryotes. Springer US, New York, NY, pp 823–835. doi:10.1007/0-387-30744-3_28
22. De Maesschalck C, Van Immerseel F, Eeckhaut V, De Baere S, Cnockaert M, Croubels S, Haesebrouck F, Ducatelle R, Vandamme P (2014) Faecalicoccus acidiformans gen. nov., sp. nov., isolated from the chicken caecum, and reclassification of Streptococcus pleomorphus (Barnes et al. 1977), Eubacterium biforme (Eggerth 1935) and Eubacterium cylindroides (Cato et al. 1974) as

Faecalicoccus pleomorphus comb. nov., Holdemanella biformis gen. nov., comb. nov. and Faecalitalea cylindroides gen. nov., comb. nov., respectively, within the family Erysipelotrichaceae. Int J Syst Evol Microbiol 64 (Pt_11):3877–3884. doi:https://doi.org/10.1099/ijs.0.064626-0

23. Pujo J, Petitfils C, Le Faouder P, Eeckhaut V, Payros G, Maurel S, Perez-Berezo T, Van Hul M, Barreau F, Blanpied C, Chavanas S, Van Immerseel F, Bertrand-Michel J, Oswald E, Knauf C, Dietrich G, Cani PD, Cenac N (2020) Bacteria-derived long chain fatty acid exhibits anti-inflammatory properties in colitis. Gut:gutjnl-2020-321173. doi:10.1136/gutjnl-2020-321173
24. Schippa S, Iebba V, Santangelo F, Gagliardi A, De Biase RV, Stamato A, Bertasi S, Lucarelli M, Conte MP, Quattrucci S (2013) Cystic fibrosis transmembrane conductance regulator (CFTR) allelic variants relate to shifts in faecal microbiota of cystic fibrosis patients. PLoS One 8 (4):e61176-e61176. doi:10.1371/journal.pone.0061176
25. Rau M, Rehman A, Dittrich M, Groen AK, Hermanns HM, Seyfried F, Beyersdorf N, Dandekar T, Rosenstiel P, Geier A (2018) Fecal SCFAs and SCFA-producing bacteria in gut microbiome of human NAFLD as a putative link to systemic T-cell activation and advanced disease. United European Gastroenterol J 6 (10):1496–1507. doi:10.1177/2050640618804444
26. Maity C, Gupta AK, Saroj DB, Biyani A, Bagkar P, Kulkarni J, Dixit Y (2020) Impact of a Gastrointestinal Stable Probiotic Supplement Bacillus coagulans LBSC on Human Gut Microbiome Modulation. J Diet Suppl:1–20. doi:10.1080/19390211.2020.1814931
27. Lozupone CA, Li M, Campbell TB, Flores SC, Linderman D, Gebert MJ, Knight R, Fontenot AP, Palmer BE (2013) Alterations in the gut microbiota associated with HIV-1 infection. Cell host & microbe 14 (3):329–339. doi:10.1016/j.chom.2013.08.006
28. Plugge CM, Zoetendal EG (2014) The Family Victivallaceae. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The Prokaryotes: Other Major Lineages of Bacteria and The Archaea. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 1019–1021. doi:10.1007/978-3-642-38954-2_150
29. Sánchez-Alcoholado L, Ordóñez R, Otero A, Plaza-Andrade I, Laborda-Illanes A, Medina JA, Ramos-Molina B, Gómez-Millán J, Queipo-Ortuño MI (2020) Gut Microbiota-Mediated Inflammation and Gut Permeability in Patients with Obesity and Colorectal Cancer. Int J Mol Sci 21 (18):6782. doi:10.3390/ijms21186782
30. Cornejo-Pareja I, Ruiz-Limón P, Gómez-Pérez AM, Molina-Vega M, Moreno-Indias I, Tinahones FJ (2020) Differential Microbial Pattern Description in Subjects with Autoimmune-Based Thyroid Diseases: A Pilot Study. Journal of personalized medicine 10 (4):192. doi:10.3390/jpm10040192
31. Li N, Wang X, Sun C, Wu X, Lu M, Si Y, Ye X, Wang T, Yu X, Zhao X, Wei N, Wang X (2019) Change of intestinal microbiota in cerebral ischemic stroke patients. BMC Microbiol 19 (1):191–191. doi:10.1186/s12866-019-1552-1
32. La Reau AJ, Meier-Kolthoff JP, Suen G (2016) Sequence-based analysis of the genus Ruminococcus resolves its phylogeny and reveals strong host association. Microbial genomics 2 (12):e000099-e000099. doi:10.1099/mgen.0.000099

33. Togo AH, Diop A, Bittar F, Maraninchi M, Valero R, Armstrong N, Dubourg G, Labas N, Richez M, Delerce J, Levasseur A, Fournier P-E, Raoult D, Million M (2018) Description of Mediterraneibacter massiliensis, gen. nov., sp. nov., a new genus isolated from the gut microbiota of an obese patient and reclassification of Ruminococcus faecis, Ruminococcus lactaris, Ruminococcus torques, Ruminococcus gnavus and Clostridium glycyrrhizinilyticum as Mediterraneibacter faecis comb. nov., Mediterraneibacter lactaris comb. nov., Mediterraneibacter torques comb. nov., Mediterraneibacter gnavus comb. nov. and Mediterraneibacter glycyrrhizinilyticus comb. nov. Antonie Van Leeuwenhoek 111 (11):2107–2128. doi:10.1007/s10482-018-1104-y
34. Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, McGuckin MA, Florin TH (2010) Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. Am J Gastroenterol 105 (11):2420–2428. doi:10.1038/ajg.2010.281
35. Henke MT, Kenny DJ, Cassilly CD, Vlamakis H, Xavier RJ, Clardy J (2019) Ruminococcus gnavus, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. Proc Natl Acad Sci U S A 116 (26):12672–12677. doi:10.1073/pnas.1904099116
36. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, Prieto PA, Vicente D, Hoffman K, Wei SC, Cogdill AP, Zhao L, Hudgens CW, Hutchinson DS, Manzo T, Petaccia de Macedo M, Cotechini T, Kumar T, Chen WS, Reddy SM, Szczepaniak Sloane R, Galloway-Pena J, Jiang H, Chen PL, Shpall EJ, Rezvani K, Alousi AM, Chemaly RF, Shelburne S, Vence LM, Okhuysen PC, Jensen VB, Swennes AG, McAllister F, Marcelo Riquelme Sanchez E, Zhang Y, Le Chatelier E, Zitvogel L, Pons N, Austin-Breneman JL, Haydu LE, Burton EM, Gardner JM, Sirmans E, Hu J, Lazar AJ, Tsujikawa T, Diab A, Tawbi H, Glitza IC, Hwu WJ, Patel SP, Woodman SE, Amaria RN, Davies MA, Gershenwald JE, Hwu P, Lee JE, Zhang J, Coussens LM, Cooper ZA, Futreal PA, Daniel CR, Ajami NJ, Petrosino JF, Tetzlaff MT, Sharma P, Allison JP, Jenq RR, Wargo JA (2018) Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 359 (6371):97–103. doi:10.1126/science.aan4236
37. Jin Y, Dong H, Xia L, Yang Y, Zhu Y, Shen Y, Zheng H, Yao C, Wang Y, Lu S (2019) The Diversity of Gut Microbiome is Associated With Favorable Responses to Anti–Programmed Death 1 Immunotherapy in Chinese Patients With NSCLC. Journal of Thoracic Oncology 14 (8):1378–1389. doi:https://doi.org/10.1016/j.jtho.2019.04.007
38. Limeta A, Ji B, Levin M, Gatto F, Nielsen J (2020) Meta-analysis of the gut microbiota in predicting response to cancer immunotherapy in metastatic melanoma. JCI insight 5 (23):e140940. doi:10.1172/jci.insight.140940
39. Peng Z, Cheng S, Kou Y, Wang Z, Jin R, Hu H, Zhang X, Gong J-f, Li J, Lu M, Wang X, Zhou J, Lu Z, Zhang Q, Tzeng DTW, Bi D, Tan Y, Shen L (2020) The Gut Microbiome Is Associated with Clinical Response to Anti–PD-1/PD-L1 Immunotherapy in Gastrointestinal Cancer. Cancer Immunology Research 8 (10):1251–1261. doi:10.1158/2326-6066.cir-19-1014
40. Wardill HR, Chan RJ, Chan A, Keefe D, Costello SP, Hart NH (2022) Dual contribution of the gut microbiome to immunotherapy efficacy and toxicity: supportive care implications and recommendations. Supportive Care in Cancer 30 (8):6369–6373. doi:10.1007/s00520-022-06948-0

Figures

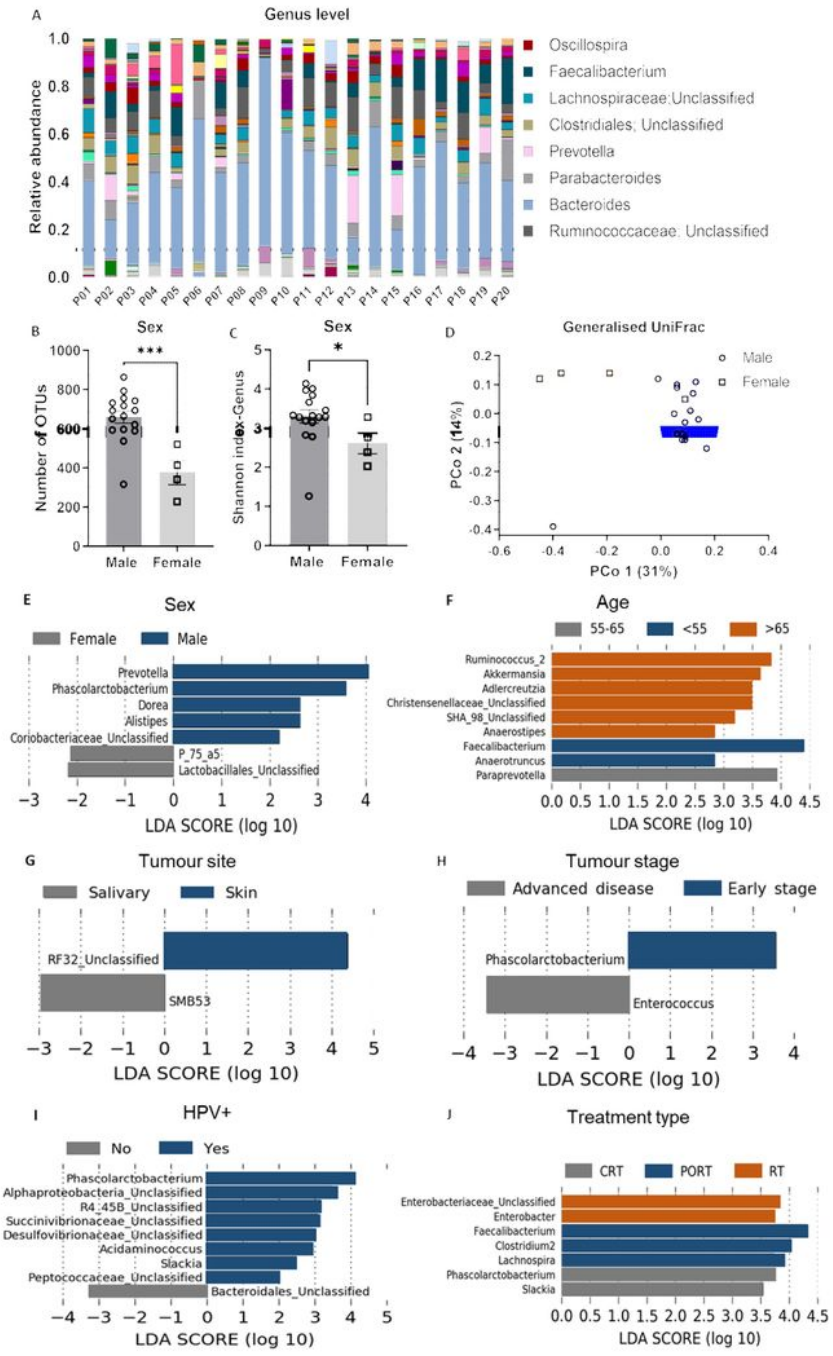

Figure 1

The gut microbiome composition of HNC patients. A) The gut microbiota relative abundance at the genus level for all patients. B-C) Male patients had a significantly higher number of OTUs (unpaired t-test) and higher alpha diversity (Mann-Whitney test) than female patients. D) Female patients have distinctive

microbial pattern compared to males. The differential microbial features according to sex (E), age (F) tumour site (G), tumour stage (H), HPV status (I), and treatment type (J). LDA, Linear discriminant analysis; CRT, Chemoradiotherapy; PORT, postoperative radiotherapy; RT, radiotherapy. *p ≤ 0.05; ***p ≤ 0.001

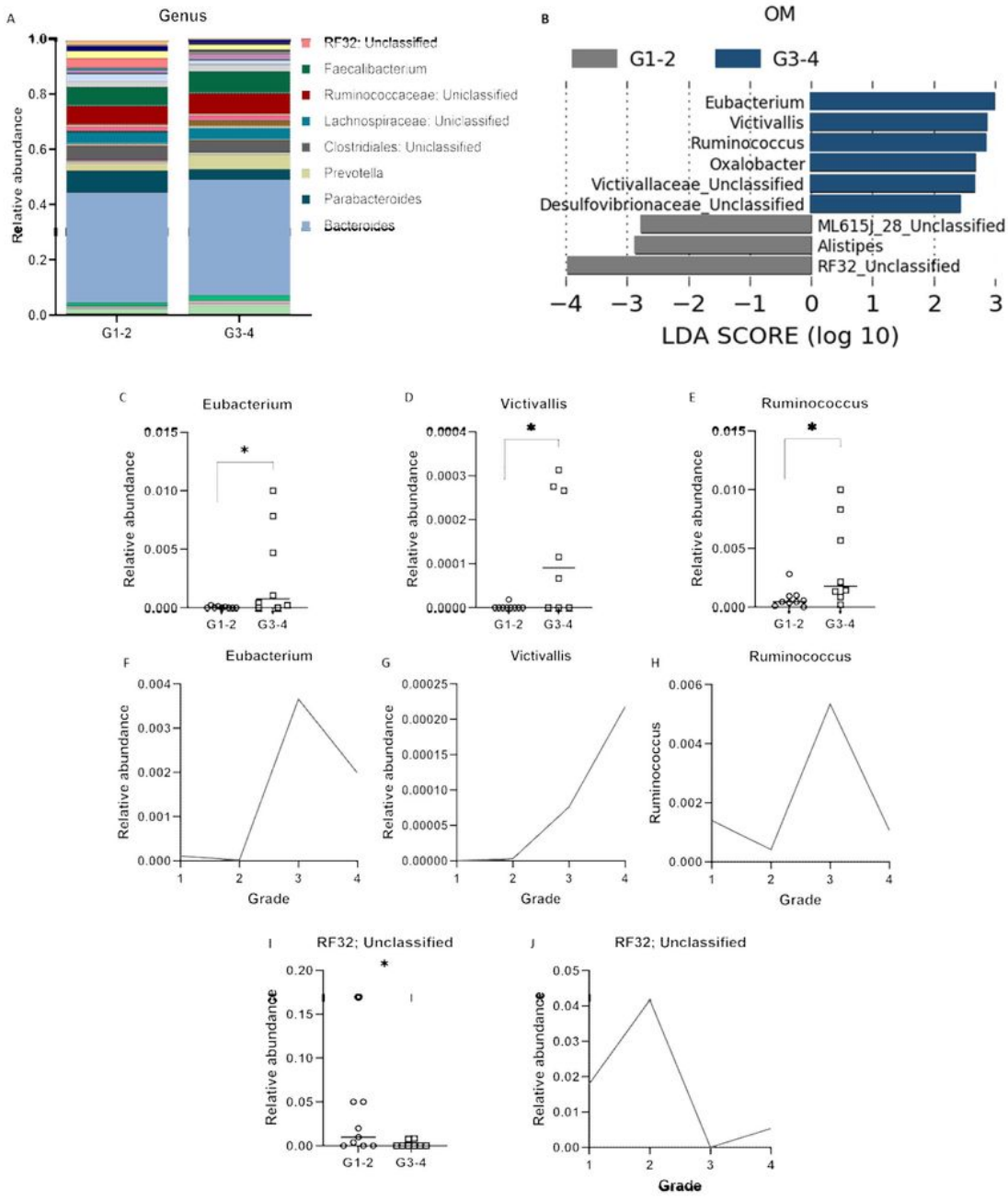

Figure 2

Association between the gut microbiota and OM severity. A) The gut microbiota relative abundance at the genus level for G1-2 and G3-4 OM. B) The differential microbial features for G1-2 and G3-4 OM. The relative abundance of Eubacterium (C), Victivallis (D), and Ruminococcus (E) was significantly higher in G3-4 group. F-H) Change in the average relative abundance of Eubacterium (F), Victivallis (G), and Ruminococcus (H) according to OM severity grade. I) The relative abundance of unclassified RF32 was significantly higher in G1-2 group. J) Change in the average relative abundance of unclassified RF32 according to OM severity grade. LDA; Linear discriminant analysis. *$p \leq 0.05$. Mann-Whitney test; Line represents the median

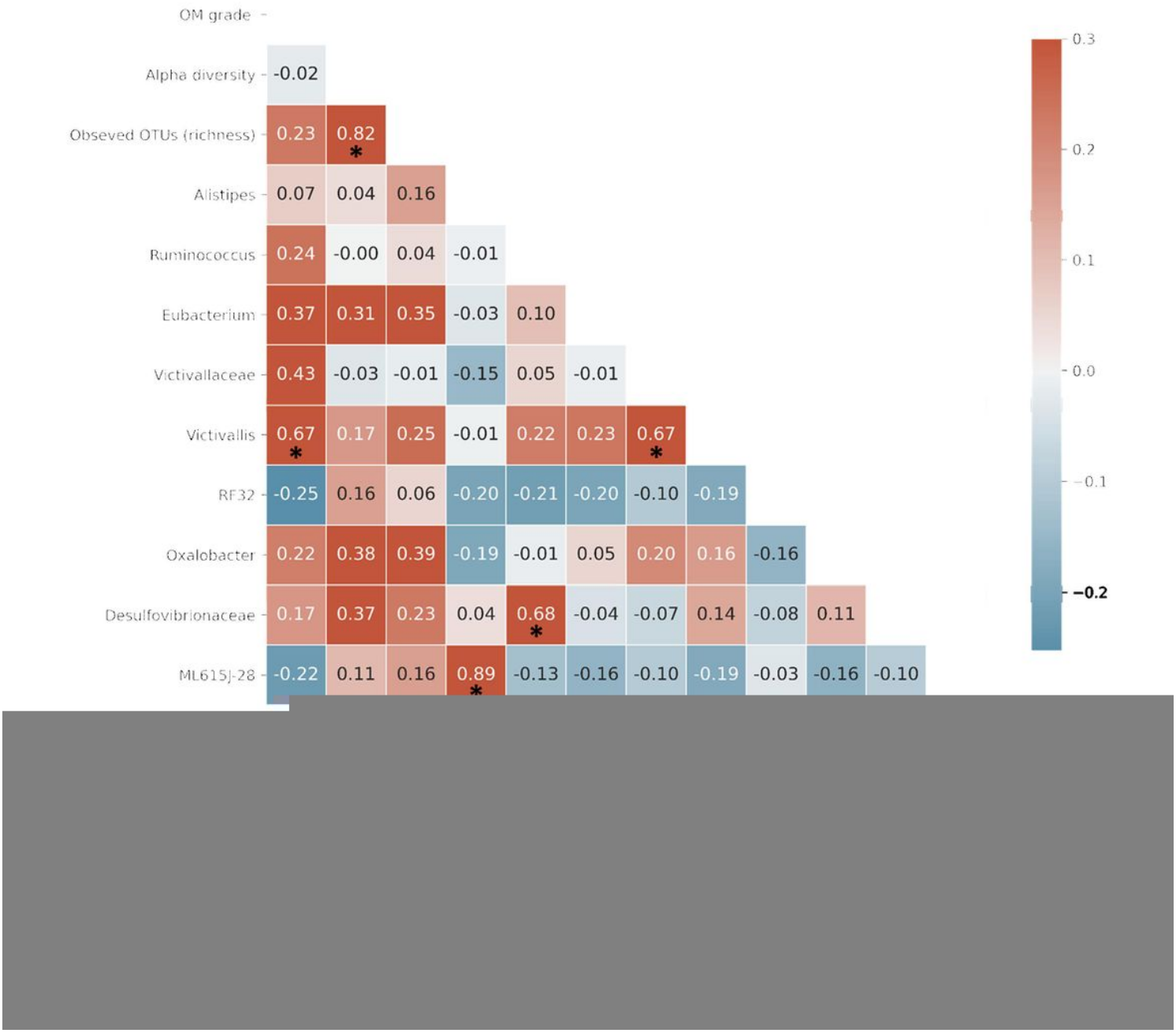

Figure 3

Correlation heatmap of the microbial richness, alpha diversity, and selected genera and OM severity grade. The colour of the cells is proportional from the negative correlation (blue) to the positive correlation (red). *p < 0.05

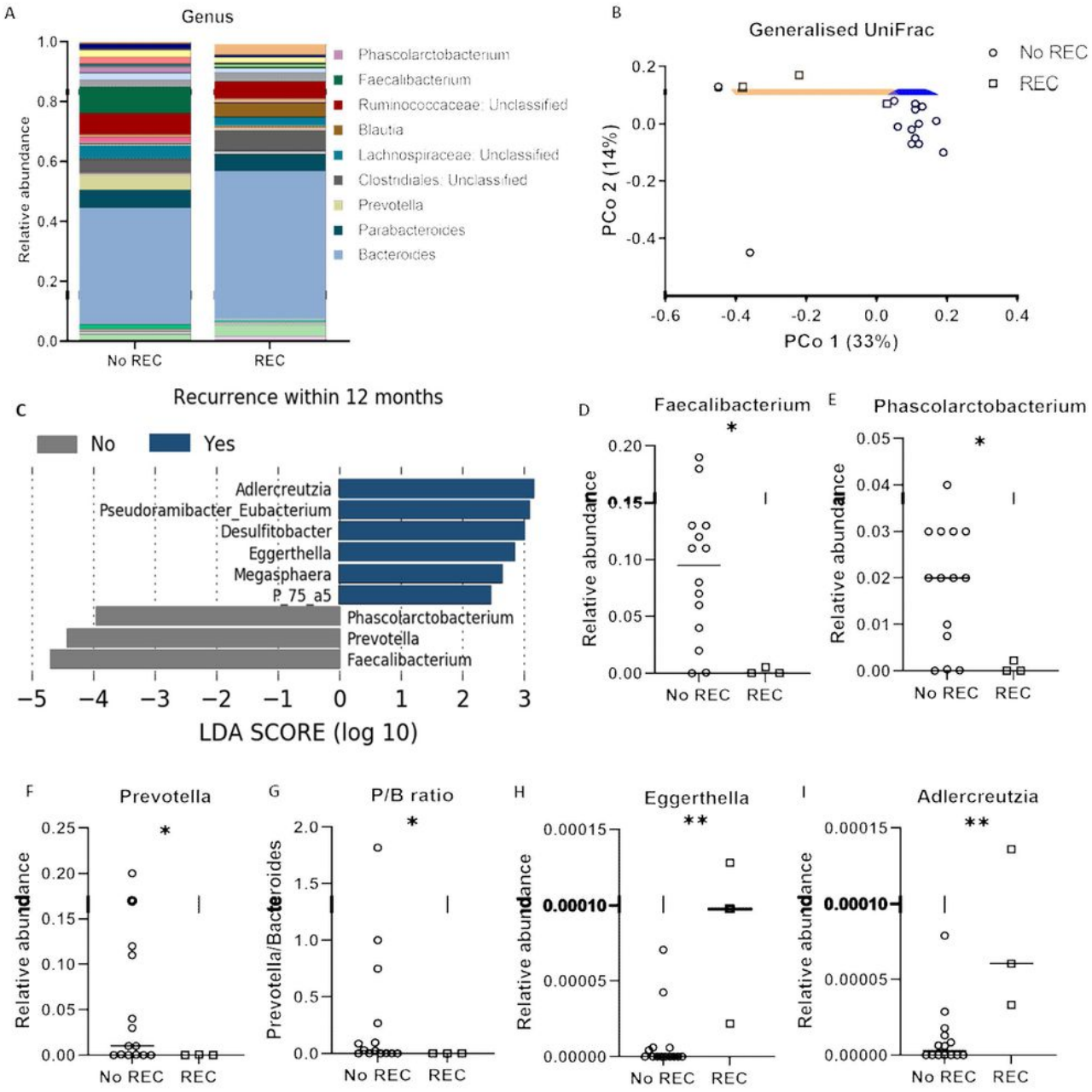

Figure 4

Association between the gut microbiota and tumour recurrence at 12 months. A) The relative abundance of the gut microbiota at the genus level for No REC and REC groups. B) PCoA of No REC and REC groups. C) LEfSe analysis showing the differential genera enriched in No REC and REC groups. D-G) The relative abundance of *Faecalibacterium* (D), *Phascolarctobacterium* (E), *Prevotella* (F), and P/B ratio (G) was significantly higher in No REC group. H-I) The relative abundance of *Eggerthella* (H) and *Adlercreutzia* (I)

was significantly higher in REC group. LDA; Linear discriminant analysis. * p≤ 0.05; ** p≤ 0.001; Mann-Whitney test; Line represents the median

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.pdf
- SupplementaryMaterials.pdf