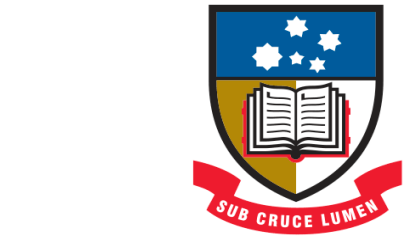

THE UNIVERSITY of ADELAIDE

Exploring the Role of the Gut Microbiome in Toxicity and Response to Radiotherapy for Head and Neck Cancer

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BSc (Hons)

A thesis submitted in fulfilment for degree of

DOCTOR OF PHILOSOPHY

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I. Table of Contents

II. Thesis abstract

Radiotherapy is a mainstay treatment modality used for the treatment of more than 80% of patients with head and neck cancer (HNC). Despite the technological advances in radiotherapy delivery, two key limitations remain a challenge for HNC radiotherapy. First, HNC radiotherapy is associated with unacceptable levels of normal tissue toxicities. One of the most frequent and troublesome toxicities is oral mucositis (OM). Radiotherapy-induced OM refers to the inflammation and/or ulceration of the oral mucosa following radiotherapy. It can affect more than 90% of patients with HNC, with varying degrees of severity. The major challenges related to OM are the lack of effective interventions to prevent or treat OM and the lack of a robust predictive marker to predict OM risk. The second limitation of HNC radiotherapy is heterogeneity in patients' response in terms of tumour control and recurrence. Currently, there are no biomarkers to identify patients with a favourable response and those at risk for primary tumour failure or tumour recurrence. Therefore, finding new targets for OM interventions and predictive biomarkers to predict radiotherapy outcomes will help address these limitations of HNC radiotherapy and improve treatment success.

Recent years have witnessed a growing interest in the role of the gut microbiome in cancer treatment efficacy and toxicity, including radiotherapy. The gut microbiome, a collection of microorganisms residing in the gastrointestinal tract, plays a central role in the modulation of systemic immune and inflammatory responses. Given that OM is an inflammatory condition and radiotherapy-induced immunogenic cell death is a key pathway by which radiotherapy kills tumour cells, we hypothesised that the gut microbiome may influence both the pathogenesis of OM and radiotherapy response through modulation of immune and inflammatory signalling. As such, this thesis aimed to investigate the impact of the gut

microbiome on the development of radiotherapy-induced OM and radiotherapy outcomes in preclinical and clinical settings. Firstly, I investigated this in preclinical studies described in chapters 3-5. In chapter 3, I successfully developed an antibiotic-induced gut microbiota depletion (AIMD) method that allows for studying the development of OM in the absence of major bacterial taxa. In chapter 4, I established a radiation-induced OM model in rats using a single radiation dose of 20 Gy. These two models were used to conduct the main animal study (chapter 5), which demonstrated that the gut microbiome is involved in the pathogenesis of OM, particularly the healing stage, through the modulation of inflammatory cytokines.

Lastly, to translate my preclinical findings to the clinical settings, I investigated whether patient pre-treatment gut microbiome is associated with the severity of OM and radiotherapy response in patients with HNC (chapter 6). The results from this clinical study showed that certain microbes in the baseline gut microbiome are associated with OM severity and tumour recurrence. Together, the results from this thesis suggest that the gut microbiome is involved in the pathogenesis of OM and is associated with radiotherapy response offering a potential target to treat or prevent OM and predict treatment outcomes.

III. Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree

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Ghanyah Al-Qadami

28/06/2021

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V. Publications arising from this thesis

Al-Qadami GH, Van Sebille YZA, Le HV and Bowen JM (2019). Gut microbiota: implications for radiotherapy response and radiotherapy-induced mucositis. Expert Review of Gastroenterology & Hepatology, 1-12.doi:10.1080/17474124.2019.1595586.

Al-Qadami GH, Verma G, Bateman E, Van Sebille, YZA, Hewson I, Le HV and Bowen JM. Antibiotic-induced gut microbiota depletion accelerates the healing of radiation-induced oral mucositis in rats. *(In preparation*)

Al-Qadami GH, Bowen JM, Van Sebille YZA, Wardill HR, Secombe KR and Le HV. Association between the Pre-treatment gut microbiome and radiotherapy response and radiotherapy-induced oral mucositis in patients with head and neck cancer: a pilot study (*In preparation*)

VI. Conference presentations

Oral presentations

Al-Qadami GH, Van Sebille YZA, Le HV, Hewson I and Bowen JM (2021). Antibiotic-induced depletion of the gut microbiota reduces injury area and duration of radiation-induced oral mucositis in rats. Multinational Association of Supportive Care in Cancer and International Society for Oral Oncology Virtual Annual Meeting.

Al-Qadami GH, Bowen JM, Van Sebille YZA, Wardill HR and Le HV (2021). Pre-treatment gut microbiome and radiotherapy response and toxicities among patients with head and neck cancer. Multinational Association of Supportive Care in Cancer and International Society for Oral Oncology Virtual Annual Meeting.

Al-Qadami GH, Bowen JM, Van Sebille YZA, Wardill HR and Le HV (2021). Association between the Pre-treatment gut microbiome and radiotherapy response and radiotherapy-induced oral mucositis in patients with head and neck cancer. Annual Australian Society for Medical Research (ASMR) SA Scientific Meeting, Adelaide, Australia.

Poster presentations

Al-Qadami GH, Van Sebille YZA, Le HV and Bowen JM (2018). The microbiome-immune axis in the development of radiation-induced oral mucositis. 12th Annual Florey Postgraduate Research Conference, Adelaide, Australia.

Al-Qadami GH, Van Sebille YZA, Le HV and Bowen JM (2018). Assessment of antibiotic-induced gut microbiota ablation in rats. The Australian Society for Medical Research National Scientific Conference, Adelaide, Australia.

Al-Qadami GH, Van Sebille YZA, Le HV and Bowen JM (2019). Antibiotic-induced depletion of gut microbiota: impact on radiation-induced oral mucositis in rats. 13th Annual Florey Postgraduate Research Conference, Adelaide, Australia.

Al-Qadami GH, Van Sebille YZA, Le HV, Hewson I and Bowen JM (2019). Antibiotic-induced depletion of gut microbiota: impact on radiation-induced oral mucositis in rats. The Clinical Oncology Society of Australia Annual Scientific Meeting, Adelaide, Australia.

Al-Qadami GH, Van Sebille YZA, Le HV, Hewson I and Bowen JM (2020). Antibiotic-induced depletion of gut microbiota: impact on radiation-induced oral mucositis in rats. 4th Meeting of the Federation of Neurogastroenterology & Motility, Adelaide, Australia.

Al-Qadami GH, Van Sebille YZA, Le HV, Hewson I and Bowen JM (2020). Antibiotic-induced depletion of gut microbiota accelerates the healing of radiation-induced oral mucositis in rats. 14th Annual Florey Postgraduate Research Conference.

VII. Other publications completed during candidature

Secombe KR, **Al-Qadami GH**, Subramaniam CB, Bowen JM, Scott J, Van Sebille YZA, Snelson M, Cowan C, Clarke G, Gheorghe C, Cryan J and Wardill HR. Guidelines for Reporting Animal Faecal Transplant (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols. (*Accepted in Gut Microbes Journal, August 2021*)

VIII. Abbreviations

HNC	Head and neck cancer	MMPs	Matrix metalloproteinases
HN	Head and neck	VEGF	Vascular endothelial growth factor
HNSCC	Head and neck squamous cell carcinomas	EGFR	Epidermal growth factor receptor
TNM	Tumour-node-metastasis	SNP	Single nucleotide polymorphism
LA	Locally-advanced	EGF	Epidermal growth factor
RM	Recurrent metastatic	GF	Germ-free
OM	Oral mucositis	Th17	T helper 17
GIM	Gastrointestinal mucositis	Tregs	Regulatory T cells
PBM	Photobiomodulation	IgA	Immunoglobulin A
ROS	Reactive oxygen species	AMPs	Antimicrobial peptides
NF-κB	nuclear factor kappa-B	TBI	Total body irradiation
IL	Interleukin	AIMD	Antibiotic-induced microbiota depletion
TNF-α	Tumour necrosis factor-alpha	OTU	Operational taxonomic unit
GI	Gastrointestinal	PCoA	Principal Coordinate Analysis
TGF-β	Transforming growth factor-beta	LEfSe	Linear discriminant analysis Effect Size
ICD	Immunogenic cell death	Gy	Gray (unit of ionising radiation)
DAMPs	Damage-associated molecular patterns	kV	Kilovolt
MAMPs	Microbial-associated molecular patterns	mA	Milliampere
APCs	Antigen-presenting cells	RGS	Rat grimace scale
DCs	Dendritic cells	VFT	Von Frey test
CRT	Calreticulin	MWT	Mechanical withdrawal threshold
HMGB1	High-mobility group box-1	TOT	Tip of the tongue
HSPs	Heat-shock proteins	BOT	Body of the tongue
PRRs	Pattern-recognition receptors	WBCs	White blood cells
LPS	Lipopolysaccharides	GCs	Goblet cells
TLRs	Toll-like receptors	HPV	Human papillomavirus
NKs	Natural killer cells	SCFAs	Short-Chain Fatty Acids

IX. Nomenclature

- **Gut microbiota**: a collection of microorganisms including bacteria, viruses, fungi, and protozoa that resides in the gastrointestinal tract.
- **Gut microbiome**: the microorganisms and their collective genome found in the gastrointestinal tract. In this thesis, gut microbiota was used when referring to the microorganisms (mainly bacteria) and gut microbiome was used when referring to the genetic profile of gut microbiota (particularly in chapter 6)
- **Dysbiosis**: the alteration of gut microbiota balance characterised by the reduction in the microbial diversity, reduction in the abundance of beneficial commensals, and the increase in the abundance of the harmful organisms (pathobionts).
- **Radiotherapy-induced oral mucositis**: inflammation and/or ulceration of the oral or oropharyngeal mucosa following radiotherapy.

X. Thesis explanation

This thesis is written in a combined conventional and publications format. It is composed of eight chapters including general introduction, literature review, four research chapters, general discussion, and references. Chapter 1 is a general introduction that explains the research background and current gaps in knowledge. Chapter 2 is a published literature review, which discusses the current evidence on the role of the gut microbiome in cancer treatment outcomes and its potential impact on radiotherapy response and radiotherapy-induced mucositis. Chapters 3, 4, and 5 are research chapters that describe the development of preclinical models and investigating the role of the gut microbiome in OM development in preclinical settings. Chapter 6 is a research paper that describes a clinical study aimed to assess the association between the baseline gut microbiome and radiotherapy response and oral mucositis severity in patients with head and neck cancer. Chapter 7 is a general discussion that explains and discusses all results and proposes future directions for this field of research. The references of all chapters were combined in chapter 8 at the end of the thesis.

Chapter one

This chapter is a general introduction of the main topics of this thesis including head and neck cancer epidemiology, treatments, treatment-associated adverse effects, and the gut microbiome, and its key functions on the host's body. It also outlines the aims and hypotheses of the thesis.

Chapter 1: General Introduction

1.1 Head and neck cancer

Head and neck cancer (HNC) is a term referring to a group of heterogeneous malignancies that arise at different sites in the upper aerodigestive tract and include the oral cavity, nasal and paranasal sinuses, larynx, pharynx, salivary glands, and HNC skin [1]. More than 90% of these tumours are squamous cell carcinomas of the mucosal lining of the head and neck (HNSCC) [2]. Together, HNC malignancies account for the sixth most common type of cancer worldwide according to the Global Cancer Observatory (GLOBOCAN), 2020 [3]. In 2012, GLOBOCAN reported an estimated 683,235 new HNC cases worldwide. This includes 300,000 lip and oral cavity cancers, 157,000 laryngeal cancers, and 229,000 pharyngeal cancers [4]. As with most cancers, we now see the number of new HNC cases increasing. In 2020, more than 930,000 new cases were reported globally (lip and oral cavity, 377,713; larynx, 184,615; pharynx, 316,020, and salivary glands, 53,583 cases) [3]. The number of deaths is also increasing with around 375,000 deaths reported in 2012 [4] compared to more than 460,000 deaths in 2020 [3]. HNC is one of the ten most common cancers in Australia and accounts for 3.6% of the total new cancer cases in 2020. According to the Australian Institute of Health and Welfare, 5,168 new HNC cases and 1,151 deaths were reported in 2020 [5].

HNC carries enormous clinical and economic burdens. First, HNC is associated with considerable mortality and morbidity. Although early-stage HNC can be cured, HNC has a poor overall survival rate. HNC has an overall five-year survival rate of less than 50% [6], depending on tumour site. Generally, lip cancer has a better prognosis, with a five-year relative survival rate of 94% compared to oral cavity (60%), oropharynx (50%), nasopharynx (57%), and hypopharynx cancer (30%) [7]. In addition, HNC has an inevitable symptom

burden, with most patients with HNC experiencing a wide range of symptoms such as pain, fatigue, difficulty chewing or swallowing, and dry mouth caused by cancer itself or treatment complications [8, 9]. Moreover, HNC is associated with high levels of psychological stressors as a result of cancer diagnosis and treatment side effects, interference with activities, compromised interpersonal relationships, and uncertainty of outcomes, that might require clinical intervention [10]. Furthermore, HNC has a substantial economic burden with an estimated cost of up to 535 billion USD between 2018 and 2030 [11]. The main contributors of HNC financial costs include direct medical costs (diagnosis, screening, treatments, management of side effects, and follow-up care) and indirect costs (reduced productivity due to lower workforce participation and early mortality) [12]. The economic burden of HNC is also high in Australia. A study by Pollaers et al. analysed the economic cost of only one type of HNC, i.e., lip and oral cavity cancers, and reported that this type of cancer cost an average of 92,958 AUD over two years of diagnosis with 92.8% of the cost spent in the first year. The analysis included both expenses spent in inpatient and outpatient treatments [13]. Overall, HNC carries a wide range of health and economic costs that can be mitigated by improving cancer diagnosis, enhancing treatment effectiveness, and reducing treatment-associated complications.

1.2 Head and neck cancer staging

Tumour staging is a critical tool for clinicians to define tumour origin and characteristics and to predict tumour prognosis. It is also an important stratification factor in clinical research [14]. One of the commonly used staging systems for HNC is the tumour-node-metastasis (TNM) staging system of the American Joint Committee on Cancer and the International Union for Cancer Control [6]. This system is used to categorise tumours based on tumour size and location (T), the involvement of regional lymph nodes (N), and the

presence of distance metastasis (M) [15]. Based on the TNM system, HN tumours are classified into four stages; early-stage disease (stages I and II), locally advanced (LA) disease (stages III, IVA, and IVB), and recurrent metastatic disease (RM), and refractory disease (stage IVC) [16]. Overall, around 30% to 40% of total patients present with early-stage disease, and 60% present with LA disease [17]. Approximately 50% of patients with LA disease develop locoregional recurrence or distant metastasis within 2 years of treatment [18]. The eighth edition of the TNM system (Table 1) is the newest edition, which became effective in 2018 [19]. The major update in this new version is the addition of a new staging system for human papillomavirus-positive (HPV+) oropharyngeal cancer [14].

Table 1: Overview of HNC TNM clinical staging system (8th edition, 2017), adapted from [19]:

	T	N	M
Lip and oral, nasal, laryngeal, salivary glands, hypopharyngeal, and oropharyngeal (HPV/p16-negative) cancers:			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IVA	T1, T2, T3	N2	M0
	T4a	N0, N1, N2	M0
Stage IVB	Any T	N3	M0
	T4b	Any N	M0
Stage IVC	Any T	Any N	M1
Oropharyngeal cancer (HPV/p16-positive):			
Stage I	T1, T2	N0, N1	M0
Stage II	T1, T2	N2	M0
	T3	N0, N1, N2	M0
Stage III	T1, T2, T3	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any T	M1
Nasopharyngeal cancer:			
Stage I	T1	N0	M0
Stage II	T1	N1	M0
	T2	N0, N1	M0
Stage III	T1, T2	N2	M0
	T3	N0, N1, N2	M0
Stage IVA	T4	N0, N1, N2	M0
	Any T	N3	M0
Stage IVB	Any T	Any N	M1

1.3 Head and neck cancer treatment

The treatment options for patients with HNC are surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, prescribed in different combinations depending on tumour primary site and tumour stage [17, 20]. In early-stage cancer, the treatment options are surgery or radiotherapy, where efficacy is comparable [21-23], thus, the treatment is chosen based on the tumour site and extension, patient preference, co-morbidities, and expertise of the multidisciplinary team [18, 24]. For oral cavity cancer, surgery is preferred to avoid radiotherapy-related toxic effects [18]. Laryngeal cancers are treated with radiotherapy, endoscopic- or open- surgery. These modalities have comparable survival rates, but radiotherapy and endoscopic surgery are preferable due to the preservation of the patients speech and voice [25]. Patients with LA disease require multimodal therapy. Patients with resectable LA disease are treated with surgery, followed by postoperative radiotherapy with or without chemotherapy. Unresectable LA tumours are treated with combined radiation and chemotherapy (as induction or concurrent) [26]. In RM disease, the standard treatment option is systemic therapy including chemotherapy, targeted therapy and immunotherapy [27].

Among targeted therapies used for HNC, cetuximab, a targeted therapy against the epidermal growth factor receptor (EGFR), has been added to the treatment regimen of both LA and RM disease [28]. Currently, the EXTREME regimen (cetuximab, cisplatin/carboplatin, and 5-fluorouracil chemotherapy) followed by maintenance cetuximab is used as first-line treatment for RM-HNSCC [29]. Recently, a number of immunotherapies have demonstrated promising results in treating HNC. In 2016, the Food and Drug Administration approved two immunotherapies based on programmed death 1 (PD-1) receptor inhibitors (pembrolizumab and nivolumab) for the treatment of RM-HNSCC

[30]. The approval was based on the positive findings of multiple clinical trials on patients with RM-HNSCC. For example, in a multi-cohort phase 1b trial, pembrolizumab was safe and demonstrated clinically significant anticancer activity with an overall response of 18% in patients with RM-HNSCC [31]. Ferris et al. also showed that nivolumab resulted in longer overall survival and reduced toxic side effects compared to single-agent chemotherapy in patients with platinum-refractory HNSCC [32]. These two immunotherapies were approved as a second-line treatment for RM-HNSCC [27]. However, more recent evidence suggests that immunotherapy i.e., pembrolizumab could be used as a first-line treatment in patients with PD-L1 positive RM-HNSCC. Results from a recently published randomised, open-labelled, phase 3 clinical trial (KEYNOTE-048), which compared the efficacy of pembrolizumab with/without chemotherapy and cetuximab plus chemotherapy for RM-HNSCC, showed that pembrolizumab alone or with chemotherapy improved the overall survival in RM-HNSCC with PD-L1 combined positive score of >1. The trial concluded that pembrolizumab and chemotherapy could be used as first-line treatment for RM-HNSCC. While pembrolizumab alone is a suitable first-line treatment for PD-L1 positive RM-HNSCC [33].

1.4 Radiotherapy for head and neck cancer

Radiotherapy is one of the key curative-intent treatments used for treating up to 80% of patients with HNC [34]. It can be used as primary, adjuvant, or palliative treatment depending on disease stage [35]. Radiotherapy can be used alone to treat early-stage disease [36] or in combination with chemotherapy or as adjuvant therapy following surgery to improve locoregional control and overall survival in advanced-stage disease [37]. The standard conventional radiotherapy regimen involves treating the primary tumour site with a total of 66 -70 Gy (2- 2.2 Gy/fraction; 5 fractions/week over five days) over six to seven

weeks. Other altered fractionated regimens, such as hyperfractionated radiotherapy (81.6 Gy, 1.2 Gy/fraction; twice daily, 7 weeks) or accelerated fractionation (66 - 70 Gy, 2.0 Gy/fraction, 6 fractions/week), can be used for some types of HNC. For the chemoradiotherapy regimen, patients are treated with a single chemotherapeutic drug, typically cisplatin, in addition to 70 Gy radiotherapy (2 Gy/fraction) delivered to the tumour primary site [35]. Radiotherapy can be administered externally or internally [38]. External beam radiotherapy involves the administration of precise radiation dose into the tumour site delivered by different techniques including intensity-modulated radiotherapy, image-guided radiotherapy, and 3-dimensional conformal radiotherapy [36]. Internal radiotherapy (brachytherapy) utilises radioactive sources that are implanted within or close to the tumour, which then are removed when the required doses have been administered [38].

Despite the recent advances in radiotherapy techniques and the introduction of novel agents, three key challenges hinder the therapeutic efficacy of HNC radiotherapy including radioresistance, tumour recurrence, and radiotherapy-associated toxicities [36]. Radiotherapy causes tumour cell death by inducing DNA double-stranded breaks. However, tumour cells can acquire radiation resistance by enhancing the efficiency of DNA repair following exposure to radiation [38]. Intrinsic radioresistance is caused by alterations in the intracellular pathways regulating DNA repair, cell proliferation, and apoptosis. Increased frequency of p53 gene mutations, increased expression of EGFR, and the upregulation of phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin signalling pathway have been linked to radioresistance in HNC [38-40].

Tumour relapse (locoregional recurrence and distant metastasis) is another challenge and can be an indication of treatment failure. Residual tumour cells that survive after irradiation

can repopulate the irradiated region resulting in local recurrence or move and cause distant metastases [38]. Depending on tumour site, type and treatment, 15% to 50% of patients with HNC develop locoregional recurrence and/or distant metastases [41]. A large scale study conducted by Chang et al. found that patients with HNC treated with radiotherapy alone or combination with surgery or chemotherapy had an overall recurrence rate of 14.44% with a recurrence rate of 15.45%, 11.05%, and 9.90% for oral cavity, oropharyngeal and hypopharyngeal cancers respectively [42]. In this study, 60% of patients who developed recurrence had advanced-stage disease (stage III and IV) [42]. Dragovic et al. showed that, among 560 patients with HNC who received radiotherapy with or without concurrent systemic therapy, 10% developed distance metastasis within 3 years after treatment. In another study by Sun et al., which included 868 patients with nasopharyngeal carcinoma treated with radiotherapy or chemoradiotherapy, a slightly higher recurrence was reported, with 22.6% developing local recurrence, and 5.4% developing regional recurrence [43]. Similarly, Lin et al. reported that 26% of patients with nasopharyngeal carcinoma treated with chemoradiotherapy, and 46% of those who received radiotherapy alone had tumour relapse within 5 years of treatment completion [44]. The main HNC metastasis sites are lung (50%), multiple sites (18%), or bone (11%) [45].

Another challenge of radiotherapy treatment is the high level of undesirable acute and late adverse effects that can impact both treatment outcomes and patient quality of life [34, 46-48]. Severe treatment toxicities can lead to unplanned treatment interruption, hence, reducing treatment success [48]. Moreover, the toxic effects of radiotherapy are associated with compromised quality of life, depression, and anxiety [49, 50]. Generally, radiotherapy is a key part of HNC management, however, current therapeutic success rates are not optimal. Therefore, further research to identify potential factors that affect radiotherapy

anti-tumour response and contribute to the pathogenesis of radiotherapy-associated normal tissue toxicities is needed to enhance treatment outcomes and minimise treatment-related complications.

1.5 Radiotherapy-related toxicities

Radiotherapy is associated with several toxicities that develop during treatment or after treatment completion. These toxicities can be classified into acute toxic effects (oral mucositis (OM), dysphagia, dysgeusia, dermatitis, infection, and pain) or late toxicities (xerostomia, dental caries, osteoradionecrosis, trismus, and fibrosis) [34, 46, 51]. The acute short-term toxicities develop during radiotherapy treatment and resolve within a few weeks to a few months after treatment completion. Late long-term side effects arise around 3 months or more following radiotherapy completion and can persist for years [34]. Often the severe, persistent, or unresolved acute complications can lead to consequential late side effects. For example, the severity and duration of acute OM are associated with a higher risk of developing late mucosal reactions (atrophy, dryness, telangiectasia, or ulceration) [52]. Moreover, acute xerostomia and dysphagia are prognostic factors for the risk of developing late dysphagia [53].

1.5.1 Oral mucositis

Radiotherapy-induced OM refers to inflammation and ulceration of the oral/oropharyngeal mucosa following radiotherapy [54]. OM develops in the second or third week after treatment initiation, peaks between 4–5 weeks, lasts around 40 days [range 7-98 days], and resolves within 4 to 6 weeks after treatment cessation [55, 56]. It is one of the most frequent complications that affect the majority of patients with HNC depending on tumour location and type of treatment received [55, 57, 58]. Trotti et al. reported that the overall incidence of OM among 6181 patients with HNC was 80% [55]. The frequency of OM among

patients treated with altered fractionated radiotherapy, conventional radiotherapy, or chemoradiotherapy was 100%, 97%, and 90% respectively. Severe OM (grade 3-4) was higher in patients who received altered fractionated radiotherapy (56%) compared to those treated with conventional radiotherapy (34%) or chemoradiotherapy (43%) [55]. A study by Vera-Llonch et al. analysed the incidence of OM among 450 patients with HNC and reported that 83% of the patients developed OM [59]. Severe OM was observed in 29% of patients and was more common among patients with naso/oropharyngeal carcinoma and those who received a higher radiotherapy dose, or those treated with chemoradiotherapy [59]. Moreover, in a retrospective study by Elting et al. that included 204 patients, 91% of the patients developed OM, with 66% presented with severe OM. Cancer site (oral cavity and oropharynx), chemoradiotherapy, and altered fractionated radiotherapy were associated with a higher risk of severe OM [57].

In addition to the high incidence of OM, it is also one of the most debilitating toxicities that significantly impact patients' quality of life. OM is associated with secondary complications such as severe pain, difficulty swallowing, weight loss, and infections [55, 57, 60, 61]. Trotti et al. reported that 69% of patients with OM experienced oral pain, 54% suffered from dysphagia, and 34% had weight loss [55]. Moreover, Elting et al. found that around 54% of patients with OM experienced severe pain and 60% had a weight loss of ≥ 5% [57]. OM is also associated with an increased risk of infections. The breakdown of the protective mucosal layer results in the translocation of oral microorganisms into the oral mucosa, and eventually the bloodstream causing a local or systemic infection that could become a life-threatening complication, especially among immunocompromised patients [62]. Among patients with HNC with OM, 43.2% had oral herpes simplex virus [60], and around 38% developed oral candidiasis [61]. Patients with severe OM often require hospitalisation or

medical intervention including feeding tube insertion and pain and infection management. Studies have reported that around 19% of patients need feeding tube insertion and 16% to 33% required hospitalisation due to severe OM [55, 59].

OM is also a dose-limiting toxicity that often leads to treatment beaks or modification [63]. Around 24%, 32%, and 60% of patients experience unplanned beaks or delays in radiotherapy due to mild, moderate, and severe OM respectively [59]. Treatment interruption or dose reduction strongly correlates with reduced locoregional control and survival rates [48, 63]. Groome et al. reported that radiotherapy interruption for >3 days and treatment break at the end of the treatment course were associated with a higher risk of local failure in patients with glottic cancer [64]. Moreover, long-duration (>10 days) of unplanned radiotherapy gaps are associated with reduced 5-year recurrence-free survival and increased risk of locoregional recurrence (relative risk ratio of 1.016 per day of a gap) [65].

In addition to its clinical impact, OM is also associated with a significant economic burden [55, 57]. A recent study by Elting reported that the management of OM in patients with HNC treated with radiotherapy or chemoradiotherapy had an incremental cost between 5,000–22,000 USD depending on OM severity [66]. The economic burden of OM management in Australia has not been studied, however, a study by Corry et al. reported that feeding tube (percutaneous endoscopic gastrostomy) insertion for patients with HNC costs approximately 736 AUD per patient [67]. Overall, OM is one of the most common and troubling side effects of radiotherapy treated HNC and imposes significant clinical and economic consequences.

1.5.2 Oral mucositis grading

The severity of OM varies from mild erythema to severe life-threatening ulcers. There are several scales for OM grading. The most clinically relevant scales are the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) and World Health Organisation scales. According to the NCI-CTCAE scale, OM is classified into five grades (Table 2) [68]. Moreover, several quantitative scoring scales for assessment of patient-reported outcome measures (PROMs), like the Oral Mucositis Weekly Questionnaire-Head and Neck Cancer, are being used to evaluate OM severity [69]. Since the patient-reported outcomes are often different from those reported by clinicians, scales based on PROMs are essential to capture patient perceptions of OM severity, symptom burden, and its impact on oral functions and life quality [70]. Moreover, PROMs are critical for the assessment of OM in clinical trials and cohort studies [71].

Table 2: NCI-CTCAE classification of radiation-induced OM (Version 5.0, 2017) [68]:

Stage	Description
Grade 1 (mild)	Asymptomatic or mild symptoms; intervention not indicated
Grade 2 (moderate)	Moderate pain or ulcer that does not interfere with oral intake; modified diet indicated
Grade 3 (severe)	Severe pain; interfering with oral intake
Grade 4 (life-threatening)	Life-threatening consequences; urgent intervention indicated
Grade 5 (death)	Death

1.5.3 Oral mucositis management

Currently, there are no standard preventive or therapeutic measures for OM and the current management methods focus mainly on symptom relief and nutritional support [72]. Recent “clinical practice guidelines for the management of mucositis secondary to cancer therapy” by the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) highlighted the up to date recommendations for

the management of OM in patients with HNC treated with radiotherapy or chemoradiotherapy (Table 3) [73]. The current evidence only supports the use of benzydamine mouthwash and intraoral photobiomodulation (PBM) therapy for OM prevention in HNC patients undergoing radiotherapy or chemoradiotherapy in specific clinical settings [73]. Benzydamine (marketed as Diflam in Australia) is an anti-inflammatory drug that helps prevent the severity of OM through the inhibition of the production of pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) [74]. The current guidelines recommend benzydamine use for OM prevention in patients receiving radiotherapy doses less than 50 Gy only. It also suggests its use in patients receiving chemoradiotherapy but the strength of current evidence supporting this is limited [75]. Given that the conventional radiotherapy regimens involve the administration of radiation doses between 66 and 70 Gy [35] and the inadequate research supporting benzydamine use for chemoradiotherapy, more evidence is required for widespread use of this agent in patients with HNC. PBM involves the use of low-level light or laser therapy to stimulate wound healing and tissue regeneration and to reduce inflammation [76]. Despite showing promising results in patients receiving radiotherapy or chemoradiotherapy, the optimal PBM parameters such as device, delivery, and treatment settings have not been standardised [73]. Moreover, some have suggested that PBM may have carcinogenic impacts as it activates tumour related pathways such as proliferation and invasion [77], therefore, further studies on the safety of PBM and its impact on tumour cell behaviours are needed.

Palifermin (recombinant human keratinocyte growth factor 1) is the only agent approved by the US Food and Drug Administration to prevent OM in patients with haematological cancers undergoing chemotherapy and total body irradiation prior to haematopoietic stem

cell transplantation [78]. To date, two clinical studies have assessed the effectiveness of palifermin against OM in patients with HNC and reported that palifermin reduces the incidence and duration of severe OM among patients treated with chemoradiotherapy [79, 80]. Henke et al. demonstrated that Palifermin reduced the incidence of severe OM (grade ≥3) from 67% to 51% [79] while Le et al. reported a reduction in the incidence of severe OM from 69% to 54% after the Palifermin administration [80]. However, the high cost of palifermin [81] and modest impact on OM severity makes it an undesirable option for the management of OM in HNC. Overall, the management of radiotherapy-induced OM in patients with HNC remains a challenge, hence, further research to investigate new therapeutic targets to prevent or treat OM is warranted [82].

Table 3: MASCC/ISOO guidelines for the management of OM in patients with HNC treated with radiotherapy or chemoradiotherapy. Modified from MASCC/ISOO guidelines 2020 [73] and 2014 [83]:

Intervention type	Intervention (LoE)	Purpose	Treatment type
Recommendations in favour of an intervention			
Anti-inflammatory agents	Use of benzydamine mouthwash (I)	OM prevention	Patients with HNC receiving radiotherapy (<50 Gy)
PBM	Use of intraoral PBM therapy by low-level laser therapy (II)	OM prevention	Patients with HNC receiving radiotherapy (without chemotherapy)
PBM	Use of intraoral PBM therapy by low-level laser therapy (I)	OM prevention	Patients with HNC receiving chemoradiotherapy
Recommendation against an intervention			
Antimicrobials/ coating agents	Use of Sucralfate (combined topical and systemic) (II)	Prevention of OM-related pain	Patients with HNC receiving radiotherapy
	Use of Sucralfate (topical and systemic) (II)	OM treatment	Patients with HNC receiving radiotherapy
	Use of PTA (polymyxin, tobramycin, amphotericin B) and BCoG (bacitracin, clotrimazole, gentamicin) antimicrobial lozenges and PTA paste (III)	OM prevention	Patients with HNC receiving radiotherapy
	Use of iseganan antimicrobial mouthwash (II)	OM prevention	Patients with HNC receiving radiotherapy or chemoradiotherapy

Suggestions in <u>favour</u> of an intervention			
Basic oral care	Implementation of multiagent oral care protocols (III)	OM prevention	Patients with HNC receiving radiotherapy
Anti-inflammatory agents	Use of benzydamine mouthwash (II)	OM prevention	Patients with HNC receiving chemoradiotherapy
Analgesics	Use of 0.2% topical morphine mouthwash (III)	Treatment of OM-related pain	Patients with HNC receiving chemoradiotherapy
Natural/ miscellaneous	Use of oral glutamine (II)	OM prevention	Patients with HNC receiving chemoradiotherapy
Natural/ miscellaneous	Use of honey (II)	OM prevention	Patients with HNC receiving radiotherapy or chemoradiotherapy
Suggestions <u>against</u> an intervention			
Anti-inflammatory agents	Use of chlorhexidine (I)	OM prevention	Patients with HNC undergoing radiotherapy
Other agents	Use of orally- administered systemic pilocarpine (III)	OM prevention	Patients with HNC undergoing radiotherapy

LoE: level of evidence; HNC, head and neck cancer; OM, oral mucositis

LoE I: Evidence acquired from meta-analysis of multiple, well-designed studies.

Lof II: Evidence acquired from at least 1 well-designed experimental study.

LoE III: Evidence acquired from well-designed quasi-experimental studies.

1.5.4 Pathophysiology of OM

Ionising radiation initiates mucosal injury leading to the development of OM through a five-phase process proposed by Sonis [84-86], briefly outlined below (Fig. 1):

Initiation (Phase I) (day 0- 2): Radiation initiates basal epithelial cell death through direct DNA damage and oxidative stress leading to the production of reactive oxygen species (ROS), which causes further epithelial, submucosal, and endothelial cell damage and activates subsequent molecular pathways.

Signal upregulation and amplification (Phase II/III) (day 2-10): ROS and other damage-associated molecular patterns (DAMPs) released from the injured cells activate several signalling pathways such as nuclear factor kappa-B (NF-κB), which is one of the well-established pathways in mucositis. The activation of NF-κB induces the production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNF-α, which accelerate mucosal tissue injury. The molecular signals are amplified as TNF-α and IL-1β can further up-regulate NF-κB signalling, which in turn initiates multiple signalling pathways such as mitogen-activated protein kinase, tyrosine-kinase, and cyclooxygenase 2. These pathways activate matrix metalloproteinases (MMP-1, -3, and -9) that lead to further cell death and tissue injury [84, 87].

Ulceration (Phase IV) (day 10- 15): The ulcerative phase of OM is the most clinically significant phase in which painful and deep ulcers are formed. These ulcers are often covered with a pseudomembranous layer, which creates a thriving environment for bacterial colonisation resulting in a secondary infection. Mucosal breakdown due to ulceration facilitates bacterial translocation into the submucosa. Toll-like receptors (TLRs), like TLR4, expressed by innate immune cells interact with the translocated bacteria leading

to the activation of inflammatory cells (M1 macrophage and N1 neutrophils), production of more pro-inflammatory cytokines, and exacerbating tissue damage.

Healing (Phase V) (day 14- 21): OM is self-resolving toxicity that starting healing after treatment cessation. Signals from the submucosa and extracellular matrix induce epithelial cell proliferation and differentiation leading to the restoration of the normal tissue structure. Anti-inflammatory cells (M2 macrophage and N2 neutrophils) and activated fibroblasts produce anti-inflammatory cytokines such as IL-10 and growth factors such as transforming growth factor-beta (TGF-β) and vascular endothelial growth factor (VEGF), which help resolve inflammation and stimulate epithelial regeneration, angiogenesis, and extracellular matrix deposition [88]. Moreover, expression of some MMPs such as MMP-9 is elevated in later stages of OM suggesting a dual role of MMP-9 in tissue injury and repair [87].

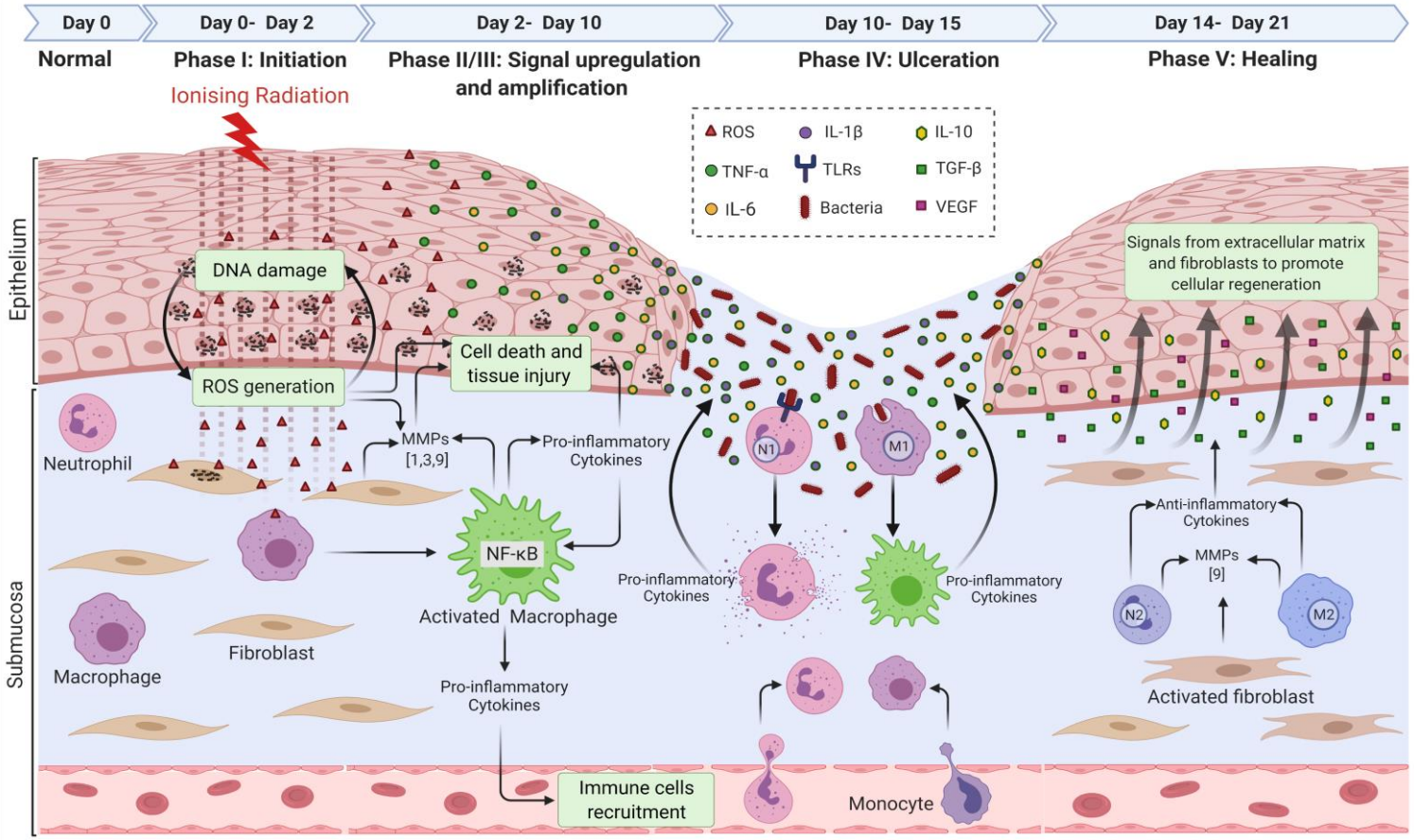

Figure 1: Five-phase model of mucositis development. The exposure to ionised radiation initiates tissue injury by DNA damage and ROS production. ROS activates NF-κB signalling pathway leading to the release of pro-inflammatory cytokines and activation of MMPs. The inflammatory and apoptotic signalling pathways are further amplified resulting in further tissue injury. This leads to the ulceration phase, in which the epithelial breakdown allows the entry of oral microbes into the submucosa. Microbes interact with inflammatory cells leading to further pro-inflammatory cytokines and infiltrates. In the healing phase, signals from the submucosa and extracellular matrix stimulate epithelial cell proliferation and differentiation leading to epithelial restoration. ROS, reactive oxygen species; NF-κB, nuclear factor kappa-B; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin 6; IL-1β, interleukin beta; MMPs, matrix metalloproteinases; TLRS, Toll-like receptors; IL-10, interleukin 10; TGF-β, transforming growth factor- beta; VEGF, vascular endothelial growth factor. *"Image created with BioRender.com."*

1.5.5 Oral microbiota and radiotherapy-induced OM

Oral microbiota refers to a collection of microorganisms that reside in the oral cavity. Overall, the oral microbiota is composed of more than 700 bacterial species and is considered the second-largest microbial community in the human body after the gut microbiota [89]. Oral microbiota plays a key role in oral health by preventing the colonisation of pathogenic bacteria. However, in the case of increased microbial load or weak immunity, oral microbes could contribute to local and systemic diseases [90]. Alteration of the oral microbiota has been found to be associated with radiotherapy-induced OM. A study by Hou et al. demonstrated that oral microbiota dysbiosis is associated with OM progression in patients with nasopharyngeal carcinoma [91]. Moreover, Vesty et al. found that the increased abundance of Gram-negative *bacilli* in saliva and buccal mucosa of patients with HNC treated with radiotherapy is associated with grade ≥2 OM [92]. Another study by Zhu et al. also reported that an increase in Gram-negative bacteria (*Actinobacillus*) correlates with the severity of radiotherapy-induced OM [93]. These findings suggest an association between the oral microbes and OM, however, the mechanisms by which microbiota contribute to OM have not been fully investigated. Furthermore, given that the use of topical antimicrobial agents is unsuccessful in preventing or reducing the severity of OM [94], this brings into doubt whether there are any causative links.

In addition to investigating the role of the oral microbiota in OM pathogenesis, previous studies have investigated whether oral microbial composition can be used as a predictive marker for OM risk [92, 93]. Analysing the oral microbiota samples collected from buccal [92] or oropharyngeal mucosa [93] showed that dynamic changes in the oral microbial community after radiation exposure could be used to predict OM progression. However,

more studies are needed to confirm these findings and to determine the most efficient sampling site e.g., saliva, buccal or oropharyngeal mucosa to be used as a standard for oral microbiota sampling for OM prediction.

1.6 Heterogeneity in radiotherapy response and toxicities and the need for biomarkers

Radiotherapy response rates and severity of radiotherapy-associated toxicities vary between patients, even among those diagnosed with the same tumour type and treated with the same treatment regimen [95, 96]. Patients who have identical tumour characteristics (size, stage, and spread) and receive similar radiotherapy doses do not have the same treatment outcomes (tumour control, recurrence rate, and survival rates) [97]. The current HNC staging system and tumour volume do not adequately explain the variation in survival and recurrence risk between patients [98]. HNC staging systems account for <30% of the variation in survival, thus, more than 70% of the variation in HNC survival remains unexplained [99, 100]. Several biomarkers including, human papillomavirus (HPV) infection, EGFR, p53 mutations, and hypoxia have been linked to radiotherapy response in HNC, however, the only HPV is being routinely used as a prognostic biomarker in clinical practice [101]. Therefore, research is needed to validate the current biomarkers and identify new and more precise predictive and prognostic markers.

Similar to the variation in tumour response, the degree of normal tissue toxicities caused by radiotherapy vary between patients. For example, the frequency and severity of radiotherapy-induced skin toxicities and OM differ from one patient to another and the reason behind this is still largely unexplained [102, 103]. Patient-related factors (age, smoking, co-morbidities) and treatment-related factors (single or combined modality,

treatment dose, and site) only explain 30% of patient-to-patient variability in radiotherapy acute and late skin toxicities [103]. Furthermore, the current factors are inadequate to identify patients at a higher risk of OM [104]. Genomic and proteomic-based approaches have been used to identify genetic variants or alterations in levels of certain proteins involved in OM development [81]. Genetic analysis has found that single nucleotide polymorphisms (SNPs) in DNA repair-related genes like *XRCC1*, *XRCC3*, and *RAD51* have been linked to severe OM [81, 96]. However, the results of these studies were not consistent and the current evidence is not adequate to support the use of SNPs as predictive markers for OM [96]. Studies also have investigated the correlation between the severity of OM and protein levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), TGF-β, epidermal growth factor (EGF), and C-reactive protein [81, 96]. These biomarkers are not ideal as they are not specific to OM and could be influenced by stress caused by many other conditions. Hence, there is a need for specific, easily measured, and non-invasive biomarkers to predict the risk of OM and thus design personalised interventions for individual patients [81]. There is growing evidence of the importance of tumour immunogenicity and radiotherapy-induced anti-tumour immune response as determinates of radiotherapy efficacy. Moreover, inflammation plays a major role in radiotherapy-induced toxicities, particularly OM (*further discussed in chapter 2 of the thesis*). Therefore, factors that may influence an individual's immune system such as the gut microbiota may play a role in the anti-tumour activity and pathogenesis of inflammatory toxicities of radiotherapy.

1.7 Gut microbiota

The human gastrointestinal (GI) tract harbours trillions of microorganisms (bacteria, viruses, fungi, and protozoa). These microorganisms are collectively known as "gut microbiota"

while the term “gut microbiome” refers to the microorganisms and their genome present in the GI tract [105]. The term “microbiome” was first coined by Joshua Lederberg “to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space” [106]. In recent years, gut microbiome research has gained increasing attention and gut microbiota functions and impacts on several physiological and pathological conditions have been explored [107]. This gaining momentum in gut microbiome research has established that the gut microbiota plays a key role in the development and modulation of the host’s physiological processes including the development of the immune system, maintenance of intestinal homeostasis, and regulation of host metabolism [108].

1.7.1 Gut microbiota and Immune development:

Gut microbiota plays a key role in shaping the intestinal and systemic immune response in health and disease. It is involved in the development and maturation of the immune system by the modulation of immune cell differentiation and the regulation of immunoglobulin A (IgA) and cytokine production [109]. The gut microbiota is important for the development of immune cells such as T helper 17 (Th17) and Treg cells. It has been demonstrated that germ-free (GF) mice, mice born and raised in sterile conditions, lack intestinal Th17 and have a reduced number of Treg cells in the colon [110, 111]. Colonising GF mice with segmented filamentous bacteria or Clostridium (clusters IV and XIVa) enhanced the generation of intestinal Th17 and increased the accumulation of Treg cells in the lamina propria of the colon respectively [110, 111]. Moreover, the gut microbiota is important for the maturation of gut-associated lymphoid tissues (mesenteric lymph nodes and Peyer's patches) [108, 109]. Furthermore, these microbes can modulate the production of immune mediators such as cytokines, chemokines, and IgA [108]. Systemically, gut microbes have

been found to modulate the production of pro-inflammatory cytokines impacting inflammation in sites other than the gut such as the lung [112] *(the role of the gut microbiome in modulating mucosal and systemic immune response is further discussed in chapter 2 of the thesis).*

1.7.2 Gut microbiota and intestinal homeostasis and intestinal barriers:

The nature of the intestinal environment requires a complex system to maintain the balance between the trillions of microbes residing in the GI tract and the host's mucosal immune system. The failure to maintain this balance negatively impacts both intestinal and systemic health. The Intestinal barriers including physical (epithelium and mucus layer) and biochemical barriers (antimicrobial peptides (AMPs) and IgA) are critical for the modulation of intestinal homeostasis [113]. The intestine is lined with a single layer of epithelial cells joined by tight junction proteins that regulate the flow of antigens and molecules across the epithelium. Specialised intestinal cells and immune cells produce and secrete molecules such as mucins, AMPs, and IgA that are important for clearing and preventing the invasion of intestinal pathogens. Furthermore, the epithelial and mucosal immune cells express pattern recognition receptors, such as TLRs that recognise a variety of microbial antigens and help modulate mucosal immune responses [114]. Accumulating evidence shows that gut microbiota is involved in the modulation of epithelial structure maturation [115], tight junction proteins reorganisation, AMPs and IgA production [108], and mucus layer fortification [115]. Dysbiosis, imbalanced gut microbial composition, could lead to intestinal barrier dysfunction and facilitates bacterial translocation. This will result in the activation of both local and systemic immune responses contributing to the onset or the augmentation of intestinal and extraintestinal inflammatory conditions [109].

Therefore, the gut microbiota is a key regulator of intestinal homeostasis and healthy intestinal barrier functions.

1.7.3 Gut microbiota as a biomarker and therapeutic target

Recent studies have revealed distinctive microbial features associated with different disease states [116, 117]. Therefore, the gut microbiota or its metabolites have been investigated as potential predictive, diagnostic, or prognostic biomarkers for conditions such as inflammatory bowel disease [118], obesity [119], chronic kidney disease [120], and cognitive impairment [121]. Moreover, a distinctive gut microbiome profile has been associated with the development of colorectal cancer [122], hepatocellular carcinoma [123], and lung cancer [124], suggesting that gut microbiota composition could be used as diagnostic markers for these cancers. Furthermore, recent evidence demonstrated that the gut microbiome can be used as a predictive marker of the efficacy and toxicities of anti-cancer treatments including chemotherapy, immunotherapy, and radiotherapy [125]. (*The potential role of gut microbiota on radiotherapy response and gastrointestinal toxicities is reviewed in chapter 2 of this thesis*).

Despite the current evidence linking specific microbial patterns to different diseases, the gut microbiome is yet to be used as a biomarker in clinical settings. Therefore, further research is needed to develop a standardised system of microbiome sampling and analysis and investigate the exact mechanisms by which the gut microbiota influences each pathological condition. This will allow the implementation of gut microbiome-based biomarkers in personalised medicine for disease diagnosis, severity assessment, and treatment response prediction to improve therapeutic outcomes [126]. The gut microbiome offers a unique, non-invasive, and targeted biomarker as it can be manipulated by various methods such as diet, pre-and probiotics, or faecal microbiota transplant [126].

Therefore, it may be modified to prevent the onset of diseases or improve therapeutic outcomes.

1.8 Thesis rational

Based on the evidence mentioned above, radiotherapy is a crucial treatment modality for HNC. However, the efficacy and toxicity of radiotherapy vary between patients and there is a need for new predictive markers and biological targets to enhance the radiotherapy anti-tumour response and minimise radiotherapy-associated complications. In recent years, the impact of the gut microbiota on the modulation of anti-tumour response through immunomodulation has gained increased attention. The gut microbiota has been widely explored in the context of immunotherapy and chemotherapy, however, little is known about how the gut microbiota impacts radiotherapy outcomes. It is well established that radiotherapy can induce immune-mediated cell death. Moreover, inflammation plays a central role in the pathogenesis of radiotherapy-associated OM. Given that each patient harbours a distinct gut microbial composition and that the gut microbiota is a key regulator of systemic inflammatory and immune responses, it can be hypothesised that gut microbiota is a determinant of radiotherapy outcomes in terms of both efficacy and toxicities.

1.9 Hypotheses and Aims

In this thesis, two distinct approaches were used to explore the relationship between the gut microbiota and radiotherapy outcomes: 1) A rat model of radiation-induced oral mucositis; 2) A clinical observational study.

Approach 1: the overall aim of this approach was to assess whether antibiotic-induced gut microbiota ablation impacts the development and severity of radiation-induced OM in rats.

In this study, I hypothesised that board-spectrum antibiotic treatment will ablate the gut microbiota resulting in a reduction in inflammatory signals and cytokine production and thus reducing the severity of radiation-induced OM. To test this hypothesis, I conducted three studies to 1) validate the gut microbiota ablation method using antibiotics, 2) develop a moderate radiation-induced OM model in rats, and 3) assess the role of gut microbiota ablation in the severity of radiation-induced OM.

1. **Aim 1a**: Assess palatability and efficacy of an antibiotic cocktail to ablate the gut microbiota in rats.
2. **Aim 1b**: Establish a moderate self-limiting single-dose radiation-induced OM model in rats.
3. **Aim 1c**: Evaluate the role of gut microbiota on the development and severity of radiation-induced OM in a rat model.

Approach 2: To translate the preclinical findings into a clinical setting, an observational study that included patients with HNC, was conducted. In this study, I hypothesised that; a) an individual's gut microbiome composition plays a role in their response to radiotherapy and the severity of radiotherapy-induced OM, and b) Patients with altered and less diverse gut microbiome will have increased severity of OM and unfavourable radiotherapy outcomes.

1. **Aim 2a**: Assess whether an individual's gut microbiome composition impacts the severity of radiotherapy-induced OM in patients with HNC.
2. **Aim 2b**: Assess whether an individual's gut microbiome composition impacts tumour response and recurrence in patients with HNC treated with radiotherapy.

Chapter two

This chapter is a literature review that discusses the potential impact of the gut microbiota on tumour response to radiotherapy and its role in oral and gastrointestinal mucositis. The chapter has been published as a review paper and is presented here in its original published format with few modifications including changing citation numbers, changing spelling to Australian spelling, and excluding the reference section, which is included in chapter 8.

Statement of Authorship

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Name of Principal Author (Candidate)	Ghanyah Al-Qadami		
Contribution to the Paper	I am the first author and was responsible for manuscript preparation, writing, and editing.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/06//2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 2: Gut microbiota: Implications for radiotherapy response and radiotherapy-induced mucositis

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2.1 Abstract

Introduction: Radiotherapy is a mainstay of solid tumour management but can be associated with unacceptable levels of off-target tissue toxicity which impact treatment outcomes and patients' quality of life. Tumour response to radiotherapy and the frequency and severity of radiotherapy-induced toxicities, especially mucositis, varies among patients. Gut microbiota has been found to modulate both the efficacy and toxicity of some types of cancer chemotherapies and immunotherapies but has yet to be investigated thoroughly in the setting of radiotherapy.

Area covered: In this review, we discuss the potential role of gut microbiota in modulating radiotherapy-induced oral and gastrointestinal mucositis and the anti-tumour response to radiotherapy through modulation of immune responses.

Expert commentary: The gut microbiota plays a major role in the modulation of systemic immune responses, which influence both radiotherapy response and gastrointestinal toxicities such as mucositis. Hence, investigating the gut microbiota link to the variation in radiotherapy responses and toxicities among patients is warranted. Future targeting of these responses with a patient-tailored restoration of optimal microbial composition could lead to a new era of mucositis prevention and enhanced tumour responses.

Keywords: Immunomodulation, Microbiota, Mucositis, Radiotherapy

2.2 Introduction

Radiotherapy is a core modality used for the treatment of brain, head and neck, breast, lung, abdominal and gynecological cancers [127]. Around 50% of all patients with cancer are treated with radiotherapy during the course of their disease [128] and 60% of these are treated for curative purposes [129]. It is a cost-effective method that accounts for only 5% of the total cancer care expenses [130]. It is also a minimally invasive modality that allows flexible adjustment of the dose regimen required for each disease stage and for each individual patient [131]. Despite technological advances in radiotherapy, there are a number of limitations that affect radiotherapy treatment success. Currently, tumour radioresistance and recurrence are major clinical challenges of radiotherapy [132]. Tumours vary in their response to radiation and recurrence rate from one patient to another. Some of these variations can be explained by clinical factors such as tumour size, stage of disease, or failure to determine the exact tumour spread leading to reduced local control. However, these variations do not adequately explain the differences in treatment response considering that among tumours with the same size, stage, and grade and treated with same radiation dose, some will have a recurrence and some will not [97]. Furthermore, there is accumulating evidence that biological factors such as intrinsic radioresistance [133], hypoxia [134], inflammatory cell infiltration [135], and recruitment of bone marrow-derived cells [136] into the tumour microenvironment can also affect radiotherapy outcomes.

Radiotherapy is also associated with several toxicities that impact patients' quality of life. These toxicities can be acute; developing during or immediately following radiotherapy and may affect the likelihood of treatment course completion, including mucositis, dermatitis, cystitis, and bone marrow suppression. In contrast, chronic toxicities appear months or

years after the completion of the treatment and include fibrosis, vascular damage, or atrophy of the affected tissue or organ [129]. Late chronic toxicities may develop as consequential effects of severe non-healing acute toxicities, for instance, prostate cancer patients treated with radiotherapy who experience acute gastrointestinal (GI) toxicities are significantly more likely to develop late GI toxicities such as long-term diarrhoea [137]. However, there is a variation in the incidence and severity of radiotherapy-induced toxicities between patients [138]. Among patients with identical tumour size, site and stage, who receive the same treatment schedule, some patients will develop severe toxicities while some will not [139]. Among the identified risk factors of developing toxicities are therapy-related factors (radiation dose, volume, fraction and site, and concomitant therapies) and patient-related factors (age, gender, smoking, and comorbidities) [138]. In addition, studies have shown that genetic variations also may contribute to the risk of severe toxicities following radiotherapy [140]. However, these factors still poorly estimate the risk of toxicities and there is a need for more accurate and sensitive predictive markers.

In recent years, we have witnessed a growing interest in the impact of the gut microbiota on modulating cancer treatment efficacy and toxicity. Gut microbiota refers to a collection of microorganisms such as bacteria, viruses, archaea, and eukarya that reside in the human GI tract [141]. It is estimated that more than 100 trillion microbes, mainly bacteria, with 500–1000 different species, are found in the GI tract [109, 142]. These microorganisms can be found in the mouth, stomach, small and large intestine but the diversity and load substantially increase distal to the ileocecal junction [143]. The microbiota composition varies along the different sites of the GI tract and the dominant phyla of gut microbiota are *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria,* with around 90% of them belonging to *Firmicutes* and *Bacteroidetes* [143]. However, there is a

significant interindividual variation in the gut microbiota composition and even a single individual's microbes composition changes over time [144]. Host genetics [145] and several environmental factors such as age, diet, disease, medications, and lifestyle can influence the composition of gut microbiota [146]. The gut microbiota exists in a mutualistic symbiotic relationship with the host, in which the host provides nutrients and environment necessary for the microorganisms' survival. In turn, microbiota contributes to several physiological processes of the host such as digestion, carbohydrate fermentation, immune response modulation and prevention of pathogenic colonisation [109].

The alteration of the gut microbiota composition, also known as dysbiosis, has been linked to the pathogenesis of intestinal diseases, such as inflammatory bowel disease and celiac disease, and extra-intestinal diseases including obesity, allergies and type 2 diabetes [147, 148]. The microbiota contributes to the development of these disorders primarily through the interaction between the microbiota or their metabolic products and the host immune system. For instance, inflammatory bowel disease is associated with alterations in the abundance and diversity of gut microbiota. Changes in the microbial composition can lead to disruption in metabolites production, intestinal barrier and intestinal immune and inflammatory responses, therefore, contributing to inflammatory bowel disease pathogenesis [149]. Moreover, dysbiosis can enable overgrowth of pathogenic microorganisms such as *Clostridium difficile.* The loss of colonisation resistance and imbalanced immune responses during dysbiosis contributes to the pathogenesis of *Clostridium difficile* infection [150]. Recently, it has been revealed that gut microbiota influences tumour response [151-154] and severity of cancer treatment-induced GI toxicities [155-158]. The gut microbiota has been shown to affect both the efficacy and toxicity of various chemotherapies and immunotherapies via several mechanisms,

primarily through the modulation of immune responses [125]. However, little is known about the role of the microbiota in response to radiotherapy [159]. In this review, we will explain how immune responses affect cellular responses to radiation. In addition, we will discuss the potential impact of gut microbiota on radiotherapy anti-tumour activity and its role in radiotherapy-induced GI mucositis through immunomodulation.

2.3 Effect of immune signalling on cellular response to radiotherapy

Radiotherapy causes tumour cell death by depositing high energy radiation in the cell which induces DNA damage. Ionising radiation causes DNA damage directly or indirectly through the production of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [160]. Exposure to ionising radiation can induce tolerogenic or immunogenic cell death depending on radiation dose and cell type. Exposure to low dose radiation (< 1 Gy) causes tolerogenic apoptosis in which dying cells are engulfed by macrophages and is associated with the release of anti-inflammatory cytokines including Transforming growth factor-beta 1 (TGF-β), interleukin-10 (IL-10) and prostaglandin E2. Conversely, the exposure to a higher radiation dose, like doses received during cancer treatment, can lead to immunogenic cell death which is associated with the release of pro-inflammatory cytokines (e.g., IL-1, IL-6, tumour necrosis factor-alpha (TNF-α)) [161, 162]. The activation of inflammatory responses prolongs the radiation response by generating more ROS, cytokines and growth factors [163]. This has been implicated in both radiotherapy-induced tumour cell death and normal tissue toxicities.

2.3.1 Radiation-induced immunogenic cell death

Radiation can induce tumour cell death via apoptosis, senescence, mitotic catastrophe and necrosis [164]. Generally, apoptosis is considered to be non-immunogenic, however accumulating evidence has demonstrated that, in some settings, apoptosis can be immunogenic [165]. In animal models, it has been found that ionising irradiation and specific chemotherapy agents such as anthracyclines and oxaliplatin can induce an anti-tumour immune response and result in immunogenic cancer cell death [166-169]. Immunogenic cell death (ICD), or immunogenic apoptosis, involves stimulation of the immune response against dying tumour cells and is mediated by damage-associated molecular patterns (DAMPs), which are molecules released or expressed by dying tumour cells [170]. Expression or release of these molecules leads to the activation of antigen-presenting cells (APCs), particularly dendritic cells (DCs). Activated APCs then engulf tumour cell antigens, process them and present them to cytotoxic T lymphocytes and subsequently induce a tumour-specific immune response [171] (Figure 1).

The best-characterised DAMPs include ATP, genomic DNA, calreticulin (CRT), high-mobility group box-1 (HMGB1), heat-shock proteins (HSPs), and pro-inflammatory cytokines such as IL-1α and IL-6 [170]. These molecules interact with vesicular or membrane-bound pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) resulting in activation of the immune response [172]. Gameiro et al. investigated radiation-induced immunogenic modulations *in vitro* and *in vivo* settings. The irradiation of three different tumour cell lines (lung, breast, and prostate human carcinoma) induces the release of ATP and HMGB1 from dying and surviving cancer cells. In addition, when injecting nude mice with prostate carcinoma cells, radiation increases the expression of antigen-processing machinery and CTR in tumour cells which enhances their sensitivity to cytotoxic T-cell killing [173].

Moreover, radiation can induce the production, cell surface expression and extracellular secretion of HSPs leading to the stimulation of anti-tumour immune response through the activation of T and natural killer cells (NKs) as reviewed by Multhoff et al. [174]. Furthermore, Yoshimoto et al. demonstrated that local radiotherapy induces a systemic tumour-specific immune response and that anti-tumour immunity is essential for radiotherapy efficacy. In tumour-bearing mice, they found that depletion of $CD8^+$ cells significantly reduces radiotherapy-induced delay in tumour growth and mice survival time, hence reducing radiotherapy efficacy [166].

TLRs are a class of PRRs that play a key role in ICD and mediate the interaction between DAMPs and immune cells [175]. They are transmembrane proteins expressed by different types of cells including DCs, macrophages and epithelial cells. TLR signalling is mediated through myeloid differentiation primary response protein-88 (MyD88)-dependent pathway or MyD88-independent pathway. The MyD88-dependent signalling pathway is used for all TLRs except TLR3 and is required for the production of pro-inflammatory cytokines. The Myd88-independent signalling pathway is mediated by TIR-domain-containing adaptor protein inducing interferon-β (TRIF) and is required for TLR3 and TLR4 signalling and leads to the production of type I interferons (IFN) [176]. TLR4 is a cell surface homodimer protein that recognises microorganism-derived 'microbial-associated molecular patterns' (MAMPs) such as lipopolysaccharides (LPS) or DAMPs such as HMGB1, resulting in the release of pro-inflammatory cytokines [177, 178]. One study has found that that TLR4 expression by DCs is essential for the efficient presentation of dying tumour cells antigens to immune cells. The release of HMGB1 by cancer cells and activation of the TLR4–MyD88 dependent pathway enhances anti-tumour immune response and radiotherapy and chemotherapy efficacy [178]. However, this study demonstrated that mice with deleted TRIF have a similar

response to chemotherapy as wild-type mice [178]. Moreover, in another study, it has been shown that radiotherapy increases the production of type I IFNs which enhances the cross-priming ability of DCs and improve tumour response to radiotherapy. However, the increase in IFN-β production was independent of TRIF-dependent TLR signalling [179]. Together this may suggest that TLR4–MyD88 dependent but not TLR4–MyD88 independent pathway has an impact on tumour response to cancer treatments.

Radiation-induced ICD indicates that the immune system plays a role in radiotherapy activity and tumour control, thus, the efficacy of radiotherapy may be improved through the modulation of immune responses and communication between tumour cells and the immune system. Given this, research on factors that may influence immune responses to radiotherapy, such as interaction with microbial factors, is warranted and may lead to improving an individual's response to radiotherapy.

Although immune responses can improve radiotherapy responses through ICD, some studies have found that inflammatory cell infiltration and secreted inflammatory mediators may contribute to tumour progression by promoting tumour growth and angiogenesis [180]. Different types of immune cells are recruited to the tumour microenvironment including NKs, tumour-infiltrating lymphocytes ($CD4^+$ and $CD8^+$), B cells, and a range of myeloid-derived cells (tumour-associated macrophages (TAM), DCs, and neutrophils) [181]. Infiltrating lymphocytes and NKs are important for anti-tumour immune responses. However, myeloid-derived cells can enhance or reduce radiation anti-tumour immune responses. DCs have a critical role in the cross-priming of tumour antigens to cytotoxic $CD8^+$ T cells leading to tumour-specific immune response [182]. A recent review has demonstrated that although some evidence indicates that radiation induces pro-tumourigenic phenotypes of TAM that enhance angiogenesis, tumour growth and invasion,

others have shown that radiation induces the programming of TAM toward pro-inflammatory phenotypes that enhance the anti-tumour response [183]. Therefore, to maximise radiation response, we need to overcome the pro-tumorigenic effect of TAM and enhance the communication between tumour and APCs.

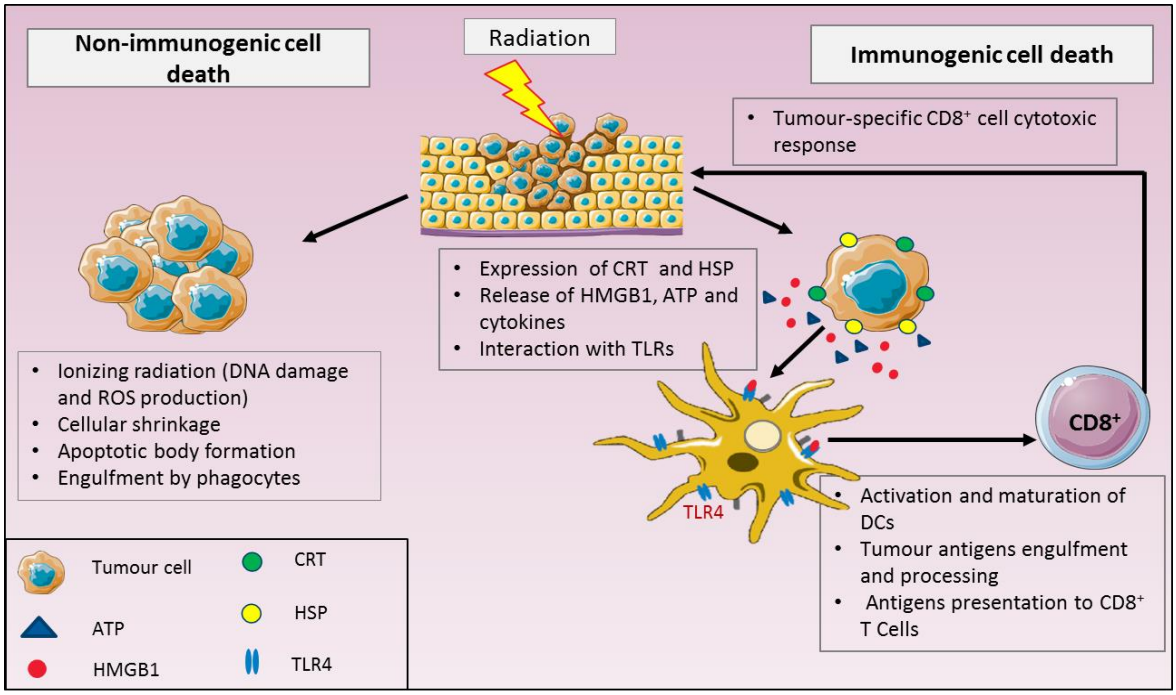

Figure 1: Immunogenic and non-immunogenic tumour cell death caused by radiation. For non-immunogenic cell death, radiation causes direct cell death through DNA damage and ROS production leading to cellular shrinkage and apoptotic body formation. Cellular debris is engulfed by phagocytes without eliciting an immune response. In immunogenic cell death, radiation causes cellular injury leading to the release of DAMPs that interact with TLRs resulting in DC activation. Activated DCs stimulate CD8+ cells, which in turn induce a tumour-specific anti-tumour response. (HAMG1: high-mobility group box-1; CRT: calreticulin; HSP: heat-shock proteins; TLR4: Toll-like receptor 4)

2.3.2 Immune signalling and radiation-induced normal tissue toxicities

Immune signalling pathways, primarily inflammatory responses, are involved in acute and late radiotherapy-induced normal tissue toxicities including gastrointestinal mucositis, pneumonitis, and fibrosis [161, 184]. The development of these toxicities is caused by direct exposure to ionising radiation, ROS production, and release of inflammatory cytokines. Inflammatory mediators cause amplification of ROS production through stimulation of ROS and RNS-producing enzymes such as cyclooxygenase-2 (COX-2), NADPH oxidase, Nitric oxide synthase (NOS) [185]. Overproduction of free radicals causes oxidative damage to both irradiated and non-irradiated bystander cells and contributes to acute and chronic complications [186]. Thus, targeting inflammatory responses and redox system pathways is a potential approach to ameliorate normal tissue toxicity following radiotherapy.

2.4 Gut microbiota modulates intestinal and systemic immune responses

Gut microbiota plays a critical role in the development and modulation of both mucosal and systemic immune responses. Germ-free (GF) mice are associated with many defects in the intestinal immunity at the tissue, cellular and molecular levels [187]. GF mice have significantly small Peyer's patches and reduced numbers of IgA-producing plasma cells and $CD4^+$ T cells in the lamina propria [188]. Moreover, they have a systemic T-cell deficiency, reduced production of IFN-γ, and $CD4^+$ T cells are biased toward T helper 2 (Th2) cells [189]. Furthermore, the secondary lymphoid organs such as peripheral lymph nodes and spleen of GF mice are less cellular and have poorly developed T and B cells zones [190]. Gut microbiota modulates intestinal immunity through the interaction with PRRs, mainly TLRs. In the intestine, TLRs that are expressed by enterocytes and DCs, recognise several MAMP molecules on the bacterial cell surface such as LPS and peptidoglycan [191]. The

microorganisms maintain intestinal homeostasis by regulating the balance between anti-inflammatory and pro-inflammatory signals. For instance, it has been demonstrated that commensal Bacteroides fragilis facilitates the development of Regulatory T (Treg) cells, which produce anti-inflammatory IL-10 [192]. Conversely, the segmented filamentous bacterium (SFB) induces the differentiation and generation of Th17 cells, which produce pro-inflammatory cytokines in the small intestine [110].

Systemically, microbiota metabolites such as short-chain fatty acids (including butyrate and propionate) have been found to promote Treg extrathymic generation, suggesting that by-products can mediate the gut microbiota communication with the immune system and modulate the pro-and anti-inflammatory response equilibrium [193]. Furthermore, gut microbial dysbiosis is associated with several immune-mediated disorders such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis, as well as obesity, autism, and type 2 diabetes [148, 194]. In GF mice, arthritis was attenuated due to the reduction in autoantibodies, splenic Th17, and autoantibodies-secreting cells. However, the introduction of SFB into GF mice led to the development of arthritis and restored Th17 cells and autoantibody production [195].

2.5 Gut microbiota and radiotherapy efficacy

The gut microbiota has recently been suggested to play a major role in cancer pathogenesis and response to cancer treatment [159, 196]. Gut microbiota interacts with cancer treatments in a bidirectional manner. Anticancer treatments disrupt intestinal microbiota composition and promote dysbiosis. Kim et al. characterised the mouse gut microbiota and revealed that radiation causes significant alteration in both the abundance and diversity of microbiota with an increase in *Alistipes* and a decrease in *Mucispirillum* genus [197]. In addition, a clinical study showed that exposure to pelvic radiotherapy results in the

remodeling of overall gut microbiota composition, with a 10% decrease in phylum Firmicutes and a 3% increase in phylum *Fusobacterium* [157]. Generally, the most significant alterations in the gut microbiota associated with cytotoxic chemotherapy or radiotherapy are the increase in *Bacteroides* and *Enterobacteriaceae* and the decrease in *Bifidobacterium*, *Faecalibacterium prausnitzii* and *Clostridium cluster XIVa* [198].

Evidence also indicates that gut microbiota affects anticancer treatment activity and side effects. The gut microbiota modulates chemotherapy efficacy through 'TIMER' mechanisms which include "Translocation, Immunomodulation, Metabolism, Enzymatic degradation, and Reduced diversity" [125]. Immunomodulation is an important mechanism by which gut microbiota influences the response to chemotherapies (cyclophosphamide and oxaliplatin) and immunotherapies (CpG-oligodeoxynucleotides, CTLA-4 inhibitors, and anti-PD-L1) [125]. Viaud et al. demonstrated that Gram-positive gut bacteria induces Th17 and Th1 cell immune responses which are critical for anti-tumour activity of cyclophosphamide in tumour-bearing mice [151]. Another study, in mouse models, demonstrated that the disruption of gut microbiota impacts the response of subcutaneous tumours to oxaliplatin, due to reduced production of ROS by myeloid cells, and to CpG-oligonucleotide due to the poor response of myeloid-derived cells and reduction in cytokine production [152]. Moreover, gut bacteria are critical for CTLA-4 blockade (ipilimumab) anti-tumour response. Vétizou et al. showed that in antibiotic-treated and GF mice, Ipilimumab anti-tumour activity was compromised compared to specific pathogen-free mice. However, treating mice with *Bacteroidales spp* induces immunostimulatory effects, mediated by IL-12–dependent Th1 immune response, resulting in better tumour control [153]. Furthermore, commensal *Bifidobacterium* induces a tumour-specific immune response and enhances the response to anti-PD-L1 immunotherapy in melanoma mouse models. This effect is

mediated by enhancing DC function which increases the accumulation of $CD8^+$ T cells in the tumour microenvironment [154]. Clinically, the use of antibiotics against Gram-positive bacteria impairs patients' response to cisplatin and cyclophosphamide chemotherapy. Among patients with chronic lymphocytic leukemia and relapsed lymphoma, the use of Gram-positive active antibiotics was associated with early tumour progression and lower overall survival [199].

Currently, no studies have investigated the impact of gut microbiota on radiotherapy efficacy. However, some studies have found that gut microbiota impacts normal tissue radiosensitivity [200-202]. Crawford and Gordon (2005) investigated the role of gut microbiota in intestinal radiosensitivity and found that the small intestine of GF mice is resistant to radiation injury. The deficiency of fasting-induced adipose factor, which is normally suppressed by microbiota, enhances the villus endothelium and lymphocytes radiation-induced cell death [200]. Moreover, a study conducted by Cui et al. demonstrated that gut microbiota disruption impacts radiosensitivity in conventional mice. It was found that disruption of the circadian rhythm of mice is associated with a reduction in the abundance of gut microbe species and that the change in the gut microbiota composition increases mouse sensitivity to gamma-ray irradiation. When mice were exposed to 5 Gy total body irradiation (TBI), those with altered circadian rhythm have a lower survival rate compared to those housed in a 12 h dark/12 h light cycle. This suggests that circadian rhythm may have a gut microbiota-dependent effect on the radiation response [201]. Evidence from clinical studies also showed that circadian rhythm may impact radiotherapy local control and toxicities. Patients treated in the morning have better local control and less severe toxicities [203]. Moreover, a preclinical study showed that gut microbiota is important for the efficiency of TBI preconditioning. In this study, mice were injected with

melanoma cells and exposed to 5 Gy of TBI followed by adoptive transfer of pmel-1 $CD8^+$ T cells, which are splenocytes activated *in vitro* in the presence of hgp100 tumour antigen that is highly expressed in human melanomas. Lymphodepletion using TBI before adoptive transfer of tumour-reactive $CD8^+$ T cells enhances tumour control. However, depletion of gut microbiota with antibiotics, LPS neutralisation, or TLR4 deletion, reduces the effectiveness of TBI, subsequently decreasing the efficiency of adoptively transferred $CD8^+$ T cells to attack tumour cells. TBI induces the translocation of the gut microbiota, and elevates LPS which interacts with TLR4 leading to innate immune activation; hence, enhancing $CD8^+$ T cell activation and improving tumour regression [202].

Although this work does not prove direct causality in terms of microbiota and radiotherapy efficacy, it does indicate a biological signal needing further exploration. Therefore, based on the current evidence and given that immune responses are involved in the radiation-induced cell death, it can be hypothesised that the gut microbiota plays a role in radiotherapy immunostimulatory effect hence, impacting the tumour response to radiotherapy. This immunostimulatory effect of gut microbiota is potentially mediated by Th17 and $CD8^+$ T immune responses (Figure 2).

2.6 Gut microbiota and radiotherapy-related GI toxicities

Preclinical and clinical studies have revealed that the gut microbiota contributes to the pathogenesis of chemotherapy, radiotherapy, and immunotherapy-related GI toxicities. The exposure to cytotoxic agents or radiation therapy causes intestinal crypt cell apoptosis, disruption of the mucosal barrier and changes the microbiota composition. This results in bacterial translocation and subsequently immune system activation and gut inflammation. The gut microbiota has been found to play a role in chemotherapy and immunotherapy-related GI toxicities such as irinotecan-induced diarrhoea and ipilimumab-induced colitis

[125]. Furthermore, gut microbiota plays a role in radiation-induced toxicities including alimentary tract mucositis.

2.6.1 Radiotherapy-induced alimentary tract mucositis

Mucositis is defined as an inflammation or ulcerative lesions that affect the mucosa of the GI tract or oral cavity [204]. Radiotherapy can cause both GI mucositis (GIM) and oral mucositis (OM) depending on the structures receiving radiation. The exposure to ionising radiation leads to the initiation of GIM and OM which develop through a five-stage model [85]. Mucositis pathobiology has been described elsewhere extensively [54, 85] but involves in brief; the initiation of tissue injury by radiation followed by inflammation upregulation and amplification which involve the production of pro-inflammatory cytokines such as IL-1β and IL-6 and TNF-α. This leads to ulceration and enhanced inflammation due to interactions with microbial products crossing the breached epithelium. Healing is the final stage which involves extracellular matrix signalling and the proliferation of epithelial cells restoring mucosal integrity. Three signalling pathways have been implicated in radiation-induced mucositis; 1) nuclear factor-κB (NF-κB), 2) NLR-related protein 3 nucleotide-binding domain leucine-rich repeat containing receptor-related protein 3 (NLRP3) inflammasome, and 3) mitochondrial dysfunction. Ionising radiation causes the production of ROS which activates the NF-κB pathway leading to the release of pro-inflammatory cytokines (IL-1β and IL-6 and TNF-α) and hence inducing an inflammatory response. This inflammatory response and ROS contribute to mitochondrial dysfunction which leads to amplified ROS production from impaired mitochondria resulting in the activation of NLRP3 inflammasome pathways. Activated NLRP3 leads to the production of more IL-1β through the activation of caspase-1 [205].

The frequency and severity of mucositis differ from one patient to another. Currently, conventional patient-related factors such as genetics, age, gender, lifestyle or therapy-related factors, like treatment type, dose, and schedule, are used for the prediction of those at higher risk of developing GIM and OM. These factors are unreliable and still underestimate the toxicity risk, hence new alternative risk predictive markers are needed [104]. The gut microbiota has been found to play a role in the pathogenesis of radiation-induced GIM [198]. This impact is potentially mediated through influencing and modulating the oxidative stress and inflammatory process, intestinal permeability, mucus layer composition, epithelial repair and harmful stimuli resistance, and expression and release of immune effector molecules in the intestine [206]. The gut microbiota may contribute to radiation-induced GIM through two mechanisms; translocation and dysbiosis. Radiation disrupts the intestinal barriers and mucus layer and causes bacterial translocation resulting in inflammatory response activation. Moreover, dysbiosis due to radiation or due to other factors can influence both local and systemic immune responses (Figure 2).

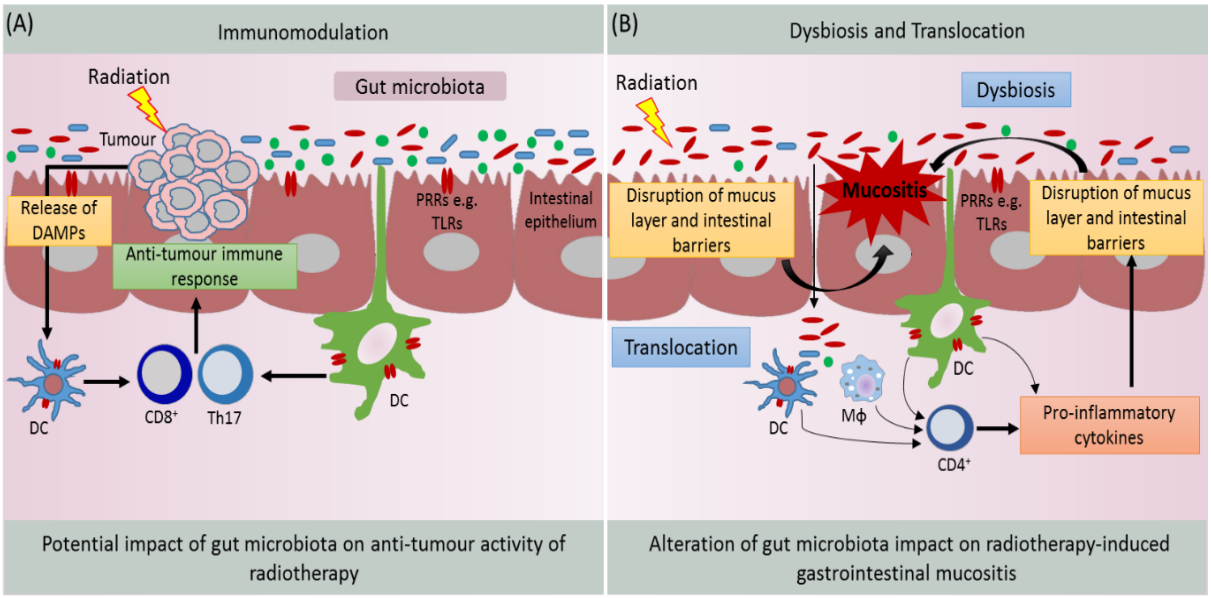

Figure 2: Gut microbiota impact on radiotherapy response and radiotherapy-induced gastrointestinal mucositis through modulation of immune responses. A) Gut microbiota potentially impacts the response to radiation by interacting with immune cells (e.g., DCs) in the intestine and enhancing the innate immune responses; hence improving the anti-tumour immune response which is mediated by Th17 and CD8+ cells. B) Radiation causes gut microbiota translocation and dysbiosis which disrupt intestinal immune homeostasis and release of pro-inflammatory cytokines. This leads to further damage of intestinal barriers; therefore, enhancing radiation-induced gastrointestinal mucositis. (DC: dendritic cells; Mϕ: macrophages; PRRs: pattern recognition receptors, TLRs: Toll-like receptors; DAMPs: damage-associated molecular patterns; Th17: T helper 17 cells)

2.6.2 Radiotherapy-induced gastrointestinal mucositis

GIM is a debilitating side effect of radiotherapy that significantly impacts patients' quality of life [207], and may lead to treatment delays or dose reductions, compromising treatment outcomes. GIM is associated with several symptoms including abdominal pain, rectal bleeding, diarrhoea, fatigue, and infections [198]. One of the most common symptoms of radiotherapy induced-GIM is diarrhoea. It affects more than 80% of cancer patients receiving pelvic radiotherapy [208]. Interestingly, some cancer patients develop severe diarrhoea following radiotherapy and some do not [156]. One of the investigated factors of radiotherapy-related diarrhoea is gut microbiota dysbiosis (Table 1). In a pilot clinical study, which involved 10 patients receiving pelvic radiotherapy and 5 healthy controls, the development of diarrhoea (in 6 patients) was associated with a significant modification in gut microbiota composition compared to patients who did not develop diarrhoea. *Firmicutes* phylum diversity was increased among patients with diarrhoea, however, *Actinobacteria* phylum was not detected in the samples of the patients who didn't develop diarrhoea. In addition, the analysis of the pre-radiotherapy stool samples of those who developed diarrhoea indicated that their microbiota composition was different from the control group and patients who did not develop diarrhoea. This suggests that pre-radiotherapy microbiota composition could be a predictive marker for radiation-induced diarrhoea [156]. In another small clinical study, Nam et al. investigated the impact of gut microbiota composition in 9 patients receiving pelvic radiotherapy for gynecological cancers. This study revealed that there are significant differences in the gut microbiota of patients and healthy individuals before radiotherapy. This study also showed that, following radiotherapy, the 8 patients that developed diarrhoea had a significant change in their gut microbiota composition [157]. Furthermore, Wang et al.'s study of 11 patients receiving pelvic radiotherapy and 5 healthy controls found that gut microbiota dysbiosis

both pre-and post-radiotherapy was associated with an increased risk of fatigue and diarrhoea. Moreover, significant differences in the relative abundance of selected genera such as *Veillonella, aecalibacterium,* and *Clostridia clusters XI and XVIII* was observed between patients who developed diarrhoea and those who did not. Patients with low microbiota diversity and high Firmicutes/Bacteroides ratio are more likely to develop diarrhoea [158]. Although these are small studies, they clearly indicate that gut microbiota composition is involved in the pathogenesis of radiation-induced GIM.

Furthermore, evidence from clinical studies has shown promising results for the use of different probiotic preparations to prevent or alleviate radiotherapy-induced GIM, mainly diarrhoea [208-210]. Moreover, MASCC/ISOO guidelines support the use of probiotic preparations of *Lactobacillus* species for the prevention of radiotherapy-induced diarrhoea [211]. However, the findings of a recent meta-analysis indicated that the current evidence shows no benefits of the use of probiotics for the prevention of radiotherapy-induced diarrhoea and suggests that future research should focus on pairing the GI toxicities with certain microbial phenotypes to allow targeted microbiota manipulation [212].

2.6.3 Toll-like receptors and radiotherapy-induced gastrointestinal mucositis

The gut microbiota interacts with TLRs expressed on epithelial and immune cells to maintain intestinal homeostasis. The depletion of gut microbiota using broad-spectrum antibiotics in mice has been associated with increased susceptibility to methotrexate-induced gastrointestinal injury, which is suppressed by the administration of TLR2 ligands [155]. Conversely, the knockout of TLR4 in mice has been shown to reduce irinotecan-associated pain and gut toxicity [213]. Additionally, in mice, the administration of LPS (a component of Gram-negative bacteria membrane) before radiation, protects intestinal crypts via induction of COX-2 and the production of prostaglandins [214]. LPS stimulates

TLR4-expressing cells, leading to the release of TNF-α which interacts with the TNF receptor on the surface of subepithelial fibroblasts, leading to the production of prostaglandins that reduced radiation-induced apoptosis of epithelial stem cells [215]. Another potential mechanism of TLR protection from radiation is the activation of NF-κB pathway [206]. Egan et al. showed that the activation of NF-κB signalling was essential for the protection of the gut against radiation-induced apoptosis. In addition, this study showed that NF-κB activation mediates LPS radioprotection [216]. This suggests that TLRs may impact the intestinal response to radiation-induced epithelial damage through the NF-κB pathway.

2.6.4 Radiotherapy-induced oral mucositis

Oral mucositis (OM) is a common adverse effect of cancer treatments and it impacts 20% to 100% of cancer patients depending on the type of treatment they receive [217]. Among head and neck cancer (HNC) patients receiving radiation doses between 50–54 Gy, more than 90% develop OM, and about 60% of these develop severe OM (grades 3 and 4) [57]. OM negatively affects patients' quality of life, therapy, and economic outcomes. Most patients with OM cannot eat by mouth due to severe pain, and often require parenteral nutrition, subsequently, patients also experience severe weight loss [55, 57]. Furthermore, OM is dose-limiting and it may cause therapy interruption which negatively affects tumour outcome and patient survival. Some patients with OM may require extended hospitalisation; hence, increasing economic cost. In one study, 11% of the HNC patients treated with radiotherapy had unplanned treatment interruption and 16% of them required hospitalisation due to OM [55].

To date, there is no broadly effective treatment or preventive measure for OM. Management of OM is mainly supportive care to reduce pain, provide patients with nutritional support, and treat secondary infections [72]. Several risk factors have been

found to impact the incidence and severity of OM including patient-related factors (genetics, age, gender, body mass index (BMI), oral hygiene, and tobacco use) and therapy-related factors (radiation site, dose, and fractionation, and combined therapies) [218]. Recent studies have investigated the genetic polymorphisms associated with the risk of OM development. Single nucleotide polymorphisms (SNPs) in radiosensitivity gene (*XRCC1*) [219], DNA repair gene (*Ku70*) [220], TNF receptors gene (*TNFRSF1A*) [221] and transcription factor gene (*ZNF24*) [222] were associated with increased risk of OM among HNC patients. Although many factors have been identified, there is still a lack of clear predictive markers for OM that can be modified to reduce incidence and severity.

For radiation-induced OM, no study has investigated the impacts of gut microbiota composition in OM incidence and severity. However, few studies have examined the association between oral microbiota and OM development. It has been found that radiation changes the diversity and functional behaviors of the oral microbiota in patients with radiotherapy-induced OM [223]. In addition, Zhu et al. found that alteration of the oral microbiota is associated with the progression and severity of OM. Their study included 41 nasopharyngeal carcinoma patients receiving 3D-conformal radiation therapy. The oral microbiota analysis revealed that the Gram-negative bacteria relative abundance increased as mucositis reached peak severity, and those with severe OM had lower bacterial alpha diversity and higher abundance of *Actinobacillus spp.* [93] (Table 1). However, the use of antibiotics, in the form of paste [224] or lozenges [225], to selectively eliminate oral microbiota have failed to reduce or prevent radiation-induced OM among HNC patients who were treated with radiotherapy. Moreover, the use of topical (lozenges/paste) antimicrobial agents is not recommended for the prevention of OM [226]. However, Sharma et al. demonstrated that the use of lozenges of *Lactobacillus brevis* CD2 in HNC

patients receiving chemo-radiotherapy is associated with reduced OM incidence and higher treatment completion rate [227]. This may suggest that probiotics may have a better impact on alleviating OM compared to antibiotics, and further studies are warranted.

Oral microbiota may play a role in the pathogenesis of radiotherapy-induced OM, however, it is not an ideal predictive marker for the OM risk. There are several challenges for the use of oral microbiota composition as a predictive marker for radiation-induced OM. First, different sites of the mouth harbour different microbiota species which results in sampling variations and biases and makes it difficult to determine which species are actually associated with OM. Additionally, radiation changes saliva composition and volume which impacts microbiota diversity. Salivary glands are radiosensitive and the exposure to radiation therapy can cause gland damage, resulting in hypofunction and xerostomia [228]. Radiotherapy in patients with HNC is associated with hyposalivation, reduction in saliva buffering pH and an increase in *Lactobacilli* and *Candida* species [229]. The changes in saliva quality and quantity result in less flushing, buffering, and immune function. Moreover, radiotherapy-induced xerostomia reduces the oral environment proteins and immunological properties and increases the acidity leading to an increase in the acidogenic microbes such as *Lactobacillus spp, Actinomyces Streptococcus mutans*, and a decrease of other types of microorganisms such as *Fusobacterium, Neisseria* and *Streptococcus sanguis* [230]. Therefore, oral microbiota is not a good marker for predicting the risk of radiation-induced OM.

Table 1: Clinical studies to investigate impact of microbiota in the pathogenesis of radiotherapy toxicities

Gut microbiota				
Study	**Study subjects**	**Treatment type**	**Toxicity**	**Key findings**
Manichanh et al. [156]	10 patients with abdominal cancer 5 healthy controls	Pelvic radiotherapy (1.8–2.0 Gy/ day, 5 times/week, 5 weeks)	Diarrhoea	Controls and patients without diarrhoea had a stable microbial diversity over 7 weeks period Patients who developed diarrhoea had a significant modification in their microbial diversity *Actinobacteria* phylum not detected in patients without diarrhoea Higher diversity in the *Firmicutes* phylum in patients with diarrhoea
Nam et al. [157]	9 female patients with gynaecological cancer Data of six healthy controls	Pelvic radiotherapy (50.4 Gy/day, 5 times/week, 5 weeks)	Diarrhoea	Significant differences in gut microbiota between cancer patients and controls Patients who developed diarrhoea had a marked change in gut microbiota after radiotherapy
Wang et al. [158]	11 patients with colorectal, anal, cervical cancer 4 healthy controls	Pelvic radiotherapy (1.8–2.0 Gy/day, 5 times/ week, 5 weeks)	Fatigue and diarrhoea	Significant differences in relative abundance of some genera between patients who developed or those who did not Patients who developed diarrhoea had lower alpha diversity and higher *Firmicutes to Bacteroides* ratio
Oral microbiota				
Zhu et al. [93]	41 patients with Nasopharyngeal carcinoma 49 healthy controls	Radiotherapy (2.0 Gy/day, 5 times/ week, 6–7weeks) (alone/with concomitant therapies)	Oral and oropharyngeal mucositis	Healthy controls have more diverse and more similar bacterial composition Increase in relative abundance of Gram-negative bacteria (mostly belong to phylum *Proteobacteria*) as mucositis develop to peak severity Patients with severe mucositis had a significantly higher abundance of *Actinobacillus spp* and lower bacterial diversity

2.7 Conclusion

Gut Microbiota has been found to contribute to cancer chemotherapy and immunotherapy response by modulation of anti-tumour immune responses. However, how it impacts radiotherapy response is yet to be explored. Radiotherapy can induce an anti-tumour immune response, hence, there is a potential that gut microbiota impacts the variation on radiotherapy response through immunomodulation. Moreover, gut microbiota has been shown to contribute to the pathogenesis of radiotherapy-induced GIM by influencing intestinal barriers and modulating inflammatory responses in the intestine. In addition, it potentially impacts the severity of radiotherapy-induced OM by influencing inflammation and ulceration stages of OM, therefore, future research to investigate this effect is warranted.

2.8 Expert commentary

Radiotherapy is an essential cancer treatment modality, but the variation in tumour response and recurrence among patients is still largely unexplained. The identification of potential factors that contribute to this variation and their eventual modulation may help to improve tumour response to radiotherapy and reduce radiotherapy toxicities. Gut microbiota composition is a potential factor that may be a determinant of tumour response to radiation. First, previous work has revealed that radiation can induce immunogenic cell death through stimulation of tumour-specific immune responses that enhance tumour control. Second, studies have demonstrated that gut microbiota is critical for the anti-tumour activity of some chemotherapies and immunotherapies which also induce immunogenic cell death. Finally, animal studies have found that gut microbiota is an important determinant of intestinal radiosensitivity. Therefore, future preclinical and

clinical studies should be conducted to investigate how an individual's gut microbiota may impact tumour response to radiotherapy.

Furthermore, OM is one of the most frequent side effects of radiotherapy and chemotherapy. To date, there are no clear predictive markers for OM risk. Previous research has focused on oral microbiota and its impact on OM. However, since radiation causes substantial damage to the salivary glands and induces xerostomia, the changes in the oral microbiota may be due to hyposalivation and reduced immune functions of saliva leading to changes in the oral local environment. Thus, oral microbiota is not an ideal predictive marker of OM. The gut microbiota may offer a better marker because the variety and abundance of bacteria are greatly increased in the distal intestine. Furthermore, the impact on the systemic immune response is more related to the gut microbiota compared to oral changes. Hence, local inflammation in the mouth may be modulated more profoundly by gut microbiota impacts on the immune system compared to oral microbiota. Future studies are needed to study the impact of gut microbiota on OM risk and severity.

Due to advances in cancer treatment modalities and technology, survival rates across many cancers are markedly increasing. However, many of these survivors are living with chronic or late toxicities which impact their quality of life. Toxicities may develop due to unnecessary treatment or due to individual risk factors. Therefore, personalised treatment planning and identification of markers to predict individual patients likely to respond to treatment or at risk of developing severe toxicities will help to provide personalised treatment options to improve treatment outcomes. Thorough investigations into the role of the gut microbiota and tumour response to radiotherapy and its impact on the development and severity of radiotherapy-induced tissue injury may help to incorporate the gut microbiota into personalised radiotherapy risk prediction algorithms. Unlike

genomic or proteomic biomarkers, gut microbiota can be modified to maximise the response to treatment and minimise adverse effects. This can be done by using personalised probiotics, prebiotics, or fecal microbial transplantation. Although some individual studies have shown promising results of the use of probiotics to prevent or reduce radiotherapy-induced gastrointestinal damage, meta-analyses found these results are not clinically significant. Therefore, future research should focus on the identification of a specific association between radiation-induced toxicities and certain microbial phenotypes to enable the individualised modification of gut microbiota composition. Moreover, research on how to improve the formulation, administration, and absorption of probiotics or prebiotics-based therapies is warranted.

2.9 Five-year view

The inter-individual variations seen in cancer treatment responses and the severity of treatment-related toxicities are major challenges to cancer treatment success. Past research has attempted to identify potential factors that may explain this variation. More recently, we have witnessed a growing interest in studying the relationship between gut microbiota and cancer treatment response and toxicities. Hence, in the coming years, research will focus on targeting the gut microbiota to enhance cancer treatment, including radiotherapy anti-tumour activity. The gut microbiota can be manipulated by various techniques, and with an understanding of what makes an optimal composition, this is an exciting avenue for novel therapies. Moreover, gut microbiota can be used as a prognostic marker to predict the risk of cancer treatment-related toxicities and can be targeted to prevent or reduce cancer treatment-induced toxicities, particularly gastrointestinal toxicities such as mucositis.

2.10 Key issues

- Radiotherapy is a key treatment modality for solid tumours, but the variation in patients' response to radiotherapy and severity of radiotherapy-induced mucositis is still largely unexplained.
- Gut microbiota plays a major role in the development and modulation of intestinal and systemic immune responses.
- Previous studies have found that gut microbiota contributes to the pathogenesis of radiotherapy-induced gastrointestinal mucositis.
- Research has revealed that gut microbiota composition can be used as a predictive marker for the development of radiotherapy-induced diarrhoea and fatigue.
- Individuals' gut microbiota composition potentially influences their response to radiotherapy through the modulation of immune responses.
- Future research to investigate gut microbiota impact on the incidence and severity of radiotherapy-induced oral mucositis is warranted with a view to modulate composition to improve cancer therapy outcomes.

2.11 Funding

This paper was not funded.

2.12 Declaration of interest

The authors report no conflicts of interest.

Chapter three

This chapter describes a pilot study aimed to assess the palatability and efficacy of an antibiotic cocktail to ablate the gut microbiota in Sprague Dawley rats. This chapter is unpublished and unsubmitted work and was written in a manuscript style.

Chapter 3: Evaluation of antibiotic-induced gut microbiota depletion method using a three-antibiotic cocktail

3.1 Introduction

Recent decades have witnessed a growing interest in exploring the role of gut microbiota in human health and disease. Murine animal models provide a powerful tool that allows studying gut microbiota impact on the host's physiology and development of diseases. Currently, there are two main methods that enable the use of animal models to investigate gut microbiota effects on physiological and pathological conditions; 1) germ-free (GF) animals, and 2) antibiotic-induced depletion of gut microbiota [231]. GF animals, mainly mice and rats, are created by either in-vitro fertilisation of an embryo to a germ-free mother or via caesarean birth and maintained in an environment free from all microbes including bacteria, viruses, fungi, and parasites throughout their lifetime [232]. Antibiotic-induced gut microbiota depletion (AIMD) is achieved by treating adult animals with broad-spectrum antibiotics to eliminate gut microbes. Both approaches have strengths and limitations. GF animals are completely free of all types of microbes in all parts of the body, however, they are cost-prohibitive, require specialised facilities and equipment to maintain their germ-free status. They also have immune-related developmental defects limiting their use in certain experiments. The high cost of GF animals in terms of resources needed for specialised facilities, skills, and labour make them inaccessible to many researchers. AIMD instead is an easy and inexpensive method, however, it does not eliminate all gut microbes [231].

One of the critical features of GF animals is the impairment of early immune development and maturation [233]. Given the pivotal role of the immune system in inflammatory conditions such as mucositis, this, therefore, impedes the use of GF animals to study how

gut microbiota influence the development and pathogenesis of such conditions. To understand the precise mechanisms by which the gut microbiota affects these immune and inflammatory-related disorders, mature, well-developed, and competent immune functions are required. Another limitation of the use of GF mice is that these animals need to be kept in a sterile environment to sustain their GF status and maintaining sterility can be a highly challenging issue [234]. For studies that require exposure to the outside environment, like experimental devices or machines, maintaining GF status is therefore not possible. Thus, for studies that involve inflammation and require procedures such as radiotherapy treatment that may lead to microbial contamination, AIMD is a better option compared to GF animals.

To deplete the gut microbiota using antibiotics, previous studies have used antibiotic combinations ranging from a cocktail of two antibiotics to a combination of 4 to 5 antibiotics over 1 to 4 weeks treatment period (previous studies are summarised in Table 1) [231]. Among the most commonly used antibiotics, the majority of these studies have used AVNM antibiotic cocktail that includes ampicillin, vancomycin, neomycin, and metronidazole, administered in drinking water for the depletion of gut microbiota in both rats and mice [235-237] (Table 1). However, some studies have reported that animals restrain from drinking antibiotic-containing water [238] and showed signs of dehydration even after the addition of artificial sweeteners [239]. This is hypothesised to be mainly due to the potent bitter taste of metronidazole [240]. To overcome this problem, researchers administered the antibiotics via oral gavage [240, 241] or a combination of gavage and drinking water [242, 243] (Table 1). This may not be suitable for all disease models. For instance, in oral mucositis (OM) models, administration of antibiotics via oral gavage may affect the oral mucosal integrity and exacerbate mucosal damage, hence, influencing the

research outcome. Therefore, providing antibiotics in drinking water is the preferred approach in models assessing injuries in the oral mucosa.

As such, this small pilot study aimed to assess the uptake and effectiveness of an antibiotic mixture of ampicillin, neomycin, and vancomycin (without metronidazole) to deplete gut microbiota in a rat model. Ampicillin, neomycin, and vancomycin antibiotic cocktail cover both Gram-positive and Gram-negative bacteria. Ampicillin is a beta-lactam broad-spectrum antibiotic effective against aerobic and anaerobic Gram-positive and Gram-negative bacteria. It is mainly used for the treatment of respiratory, gastrointestinal (GI), and urinary tract infections caused by bacteria such as *Escherichia coli*, *enterococci*, and *staphylococci* [244]. Neomycin is an aminoglycoside broad-spectrum antibiotic, mainly active against Gram-negative bacteria. It is used orally or topically to treat several infections such as eye, ear, GI, and skin infections [245]. Vancomycin is a glycopeptide antibiotic active against Gram-positive microbes. It is utilised for treating serious infections such as methicillin-resistant *staphylococci*, *Clostridium difficile* infection, and *enterocolitis* [246]. Vancomycin and neomycin are poorly absorbed in the GI tract and ampicillin has relatively low oral absorption [244-246]. Due to their broad-spectrum activity and poor GI absorption, we hypothesised that an antibiotic cocktail containing these three antibiotics will be efficient in ablating gut microbes when provided via drinking water.

Table 1: Overview of Antibiotic-induced gut microbiota depletion methods used in previous studies:

Study (ref.)	Antibiotics (concentration)	Model	Additives	Administration	Duration	Gut microbiota alterations
Hill et al. [239]	Ampicillin (1 mg/ml), gentamicin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), vancomycin (0.5 mg/ml)	Mouse	Sweetener	Drinking water/ oral gavage	4 weeks/ 10 days	- ≥ 2 log reduction in bacterial 16S rDNA copies - Decrease in the abundance of Bacteroidetes (*Bacteroidaceae*) and Firmicutes (*Lachnospiraceae*). - Increase in the abundance of Firmicutes (*Leuconostocaceae* and *Streptococcaceae*) and Proteobacteria phylum (*Enterobacteriaceae* and *Moraxellaceae*)
Reikvam et al. [240]	Drinking water: Ampicillin (1 g/l) Gavage: vancomycin (5 mg/ml), neomycin (10 mg/ml), metronidazole (10 mg/ml), amphotericin-B (0.1 mg/ml)	Mouse	N/A	Drinking water + oral gavage	3 weeks	- > 400-fold reduction in copy number of 16S rRNA gene
Bercik et al. [247]	Neomycin (5 mg/ml), bacitracin (5 mg/ml), pimaricin (1.25 µg/ml)	Mouse	N/A	Drinking water	7 days	- Increase in the abundance of *Lactobacilli* (*intestinalis, johnsonii, gasseri, plantarum*) and Actinobacteria. - Decrease in the abundance of γ-Proteobacteria (*Shigella, Klebsiella*) and Bacteroidetes (*Bacteroides*)
Carvalho et al. [248]	Ampicillin, neomycin, metronidazole (1 g/l)	Mouse	N/A	Drinking water	8 weeks	- Reduction in bacterial DNA in stool - Depletion of Bacteroidetes and Verrucomicrobia - Reduction in the abundance of Firmicutes - Increase in abundance of Proteobacteria

Hill et al. [249]	Ampicillin (0.5 mg/ml), gentamicin (0.5 mg/ml), metronidazole (0.5 mg/ml), neomycin (0.5 mg/ml), vancomycin (0.25 mg/ml)	Mouse	In cases of poor animal hydration, an artificial sweetener was added	Drinking water	4 weeks	- Reduction in the number of 16S rDNA genes copies in stool - Reduction in Firmicutes and Bacteroidetes phyla
Hu et al. [250]	Ampicillin, neomycin, metronidazole (1 g/l)	Rat	N/A	Drinking water	4 weeks	- Reduction in abundance of Bacteroidetes and Firmicutes - Increase in the abundance of Proteobacteria
Kelly et al. [241]	Ampicillin (1 mg/ml), gentamicin (1 mg/ ml), metronidazole (1 mg/ml), neomycin (1 mg/ml), vancomycin (0.5 mg/ml)	Mouse	N/A	Oral gavage	3 days	- Depletion of the majority of caecal bacteria detected by denaturing gradient gel electrophoresis of 16S gene
Yan et al. [251]	Ampicillin (1 mg/ml), vancomycin (0.5 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml)	Mouse	3% sucrose	Drinking water	4 weeks	- Reduction in the relative 16S rRNA gene copies - Elimination of 99% of faecal bacteria
Zákostelská et al. [252]	Drinking water: vancomycin (0.25 mg/ml) Gavage: metronidazole (0.4 mg), colistin (0.3 mg) and streptomycin (2 mg)	Mouse	N/A	Drinking water + oral gavage	2 weeks	- No change in the number of bacterial 16S gene copies in stool - Reduced microbial diversity - Increase in the abundance of Firmicutes (*Lactobacillales*), Proteobacteria (*Gammaproteobacteria*) - Decrease in the abundance of Firmicutes (*Clostridiales, Erysipelotrichiales*), Actinobacteria (*Coriobacteriales*) and Proteobacteria (*Campylobacterales*)

Emal et al. [253]	Ampicillin (1 g/l), metronidazole (1 g/l), neomycin (1 g/l), vancomycin (0.5 g/l)	Mouse	1% glucose	Drinking water	2 weeks	- Significant decrease in microbial community diversity
Irvin et al. [236]	Vancomycin (0.5 mg/ml), ampicillin (1.0 mg/ml), metronidazole (1.0 mg/ml), neomycin (1.0 mg/ml)	Mouse	Sweetener Equal (3.75 mg/ml)	Drinking water	2 weeks	- Decrease in the bacterial diversity
Shen et al. [254]	Ampicillin (0.5 g/l), neomycin (0.5 g/l), metronidazole (0.5 g/l), vancomycin (0.25 g/l)	Mouse	Sweetener Splenda (3 g/l)	Drinking water (oral gavage when necessary)	3 weeks	- 2 log-folds reduction in faecal bacterial load - Reduction in alpha diversity - Shift in microbial community structure - Increase in the relative abundance of Proteobacteria (*Enterobacteriaceae*)
Zhao et al. [255]	Ampicillin (1 g/l), neomycin (1 g/l), metronidazole (0.5 g/l)	Rat	N/A	Drinking water	10 days	- Reduction in the faecal DNA quality - Reduction in bacterial diversity
Zarrinpar et al. [256]	Ampicillin (100 mg/kg), vancomycin (50 mg/kg), metronidazole (100 mg/kg), neomycin (100 mg/kg), amphotericin B (1 mg/kg)	Mouse	N/A	Oral gavage	13 - 30 days	- 20-fold reduction in stool bacterial DNA - Reduction in the number of sequences from the Firmicutes and Bacteroidetes phylum - Shift microbial structure toward Proteobacteria - Decrease in the number of operational taxonomic units (OTUs) - Decrease in bacterial diversity

Guida et al. [257]	Ampicillin, streptomycin, clindamycin (1 mg/ml)	Mouse	N/A	Drinking water	2 weeks	- Decreased alpha and beta diversity - Increase in the abundance of Proteobacteria (*Enterobacteriaceae*, *Desulfovibrionaceae*) and Actinobacteria - Reduction in the abundance of Bacteroidetes (*Muribaculaceae*) and Firmicutes (*Lachnospiraceae, Ruminococcaceae*)
Wang et al. [258]	Ampicillin (1 g/l), neomycin (1 g/l), metronidazole (1 g/l)	Mouse	N/A	Drinking water	2 weeks	- Shift in microbial composition - Decrease in alpha diversity - Decrease in Actinobacteria, Bacteroidetes, and Firmicutes phylum - Increased in Proteobacteria - Increased *Enterococcus, Escherichia, Klebsiella*, and *Parasutterella*
Ogawa et al. [259]	Ampicillin (1 g/l), vancomycin (0.5 g/l), neomycin (1 g/l), metronidazole***** (1 g/l) *****Metronidazole was excluded from day 10 until the end of the experiment due to reduced water consumption	Mouse	N/A	Drinking water	4 weeks	- Reduction in anaerobic faecal bacteria load - 10^5-fold reduction in the number of intestinal microbes

3.2 Materials and Methods

3.2.1 Animals

This study was approved by the South Australian Health and Medical Research Institute (SAHMRI) animal ethics committee (Project# SAM336). The study protocol complied with the Australian Code for the care and use of animals for scientific purposes (2018). Six male Sprague Dawley (SD) rats (6-weeks old, 179-200 g) were used in this study. The animals were housed in Tecniplast individually-ventilated rat cages (3 rats per cage) in 12 hours dark/light cycle at 18 – 24 °C. They were provided with standard food pellets (Teklad global 18% protein rodent diet) and sterile water *ad libitum*.

3.2.2 Antibiotic treatment

Animals were weight-matched and divided into two groups; control (Ctrl, n=3) and antibiotic-induced microbiota depletion (AIMD, n=3). The baseline water intake was assessed for 10 days before starting antibiotic treatment (Fig. 1). On day 0, the AIMD group was provided with a water-containing antibiotic cocktail of 1 g/L Ampicillin (Ampicillin sodium salt, #A9518, Sigma-Aldrich, USA), 1 g/L Neomycin (neomycin trisulfate salt hydrate, #N5285, Sigma-Aldrich, USA), and 0.5 g/L Vancomycin (Vancomycin hydrochloride, #15327, Sapphire Bioscience, USA) while the Ctrl group was provided with sterile water. These doses of antibiotics were used because they have been frequently used in previous studies and found to be effective in depleting gut microbiota in rodents (Table 1). Water bottles were topped up daily and an equivalent amount of antibiotics were added. The bottles were covered with aluminum foil to protect the antibiotics from light. Since there was a significant decrease in water intake in the AIMD group after the first day of antibiotic treatment, 3 g/L of the artificial sweetener (Splenda) [254] was added to the water of both

groups starting from day 3 of antibiotic treatment. The antibiotic treatment, with sweetener, was continued for three weeks before animals were culled (Fig. 1).

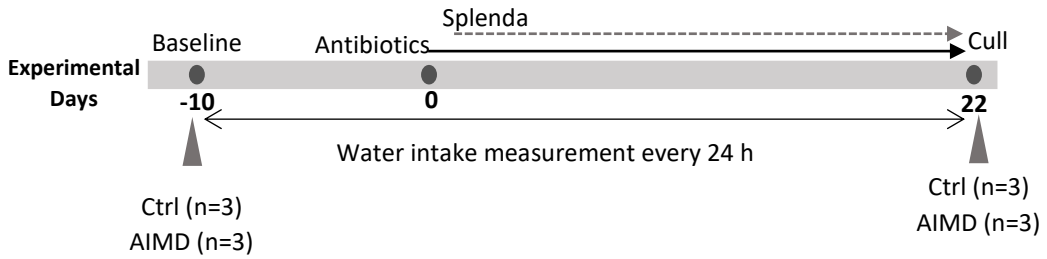

Figure 1: Study experimental design. The baseline water intake was assessed from day -10 until day -1. Antibiotic treatment started on day 0 until day 22.

3.2.3 Assessment of water intake

Animals were provided with 200 mL a day of sterile drinking water, with/without antibiotics, in two bottles (100 ml in each bottle). Water bottles were weighed twice daily at 9 am and 4 pm and 24 h water consumption was calculated. The amount of water released from the bottle within 24 h was assumed to be the amount of water consumed by rats. Rats were group-housed to minimise stress, the water consumption, therefore, was calculated per cage. As no rats in the AIMD group lost weight, which is usually an indication of a cessation of drinking water and dehydration, it was assumed that all rats consumed the antibiotic water, which was later confirmed with individual microbial analysis.

3.2.4 Clinical observations

To assess the impact of antibiotic treatment on the overall health of the animals, rats were monitored for any sign of dehydration (change in skin turgor), weight loss, diarrhoea, and any change in behaviour or activity throughout the study period. Health and clinical record sheets in the Emus database system (used as standard in the Bioresources animal facility, SAMHRI), were completed daily to record any changes in the above parameters.

3.2.5 Sample collection

At the end of the study, animals were euthanised and samples were collected. Rats were euthanised via cardiac exsanguination and death was confirmed by snipping the heart. The stomach, liver, spleen, and kidneys of each rat were dissected, weighed, and inspected for any macroscopic changes. The small and large intestines were also dissected, flushed with 1x Phosphate-buffered saline, and weighed. The internal organs weight relative to the animal body weight was calculated as follows: (Relative weight = (organ weight/body weight) x 100). To collect caecal contents, the pouched end of the caecum was wiped with ethanol, and cut and caecal contents were collected in sterile collection tubes. Samples were stored at -80 °C for further analysis.

3.2.6 Microbial diversity analysis

Caecal content samples were sent on dry ice to the Australian Genome Research Facility (AGRF, Queensland, Australia) for genomic DNA extraction and microbial diversity profiling. Genomic DNA was extracted from 250 mg of caecal content using DNeasy PowerLyzer PowerSoil Kits (Qiagen, Germany). Sequencing of the 16S ribosomal RNA (16S rRNA) gene was performed using primers targeting the 341F-806R (V3-V4) region (forward sequence: CCTAYGGGRBGCASCAG, Reverse sequence: GGACTACNNGGGTATCTAAT). Sequencing was conducted using the Illumina MiSeq platform and Illumina bcl2fastq 2.20.0.422 software was used to generate the sequences. For bioinformatic analysis, paired-ends reads were first assembled using PEAR1 (version 0.9.5) and trimmed. Trimmed sequences then were processed using QIIME 1.8, USEARCH (version 8.0.1623), and UPARSE software. This includes filtration of sequences based on quality, removal of duplicate sequences, and sorting sequences by abundance. Finally, reads were mapped back to operational taxonomic

units (OTUs) with a minimum identity of 97%, and taxonomy was assigned using the Greengenes database (v13.8) as a reference database.

3.2.7 Statistical analysis

Statistical data analyses were performed using GraphPad Prism 8.0.0. Two-way ANOVA and t-test were used to test significance depending on the type of datasets. The alpha diversity (within sample diversity) was assessed by calculating the Shannon diversity index. While Principal Coordinates Analysis (PCoA), based on generalised UniFrac distance, was performed using CLC Genomics Workbench software 21 (Qiagen, Germany) to assess the beta diversity (between sample diversity). Data were presented as mean ± SEM. $P \leq 0.05$ was considered statistically significant.

3.3 Results

3.3.1 24 h water intake

Before antibiotic treatment, the average 24 h water intake of the Ctrl and AIMD groups was similar (112.0 ± 2.3 vs 103.2 ± 2.3 mL/24 h respectively). On day 0, the AIMD group had an antibiotic cocktail administered in the drinking water. Subsequently, on day 1, the 24 h water intake dropped significantly to just 49 mL, so antibiotics were withdrawn for 24 h (day 2) and then resumed on day 3. To mask the taste of antibiotics, 3 g/L of artificial sweetener was added to the drinking water of both Ctrl and AIMD groups, starting from day 3 until the end of the study. Adding sweetener restored the water intake of the AIMD group to the pre-treatment intake (an average of 105 ± 9.4 mL/24 h). However, the water intake of the Ctrl group significantly increased after adding sweetener and the animals in this group continued to drink a higher amount of water (an average of 163.2 ± 13.4 mL/24 h) until the end of the study (Fig. 2).

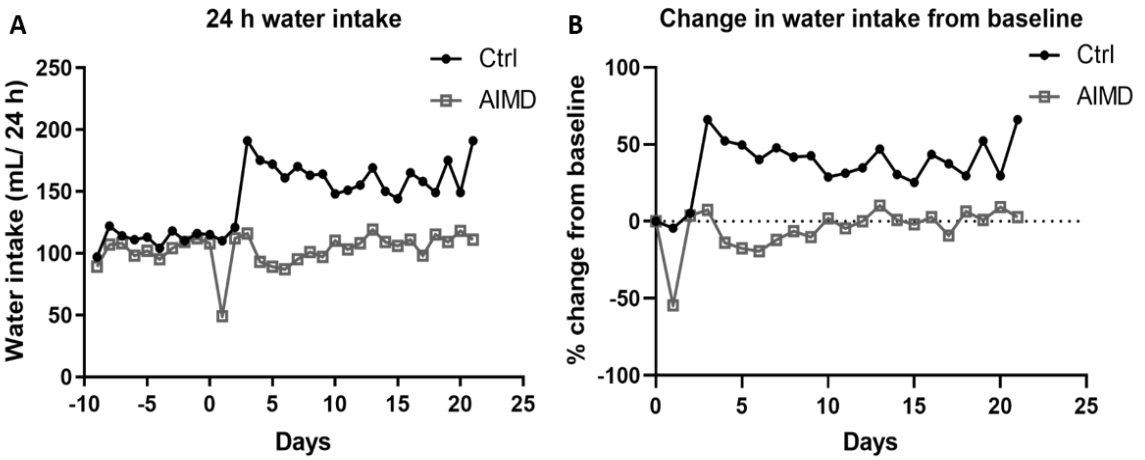

Figure 2: 24 h water intake over the period of study. **A)** Baseline water intake was similar between groups. AIMD group water intake decreased significantly on day 1 of antibiotic treatment. Adding sweetener on day 3, restored AIMD water intake, but significantly increased Ctrl water consumption. **B)** There was 54.6% decrease in the 24 h intake of AIMD group on day 1 post-antibiotic treatment. After adding sweetener, the water intake increased by 66% and 7.4% for Ctrl and AIMD group respectively. Ctrl group water intake continued to be significantly higher than the AIMD group over study period. N= 3 per group.

3.3.2 Body weight

Body weight was monitored throughout the study (Fig. 3). There was no significant difference in the body weight of the AIMD group and Ctrl group (*p = 0.236*). The body weight of animals in both groups gradually increased over the study period (Fig. 3A). Moreover, there was no statistically significant difference in the percent change in body weight from baseline between the two groups (P=0.462) (Fig. 3B).

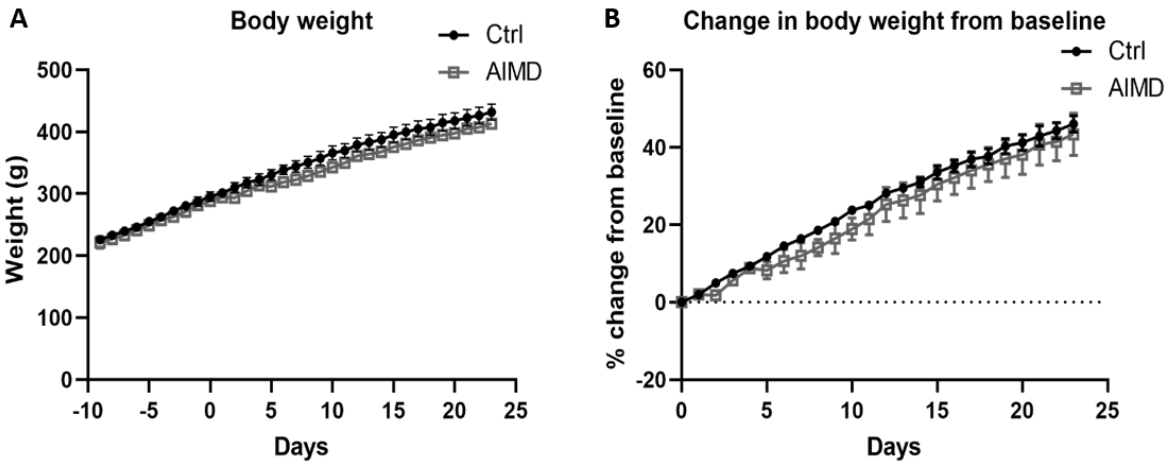

Figure 3: Body weight over the period of study. **A)** There was no significant difference in in body weight between groups throughout study period. **B)** The percent change in body weight from baseline was similar between groups. N= 3 per group; 2-way ANOVA; Data presented as mean ± SEM.

3.3.3 Clinical and necropsy observations

Throughout the study, animals were monitored daily for any change in drinking and feeding behaviours, dehydration, diarrhoea, and activity or alertness. As shown in Table 2, there was a decrease in water intake in the AIMD group when antibiotics were added to the drinking water without a sweetener. Adding sweetener increased the water intake of Ctrl group and restored the water intake of the AIMD group. There was no change in animal feeding behaviour or activity, and no signs of dehydration or diarrhoea were observed. Upon necropsy, AIMD rats were found to have a significantly enlarged caecum with increased and loose caecal contents (Fig. 4). No other features were evident on inspection for other organs.

Table 2: Key clinical observations

Observations	**Ctrl (n=3)**	**AIMD (n=3)**
Decrease in intake of antibiotic-containing water without sweetener	n/a	Yes
Change in water intake after adding sweetener	Yes	Yes
Change in feeding behaviour	No	No
Dehydration	No	No
Diarrhoea	No	No
Change in animal activity	No	No
Enlarged Caecum	No	Yes
Loose Caecal contents	No	Yes

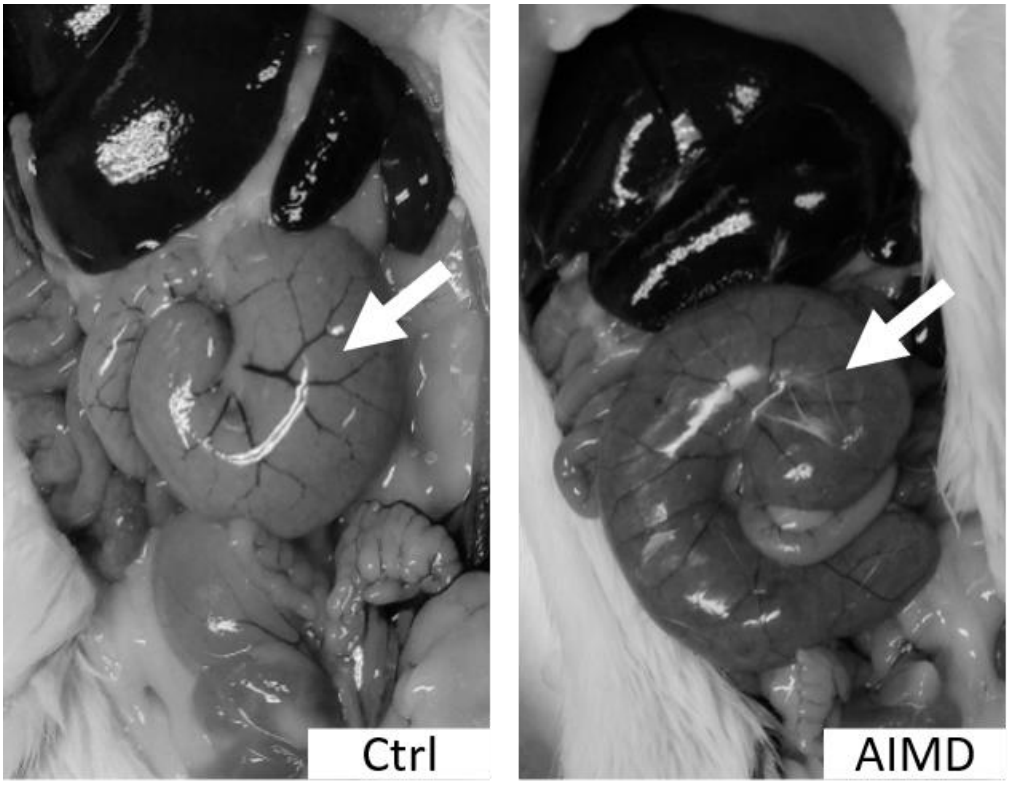

Figure 4: Pictures of the caecum (white arrows) of Ctrl and AIMD groups at the end of the study. After 3 weeks of antibiotic treatment, the AIMD group had enlarged caecum with increased and loose caecal content.

3.3.4 Organ weights

To assess the impact of antibiotic treatment on internal organs weight, the absolute and relative weight of small and large intestines, liver, spleen, kidneys, and stomach were evaluated (Fig. 5). The absolute weight represents the net weight of the organs and relative weight represents the organ's weight relative to the body weight on the cull day. There was no significant difference between Ctrl and AIMD groups in the weight of the small and large intestine, spleen, kidneys, or stomach. However, there was a significant difference in both the absolute and relative liver weight between both groups. The absolute liver weight of the AIMD group was significantly lower than the Ctrl group (13.38 ± 0.57 vs 16.36 ± 0.73 g respectively, p= 0.033). The relative liver weight was also significantly lower in the AIMD group compared to the Ctrl group (3.24 ± 0.09 g vs 3.79 ± 0.10 g respectively; p= 0.017) (Fig. 5).

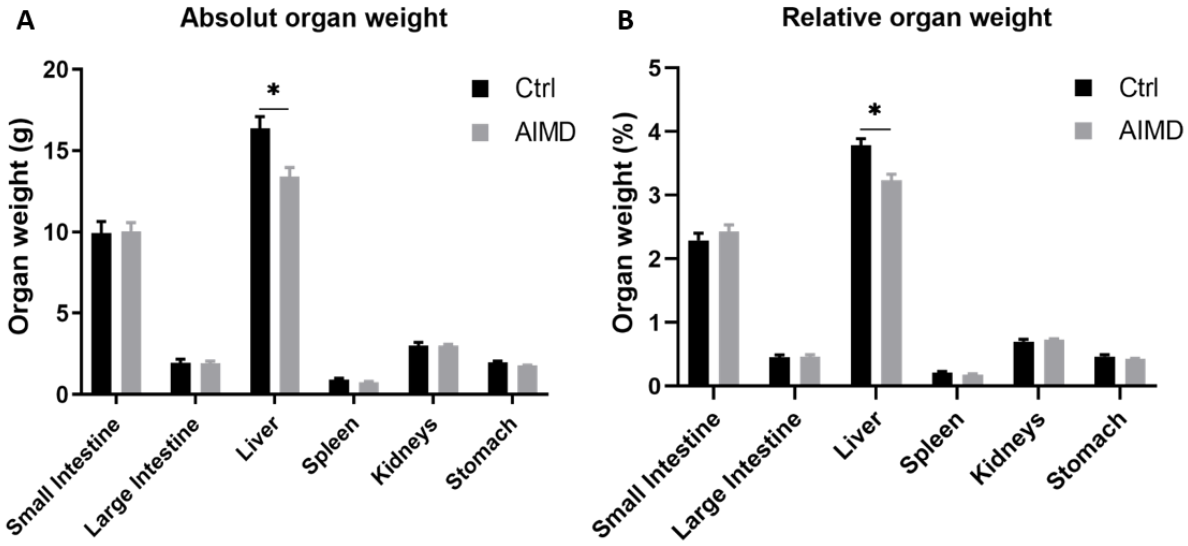

Figure 5: Internal organ weight on cull day. **A)** There were no significant differences in absolute organ weight between groups except for the liver weight, which was significantly lower in the AIMD group. **B)** Liver weight relative to body weight of AIMD group was significantly lower compared to Ctrl group. N= 3 per group; Unpaired t-test; Data presented as mean ± SEM; * P ≤ 0.05.

3.3.5 Microbial diversity profiling

Bacterial 16S rRNA gene-based microbial diversity profiling of caecal contents showed that treating rats with an antibiotic cocktail containing ampicillin, neomycin, and vancomycin caused a significant reduction in the diversity and abundance of caecal microbiota (Fig. 6). There was a significant alteration in the relative abundance of gut microbes at the phylum, class, order, and family genus and species level after antibiotic treatment (Fig. 6A-F). The main bacterial phyla detected in the Ctrl group were Firmicutes (*Lachnospiraceae* and *Lactobacillaceae* family) and Bacteroidetes (*Rikenellaceae* family). However, the AIMD group microbial composition was mainly composed of Actinobacteria phylum (*Yaniellaceae* family) and Firmicutes phylum (*Staphylococcaceae* family). Furthermore, there was a marked increase in the abundance of unclassified bacteria in two samples from the AIMD group (Fig. 6A-F). There was also a significant reduction in the microbial richness represented by the significant reduction in the number of observed OTUs in the AIMD group (Fig. 6G). The alpha diversity, which represents the diversity within each sample and measured by the Shannon diversity index, was significantly reduced following antibiotic treatment. The average Shannon index of the Ctrl rats was at species (2.71 ± 0.04), genus (2.63 ± 0.05), and family level (2.217 ± 0.01) compared to those of the AIMD group 1.09 ± 0.12, 1.04 ± 0.12, and 0.96 ± 0.13 respectively (Fig. 6H). Furthermore, the PCoA demonstrated that the antibiotic treatment shifted the microbial composition of the AIMD group and created a distinct microbial community compared to the Ctrl group (Fig. 6I).

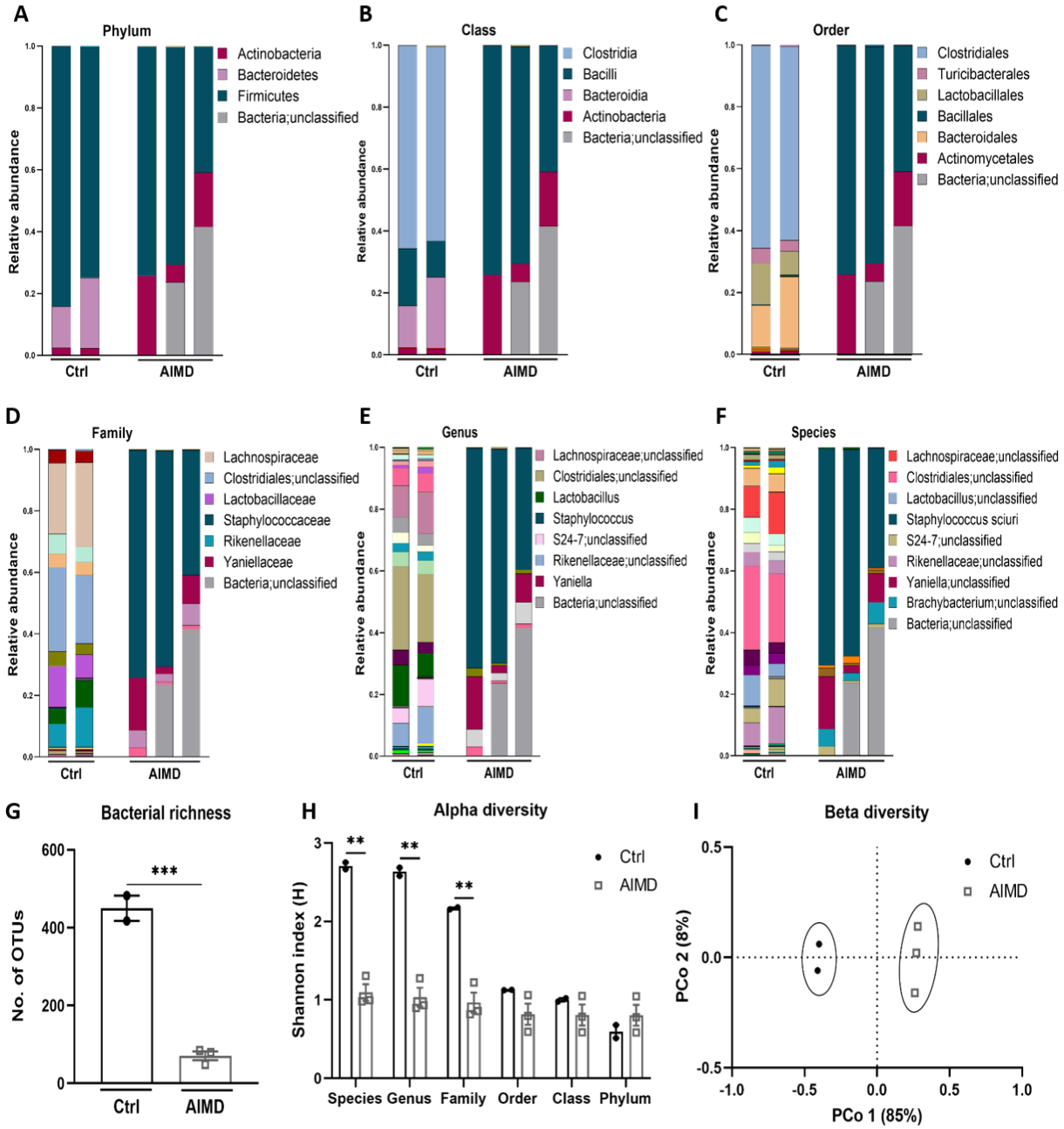

Figure 6: Microbial diversity analysis of caecal content. Relative abundance at phylum **(A),** class **(B)**, order **(C),** family **(D)**, genus **(E)**, and species level **(F)**. **(G)** The number of positive OTUs for Ctrl and AIMD groups (t- test). **(H)** Alpha diversity between groups measured by Shannon diversity index (t-test). **(I)** Beta diversity between groups assessed by PCoA. N=2-3 per group; Data presented as mean ± SEM; **P ≤ 0.01; ***P ≤ 0.001. *Please note, one Ctrl sample was excluded due to failing quality control thresholds.*

3.4 Discussion

The gut microbiota plays a critical role in the development and modulation of several host physiological processes and is implicated in the pathogenesis of inflammatory disorders. Although different AIMD methods have been previously used to study the role of the gut microbiota in different disease models, there is still a gap in understanding the best combination and duration of antibiotic treatment to effectively ablate the microbial communities in the gut. Moreover, previous studies have reported conflicting results on the palatability of drinking water-containing antibiotics in rodents. As such, this study evaluated an antibiotic mixture for ablation of gut microbiota in rats. In our study, rats were treated with an antibiotic cocktail of vancomycin, neomycin, and ampicillin for three weeks in drinking water.

Initially, the antibiotic cocktail was added alone in drinking water, but this significantly reduced the 24 h water intake of the antibiotic group on the next day of treatment. Therefore, an artificial sweetener (Splenda) was added to the water of both groups, which improved the palatability of water and restored the water intake among the antibiotic-treated group. Similarly, previous studies have reported that mice restrain from drinking antibiotic-containing drinking water leading to weight loss and dehydration [239, 240]. Therefore, sweeteners such as glucose [253], sucrose [251], or sucralose-based sweetener (e.g. Splenda) [254] are added to antibiotic-containing water to mask the taste of antibiotics. The use of sweeteners could impact the outcomes of microbiome studies as some preclinical studies have reported that the long-term (6 - 17 weeks) use of both caloric (e.g., sucrose) and non-caloric sweeteners (e.g., Splenda) can alter the composition of the gut microbiota [260, 261]. However, in the present study, a low concentration of Splenda (maximum of 3 mg/ml) was used over a short period (3 weeks), therefore, it will likely have

a negligible impact on the gut microbiota or the animal physiology. To account for any potential impacts, we provided rats in the Ctrl group with Splenda at the same concentration as AIMD rats. To confirm our assumption, future studies could test against rats that have no access to sweeteners. Non-caloric sweetener was chosen over caloric sweeteners (glucose and sucrose) because caloric sweeteners will increase the caloric intake of rats, hence inducing more profound physiological changes. Throughout the study period, no changes in rat body weight and no signs of dehydration were observed during antibiotic treatment, which indicates that all rats in the antibiotic treatment group were able to consume food and water normally and that the antibiotics had minimal impact on animals' development.

Unsurprisingly, animals treated with antibiotics have enlarged caecum with increased and loose caecal content. This has been reported in both GF [262, 263], and antibiotic-treated mice and rats [264-266]. Studies in GF rodents showed that caecal enlargement is caused by the accumulation of mucus glycoproteins that are normally degraded by gut microbiota. These glycoproteins are negatively charged macromolecules that attract water and reduce water transport out of the caecum [263]. The same mechanism could be behind the caecum enlargement after antibiotic treatment seen in our rats. Loeschke et al. reported that treating rats with bacitracin, streptomycin, neomycin, and nystatin for 2 weeks was associated with an increase in caecum surface area, dry weight, and fluid accumulation. They suggested that the reduced bacterial load results in the accumulation of non-degraded carbohydrates and proteins (from mucus or enzymes) in the lumen, creating colloid osmotic pressure hence increased water collection within the caecum [267]. Further research, however, is warranted to confirm the involvement of glycoproteins and osmotic pressure in caecum enlargement after antibiotic treatment.

Another observation in our study was the reduction in the absolute and relative liver weight of antibiotic-treated rats. Reduced liver weight has also been reported in a study by Yan et al., in which mice were treated with AVNM broad-spectrum antibiotics for 6 weeks [251]. Similarly, in hamsters fed with a high-fat diet with or without antibiotics, obese animals treated with antibiotics had significantly lower absolute and relative liver weight. The animals also had lower levels of liver enzymes, plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [268]. Furthermore, Wu et al. showed that treating mice with an antibiotic cocktail for 4 weeks, impaired liver regeneration after partial hepatectomy, however, there was no decrease in the relative liver weight. They also reported that antibiotic treatment did not elevate the levels of serum AST ALT, suggesting that antibiotics are not directly involved in the impairment of liver regeneration or cause direct liver damage [269]. Clinically, oral neomycin and vancomycin have not been linked to hepatotoxicity, while ampicillin has been associated with rare cases of liver injury [244-246]. Together, this evidence supports that reduced liver weight might be due to the ablation of the gut microbiota instead of direct antibiotic-induced liver injury. However, further research is needed to determine the exact mechanism behind the reduction in liver weight after antibiotics treatment.

The gut microbiota composition of healthy SD rats is predominantly composed of Firmicutes and Bacteroidetes phyla [270]. In the present study, antibiotic treatment resulted in a significant reduction in the abundance, richness, and diversity of the gut microbiota. Overall, the relative abundance of most of the microbial phyla was depleted, mainly Firmicutes (*Lachnospiraceae* family) and Bacteroidetes (*Rikenellaceae* family). Similar to our findings, it has been reported that antibiotic treatment significantly reduced the relative abundance of Bacteroidetes and Firmicutes phyla when animals were treated

with broad-spectrum antibiotics in drinking water or by oral gavage between 2 to 4 weeks [239, 250, 256-258]. Interestingly, these studies also have reported enrichment of certain microbes in antibiotic-treated animals. However, the bacterial families that increased after antibiotic treatment reported in these studies were different from those observed here. In this study, there was an increase in relative abundance in *Yaniellaceae* and *Staphylococcaceae* families. These families are not commonly found in the rodents' gut microbiota, however, *Yaniellaceae* have been detected in fecal samples of rats [271]. The increase in the relative abundance of the *Staphylococcaceae* family (*S. Scuiri*) in this study may be due to contamination during sample collection or processing. *S. Scuiri* is commonly found on the skin of humans and animals [272]. Conversely, most of the previous studies that used antibiotics to ablate the gut microbiota have reported an increase in the Proteobacteria phylum (mainly *Enterobacteriaceae* family) [236, 239, 248, 250, 254, 256, 257]. In this study, the abundance of Proteobacteria was higher among the antibiotic-treated animals. However, due to the presence of skin-related bacteria, the increase in Proteobacteria was not prominent. The main source of the Proteobacteria may be the rodent chow [236, 239]. To investigate why Proteobacteria phylum is enriched in antibiotic-treated animals, Hill et al. [239] extracted and analysed DNA from sterile mouse chow and found that the microbial composition of the chow is the same as stool microbial composition of antibiotic-treated and GF mice. This indicates that antibiotic treatment significantly ablates gut microbes, so only microbes from chow or cross-contamination during sample collection and processing are detected via 16S rRNA gene sequencing. Overall, the antibiotic cocktail used in the present study was able to induce significant depletion in the relative abundance of major bacterial taxa comparable to that caused by antibiotic combinations in previous studies.

Another indicator of gut microbial perturbation after antibiotic treatment is the decrease in microbial richness and diversity. In the current study, the bacterial phylotype richness represented by the number of positive OTUs was significantly reduced after antibiotic treatment. This is consistent with previous studies that have reported a reduction in the number of detected OTUs after treating mice with broad-spectrum antibiotics [256, 273]. Alpha diversity measured by the Shannon diversity index is used to determine the microbial diversity within each sample. Our results showed that, first, the Shannon index values of control rats were similar to Shannon index values of gut microbiota of normal male SD rats reported previously [274], indicating that our control animals had normal alpha diversity. Second, antibiotic-treated animals had significantly less alpha diversity represented by reduced Shannon index values. This aligns with findings of other research that has shown that broad-spectrum antibiotics significantly diminish microbial alpha diversity [254, 257, 258]. Moreover, the administration of antibiotics shifted microbial structure and formed a tightly clustered distinct pattern of microbial composition. This indicates that antibiotic treatment led to the establishment of an altered microbial signature as reported in previous studies [239, 270, 273]. Altogether, antibiotic treatment was associated with a significant reduction in microbial richness and diversity indicating successful microbiota ablation.

3.5 Conclusion

This pilot study showed that using an antibiotic cocktail containing ampicillin, neomycin, and vancomycin with artificial sweetener in drinking water was palatable to rats, as indicated by the water consumption and stable body weight throughout the experimental period. The findings also confirm that this AIMD method caused significant disruption of the abundance and diversity of the caecal microbial composition, comparable to those results obtained when a combination of 4 or 5 antibiotics was used, without causing any disruption to the welfare of rats. Moreover, the exclusion of metronidazole did not affect the efficacy of the gut microbiota ablation as the result obtained here were similar to the results of those studies that included metronidazole in the AIMD regimen. Furthermore, the present study used a cocktail of three antibiotics only, which were administered in drinking water instead of oral gavage. Therefore, the present study provides a new, simple, and cost-effective AIMD protocol that can be used for microbiota ablation in rats.

Chapter four

This chapter describes two pilot studies aimed to establish a moderate, self-limiting oral mucositis model in Sprague Dawley rats using two different doses of x-ray radiation. This chapter includes unpublished and unsubmitted work and was written in a manuscript style.

Chapter 4: Establishment of moderate radiation-induced oral mucositis model in Sprague Dawley rats

4.1 Introduction

Radiotherapy-induced oral mucositis (OM) refers to inflammation or ulcerative lesions of the mucosa of the oral cavity following radiotherapy [54]. It is one of the most common adverse effects among patients with head and neck cancer (HNC) treated with radiotherapy. Overall, it affects more than 90% of all patients with HNC, with more than half developing severe OM [55, 57]. Patients with severe OM suffer from severe pain, difficulty chewing or swallowing, and often require feeding tube placement and hospitalisation. Moreover, OM is dose-limiting toxicity that can result in treatment interruption, delay, or dose modification and hence negatively impacting treatment success [72]. The pathogenesis of radiation-induced OM is a complex, multi-phase process involving DNA damage, production of reactive oxygen species (ROS), and the activation and upregulation of inflammatory pathways, such as the nuclear factor kappa-B (NF-κB) signalling cascade, leading to ulcerative lesions formation [86]. OM normally resolves within two to four weeks of treatment cessation. This involves the restoration of tissue structure through the activation of epithelial cell proliferation and differentiation [86]. Studying the pathophysiology of OM is critical to better understand the pathways involved in OM pathogenesis and to find new targets for OM treatment or prevention.

Animal models have been extensively used to study OM. Models of OM have been successfully established using single-dose and fractionated-dose radiation in mice and hamsters [275] (Table 1). OM has also been developed in rats with the first rat model established by Cassatt et al. in 2002. In their study, female SD rats were exposed to 15.3 Gy of gamma radiation to the head and neck region and monitored for 10 days to observe the

development of OM [276]. The Golden Syrian hamster has been considered a gold-standard model for OM due to its large cheek pouch and the similarities between hamster and human oral mucosa [277]. However, all studies that used hamsters developed a radiation-induced OM model through the direct irradiation of the hamster's everted cheek pouch. Cheek pouch eversion was also done when examining the development of OM through the experiment period [278-281]. This method, therefore, is more invasive and could exacerbate oral mucosa injury during irradiation or OM examination. Mice also have been extensively used as an OM model due to the easy handling of these types of rodents. However, the small size of mice makes it difficult to establish a normative radiation-induced OM mouse model [282]. Compared to other rodents, rats have a bigger body size, hence a bigger tongue, which facilitates both the establishment of normative OM model and easier examination of OM development in the tongue [282].

Due to these advantages of rat models, several studies have developed radiation-induced OM models in rats in different settings. However, there is heterogeneity in radiation type (X-ray, ɣ-ray), radiation devices (X-ray machine/device, linear accelerator), radiation settings (voltage, current, and dose rate), radiation doses (single-dose (10 - 30 Gy) vs fractionated dose (37.5 Gy; 7.5 Gy/5 fractions)), irradiated area (direct irradiation of tongue, snout only, oral cavity, head only, neck only or both head and neck area), use of radiation shielding (no shielding, lead plates or lead blocks) and irradiation outcomes (OM peak severity point, recovery period and survival rate), reported in these studies [276, 282-287] (Table 1). Discrepancies in experimental settings and OM outcomes may impact the reproducibility of OM experiments. Therefore, here, two dose-finding pilot studies were performed to establish a moderate OM model in rats using single-dose X-ray radiation.

Table 1: Example of different preclinical models for OM using single or fractionated radiation doses

Study (ref.)	Model	Rx dose	Radiation type/delivery	Irradiated area	Shielding	Findings (OM, weight, oral intake, or survival rate)
Single-dose radiation						
Watkins et al. [288]	Male golden Syrian hamsters	40 Gy	X-ray radiation (X-ray source, 160 kVp, 18.75 mA, 3.32 Gy/ min)	Everted buccal cheek pouch	Lead shield	- OM developed on day 6, peaked on day 18, and resolved by day 28
Watanabe et al. [289]	Golden Syrian hamsters (unspecified Sex)	20, 25, 30, 40, 50 Gy	X-ray radiation (MBR-1520R-3 X-ray device, 150 kV, 20 mA, 5.1 Gy /min)	Everted buccal cheek pouch	Lead shield	- OM developed on day 8 and peaked on day 14 - No change in body weight and food intake - OM was developed at doses > 20 Gy - Optimal dose: 40 Gy
Moura et al. [290]	Male golden Syrian hamsters	10, 20, 30, and 35 Gy + mechanical abrasion on day 3 using a 22-gauge needle	γ-ray radiation (cobalt-60 machine, 0.36 Gy/ min)	Everted buccal cheek pouch	Lead shield	- 10, 20, 30, or 35 Gy without abrasion developed mild OM - 35 Gy with abrasion caused weight loss, 50% survival by day 13, and OM peaking on day 13 - A model was developed using 35 Gy with abrasion
Kamide et al. [291]	Golden Syrian hamsters (unspecified Sex)	40 Gy	X-ray radiation (X-ray apparatus, 1.0 Gy/min, 150 kV, 5 mA)	Everted buccal cheek pouch	Lead plate	- OM developed between days 15 and 20, peaked on day 16, and resolved within 4 weeks after irradiation - Weight gain suppression until day 15
Soref et al. [292]	Female golden Syrian hamsters	21 – 39 Gy	γ-ray radiation (^{137}Cs irradiator, 1.5 Gy/min)	Everted buccal cheek pouch	2.5 cm thick lead plate	- Doses of 21 – 39 Gy caused a linear increase in OM severity - 30 Gy was used to establish OM model - OM peaked on day 16 after 30 Gy radiation

Soref et al. [292]	Female ICR mice	13 – 20 Gy	γ-ray radiation (^{137}Cs irradiator, 2 Gy /min)	Head	1.5 cm thick lead plate	- Doses ≥ 17 Gy significantly reduced body weight - 20 Gy caused 20% weight loss and all animals were euthanised by day 8 - 19 Gy was used to develop OM model
Zheng et al. [293]	Female C3H mice	22.5 Gy	X-ray radiation (Therapax DXT300 X-ray irradiator, 300 kVp, 1.9 Gy/min)	Head and neck	N/A	- Oral ulcers developed on the base of dorsal tongue in ≥ 85% of mice - Severe ulceration on the dorsal tongue on day 7
Mangoni et al. [294]	Female C57B6 mice	16.5 Gy	X-ray radiation (Philips RT250 X-ray machine, 225 kV, 0.69 Gy /min)	Snout	1 cm thick lead collimator	- OM peaked on day 10 and resolved by day 22 - Thickening of the epidermis of labial mucosa and inflammatory cells infiltration in subcutaneous tissues
Sumita et al. [295]	Male C3H/HeJ mice	14, 16, 18, 20 Gy	X-ray radiation (X-ray ISOVOLT Titan 320)	Tongue	5 mm thick lead shield	- 14 Gy: not sufficient ulcers - 18 Gy & 20 Gy: too severe ulcers - 16 Gy was the optimal dose to establish OM model: OM between day 7 and 14, peaked on day 7, and weight loss between day 9 and 14
Nakajima et al. [296]	ICR mice (unspecified Sex)	20 Gy	X-ray radiation (X-ray device (MBR-1520R-3), 150 kV, 20 mA, 5.1 Gy /min)	Tongue	0.5 mm thick lead device	- Maximal decrease in body weight by day 12 - Decrease in food and water intake - OM developed on day 8 and peaked on day 12 - 70% survival by day 18
Maria et al. [297, 298]	Male BALB/c mice	10, 15, 18, 20, 25 Gy	X-ray radiation (Orthovoltage X-ray irradiator, 120 kVp,	Head	N/A	By day 9: ulceration was detectable in all doses By day 14:

			1.158 Gy /100 monitor units using)			- 15 Gy: OM healed - 25 Gy: all animals died due to extensive inflammation and dehydration - 18 & 20 Gy: in the healing process - 18 Gy was the optimal dose to establish a self-resolving OM with a 100% survival - > 18 Gy: mice could not survive after day 10
Nolan et al. [299]	Female mice (CD-1, BALB/c, B6D2F1)	Oral cavity: 9 Gy – 52 Gy Tongue: 18, 27, 36 Gy	X-ray radiation (clinical linear accelerator, 6 MV) X-ray beams (600 monitor units/min)	Oral cavity/ tongue	N/A	Oral cavity irradiation: - ≤ 12 Gy: no mucositis - ≥ 12 Gy: euthanised within 11 days due to weight loss and morbidity Tongue irradiation: - OM peak on day 10 and resolved within 14 days - Weight loss between day 9 and 12 - 33 Gy was used to develop grade 3 OM
Tao et al. [300]	C57/BL6 mice (unspecified Sex)	15, 18, 20, 25, 30 Gy	X-ray radiation (X-RAD 160)	Head and neck	Lead shield	- 25 Gy was the optimal dose to develop OM model - Ulcers developed in the posterior surface of the tongue on day 7
Yang et al. [301]	Male SPF C57BL/6J mice	16.5 Gy	X-ray radiation (RS2000 biological irradiator, 160 kVp, 25 mA, 1.325 Gy/min)	Head and neck	6 mm thick lead shield	- 45% loss of initial body weight - Significant reduction in food and water intake - All irradiated mice died within 15 days - OM peaked between day 9 and 11 - Epithelium thinning, tongue papillae flattening, ulceration, and inflammatory cells infiltration between day 9 and 11

Cassatt et al. [276]	Female Sprague Dawley rats	15.3 Gy	γ-ray radiation (^{137}Cs irradiator)	Head and neck	3 mm thick lead shield	- OM rat model was developed - OM peaked on day 10
Li et al. [287, 302]	Male Sprague Dawley rats	30 Gy	X-ray radiation (deep x-ray machine, 210 kV, 12 mA, 1.0075 Gy /min)	Tongue	2 mm thick lead device	- OM peaked on day 14 and resolved by day 35 - No animal died
Nakashima et al. [283]	Male Sprague Dawley rats	15 Gy	X-ray radiation (Small animal X-ray irradiation system, 160 Kev, 6.3 mA, 2.18 Gy /min)	Snout	Two layers of 0.5 mm thick lead plates	- Reduction in body weight - OM peaked on day 7 and resolved by day 28
Shin et al. [303]	Female Sprague Dawley rats	30 Gy	X-ray radiation (Linear accelerator, 6 MV, 2 Gy /min)	Oral cavity	N/A	- Reduced oral intake - Reduction in body weight - All animals died by day 10
Chang et al. [284]	Male Sprague Dawley rats	20 Gy	X-ray radiation (linear accelerator, 2 Gy /min)	Oral cavity	N/A	- OM peaked between day 13 and day 14 - Reduction in body weight - 86.7% (13/15) of rats died between days 7 and day 17 - A survival rate of 13.3% (2/15) on day 21
Kim et al. [286]	Male Sprague Dawley rats	18 Gy	X-ray radiation (photon 6-MV linear accelerator, 2 Gy/min)	Neck	N/A	- Damage and atrophy of the epithelium - Decrease in squamous epithelium thickness - Complete loss of epithelium on day 7
Li et al. [304]	Male Sprague Dawley rats	20 Gy	X-ray radiation (6 MV, 2.5 Gy/min)	Head	5 mm lead shield	- Weight loss between day 5 and 15 - Signs of OM appeared on day 6, peaked on day 14, and resolved by day 25
Jonsson et al. [305]	Female Sprague Dawley rats	16, 18, 20 Gy	X-ray radiation (conventional high-	Head	N/A	- All doses caused weight loss after day 5 - 4 rats (20 Gy), 1 rat (18 Gy) had >20% weight loss and were culled on day 9

			energy linear accelerator, 2300 C/D), 6 MV photons, 3 Gy/min)			- 20 Gy was used to establish OM model: OM developed between day 7 and 14, peaked on day 10, resolved by day 17
Miyamoto et al. [285]	Female F344 rats	10, 18, 30 Gy	X-ray radiation (210-kVp x-ray Machine, 10 mA)	Tongue	2 mm thick lead shielding	- 10 Gy: suppression of weight gain from day 5 - 18 Gy and 30 Gy: persistent weight loss for 11 days (30 Gy) or 9 days (18 Gy) - On day 7, epithelial ulcer after 18 Gy and 30 Gy, but no lesions detected after 10 Gy - On day 10, slight redness (10 Gy), erythematous lesions (18 Gy), extensive epithelial damage (30 Gy) were observed
Fractionated doses radiation						
Watkins et al. [288]	Male golden Syrian Hamsters	8 x 7.5 Gy (7.5/ day over 2 weeks (days 0 –3 and 7–10)	X-ray radiation (Philips X-ray source, 160 kVp, 18.75 mA, 3.32 Gy/min)	Everted buccal cheek pouch	Lead shield	- OM developed on day 6, peaked on day 18, and resolved by day 35
Zheng et al. [293], Cotrim et al. [306]	Female C3H mice	3 x 6 Gy/week, 3 x 7 Gy/week or 3 x 8 Gy/week	X-ray radiation (Therapax DXT300 X-ray irradiator, 300 kVp, 1.9 Gy/min)	Head and neck	N/A	- Lingual ulcers formed in 100% of the tongues with the 8 Gy dose by day 9 - 5× 8 Gy was used to establish OM: severe ulceration on the dorsal tongues within 8–10 days
Gruber et al. [307-310] Kowaliuk et al. [311, 312]	C3H/Neu mice (unspecified Sex)	5 x 3 Gy (3 Gy/day) over 2 weeks (days 0 – 4, 7–11)	X-ray radiation (YXLON MG325 X-ray device, 200 kV, 20 mA, 1 Gy/min)	Snout	6 mm thick lead equivalent MCP-96	- Irradiation was well tolerated by the animals - No reduction in weight loss or food intake - Mucosal ulceration in the tongue appeared after 8 days and lasted for 4 days

Gruber et al. [313], Frings et al. [314]	C3H/Neu mice (unspecified Sex)	5 x 3 Gy (3 Gy/ day) over 1 week (days 0–4) or 2 weeks (days 0–4, 7–11) + top-up doses on day 7 and day 14	X-ray radiation (200 kV, 20 mA, 1 Gy /min)	Snout	12 mm thick collimator (lead equivalent MCP-96)	- No change in body weight or food consumption - Mucosal ulcer appeared within 8 days and lasted for 3 days
Han et al. [315]	Male and female C57BL/6 mice	3 x 8 Gy (8 Gy/day for 3 days)	X-ray radiation (RS2000 irradiator, Rad Source, 1.126 Gy/min)	Head	Lead shield	- 3x 8 Gy was the minimal dose to induce OM - OM developed by day 9 post-radiation
Yang et al. [301]	Male C57BL/6J mice	3 x 8 Gy (8 Gy/day over 3 days)	X-ray radiation (RS2000 biological X-ray irradiator, 160 KVp, 25 mA, 1.325 Gy/min)	Head and neck	6 mm thick lead shield	- Lingual ulcers formed in 100% of irradiated mice by day 7 post-radiation
Ortiz et al. [316]	Male Wistar rats	5 x 7.5 (7.5 Gy/day for 5 consecutive days)	X-ray radiation (YXLON Y. Tu 320-D03 irradiator, 207.3 kV, 10.5 mA, 1.002 Gy/min)	Snout	Lead shield	- By day 14: loss of filiform papillae, ulcerations in the mucosal lining, disruption of the epithelium layer, and inflammatory cell infiltration were observed

Gy, Gray; KVp, Kilovoltage peak; kV, kilovolt; mA, milliampere; x, # of fractions (e.g., 5x is 5 fractions)

4.2 Materials and methods

4.2.1 Animals and Experimental design

This study was approved by the South Australia Health and Medical Research Institute (SAHMRI) animal ethics committee (Project# SAM336) and was performed according to the Australian Code for the care and use of animals for scientific purposes (2018). In total, 24 male SD rats were used in this study. The animals were housed in Tecniplast individually ventilated rat cages (3 rats per cage) in 12 h dark/light cycle at 18 – 24 °C, with standard rodent chow and sterile water provided *ad libitum*. As two different doses were assessed in sequential order in two experiments, they have been designated study 1 and study 2.

Study 1 (15 Gy): 12 rats (6-8 weeks old) were used. The animals were weight-matched and divided into two groups of six. During the acclimatising week, animals were weighed daily, and food and water intake were measured. In addition, the baseline rat grimace scale (RGS) and von Frey tests (VFT) were assessed on day 0. On the irradiation day (day1), one group of animals was assigned randomly as a sham group (sham, n= 6) and the other as a radiation (15 Gy, n= 6) group. Animals in the sham group were exposed to sham irradiation (0 Gy) and the radiation group was exposed to 15 Gy of X-ray irradiation. After irradiation, animals were monitored for changes in body weight and food and water intake. To assess pain behaviours post-radiation, RGS was assessed and four VFT were performed on day 3, 6, 9, and 13 post-radiation. Half of the animals were euthanised on day 7 (expected peak severity according to Nakashima et al. [283]), and the remaining animals were monitored until day 14 (expected healing time-point) when they were euthanised (Fig. 1).

Study 2 (20 Gy): Since a radiation dose of 15 Gy was not sufficient to establish a moderate OM model, another study was conducted using 20 Gy. In this experiment, another 12 rats

(6 weeks old) were used. Similar to study 1, body weight and baseline food and water intake, RGS, and VFT were assessed during the acclimatising period. On irradiation day (day 1), one group of animals was randomly assigned as the sham group and another as the 20 Gy radiation group. The radiation (Rx) group was exposed to 20 Gy of X-ray irradiation while the sham group was exposed to 0 Gy radiation. After irradiation, animals were monitored for changes in weight, and food and water intake. Pain behaviours were assessed post-radiation by RGS and VFT on day 3, 6, 8, 11, 13, and 15 post-radiation. Based on the findings of study 1, peak OM severity was observed on day 9 post-radiation, hence, in this experiment, half of the animals were euthanised on day 9. The remaining animals were monitored until day 16 when the bodyweight of all irradiated rats returned to baseline weight (weight before radiation), then they were euthanised (Fig. 1)

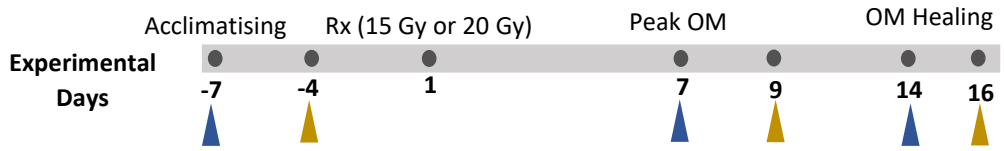

Figure 1: experimental design of study 1 and 2. In study 1 (**Blue arrows**), animals were acclimatised for 8 days and then the Rx group were exposed to 15 Gy of X-ray radiation. Half of the animals were culled on day 7 and the other half were culled day 14 post-radiation. In study 2 (**Brown arrows**), animals were acclimatised for 5 days and then the Rx group was exposed to 20 Gy of X-ray radiation. Half of the animals were culled on day 9 and half of them were culled day 16 post-radiation.

4.2.2 Irradiation

4.2.2.1 Radiation shielding

A cylinder was made using a 2-mm thick lead with six evenly placed holes (Fig. 2). The diameter of each hole was 2.5 cm, which fits the rat's snout. The lead cylinder was tested for 10 Gy, 15 Gy, and 20 Gy, and radiation outside the cylinder was measured. Residual radiation, 0.0118 Gy/min, was detected outside the lead cylinder. However, radiation at this level will not cause any biological impact on the rat body [317].

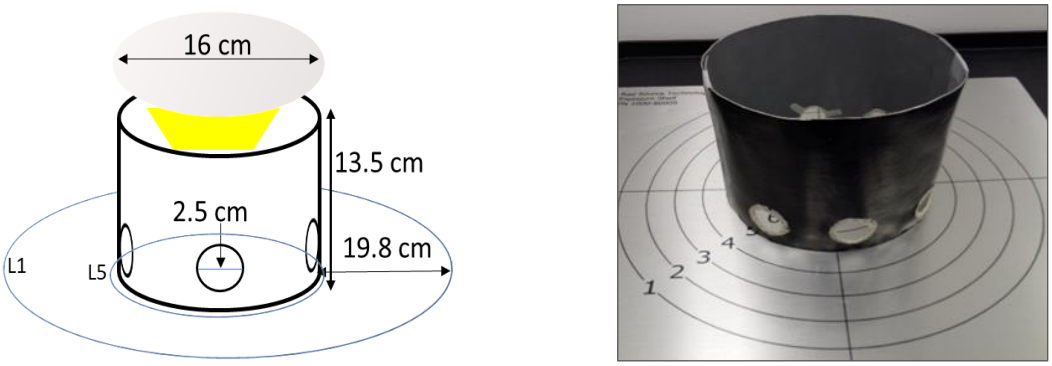

Figure 2: A lead cylinder for radiation shielding. A 2-mm thick lead plate was used to create a lead cylinder. 2.5 cm holes were made on the sides of the cylinder to fit the rats' snout.

4.2.2.2 Animal irradiation

Before irradiation, animals were anaesthetised with ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) via intraperitoneal injection. While deeply anaesthetised, animals were adjusted by placing their snouts through the holes of the lead cylinder. The snout was inserted to the level of the periorbital region with eyes were protected from radiation. Then, animals were placed on level 5 (14 cm distance from radiation source) of the irradiator (Small animal Irradiator, RS-2000, Radsource, USA). Small cardboard boxes were used to help maintain rats on their left side during irradiation (Fig. 3). Rats in the sham groups (one sham group for each experiment) were exposed to 0 Gy irradiation while irradiated groups were exposed to either 15 or 20 Gy X-ray radiation as follows:

- **Study 1 (15 Gy)**: a dose rate of 4.18 Gy/min (tube voltage of 160 kV, tube current of 25 mA for 3 min and 59 sec).
- **Study 2 (20 Gy)**: a dose rate of 2.12 Gy/min (tube voltage of 160 kV, tube current of 13 mA for 9 min and 26 sec). To achieve a dose rate comparable to that used in clinical settings, the dose rate was adjusted to 2.12 Gy/min (instead of 4.18 Gy/ min in study 1) by reducing the tube current from 25 mA to 13 mA.

After irradiation, rats were removed from the irradiator and placed in their home cages. An anaesthetic reversal agent, Atipamezole (1 mg/kg), was administered subcutaneously to reverse the sedative effect of anaesthetic drugs. The rats were monitored until they fully recovered from anaesthesia and were able to move normally. The cages were returned to the holding room and animals were provided with soaked chow pellets on the floor of the cage to facilitate access to food.

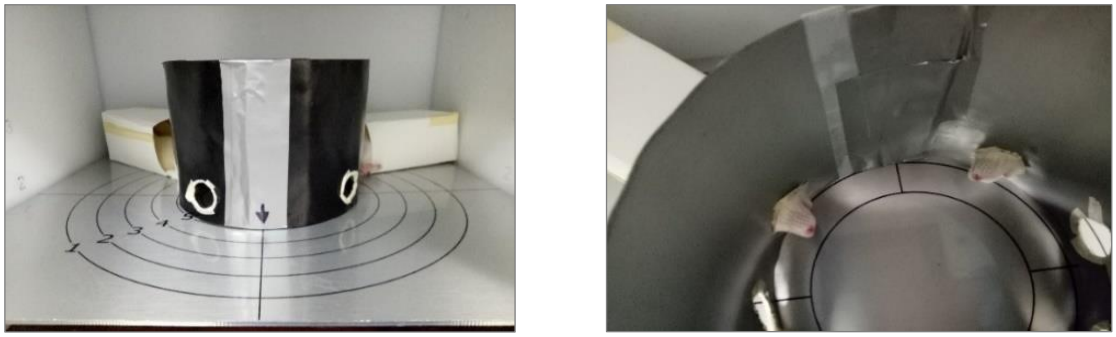

Figure 3: Animal irradiation. Rat snouts were placed into the holes on the sides of the lead cylinder. Animals were placed on their right, so the left side of the snout is irradiated. Cardboard boxes were used to maintain rats on the right position during irradiation.

4.2.3 Food and water intake assessment

Rats were provided with sterile water and standard rat chow pellets *ab libitum*. The food hopper in each cage was weighed daily and the 24 h food intake for each cage was calculated. After radiation, some food pellets were soaked in water to make soft chow and placed on the floor of the cage to facilitate food access to animals. The weight of the soaked food was also added to the total feed weight when calculating the 24 h intake. The 24 h water intake was measured by weighing the water bottles daily.

4.2.4 OM assessment

Following irradiation, rat tongues were examined daily. Each rat's mouth was open by gently pulling the cheeks. Signs of OM on the tongue were examined and scored using the Parkins OM scoring system [318]. This scoring system assesses changes in the oral mucosa

such as tongue erythema, focal desquamation, exudation, and mucosal ulceration (Table 2).

Table 2: OM scoring system [318]:

Grade	Description
0	Normal
0.5	Slight pink
1	Slight red
2	Severe reddening
4	Exudation covering less than one half of the irradiated mucosa
5	Virtually complete ulceration of the mucosa

4.2.5 Rat grimace scale

To determine the RGS scores, photos were taken daily in the morning throughout the experiment. Photos were taken once before radiation to ascertain baseline, then daily after radiation. The rats were individually caged and photos were taken for each rat. The photos were examined in a blinded fashion and scored according to the RGS that includes the assessment of orbital tightening, nose elongation, cheek flattening, ear curling, and whisker bunching (Fig. 4). Changes in facial features were scored as not present (0), moderately present (1), or obviously present (2). The average RGS score was calculated for each photo as described previously [319].

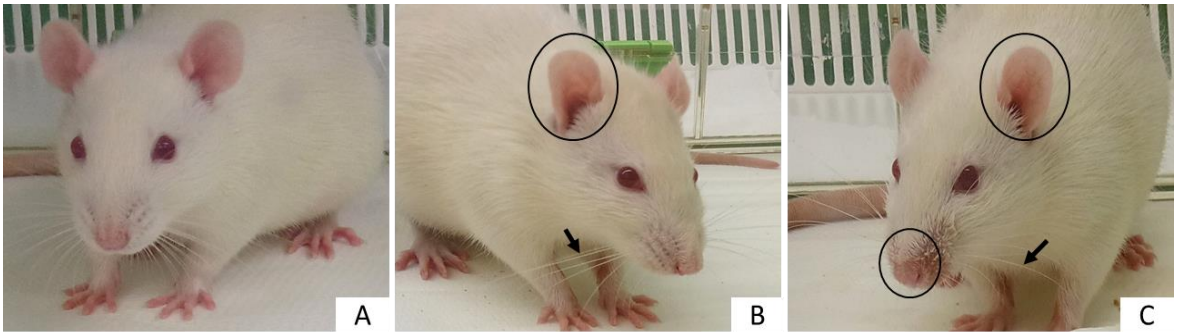

Figure 4: Example of observed changes in RGS score. **A)** Normal rat. **(B, C)** Images of irradiated rats showing ear curling, nose elongation and cheek flattening (circles) and whiskers tightening and bunching (arrows).

4.2.6 Von Frey Test

A von Frey platform was prepared by placing four clear mice cages upside down on the top of a mesh floor (hole size 0.65x 0.65 cm). Calibrated von Frey monofilaments (Stoelting Touch Test, USA) with serial forces of 2, 4, 6, 8, 10, and 15 g were used. Before conducting the test, animals were habituated to the test environment and the platform for 30 minutes for two consecutive days before the experiment. On the third day, the test was conducted using the ascending stimuli method [320]. Briefly, rats were placed individually in clear mice cages of the von Frey platform and allowed to habituate for 10 minutes. Von Frey filaments were applied to the plantar surface of the rat's hind paw (left or right hind paw) in ascending order starting with the lowest filament (2 g). Each von Frey filament was applied until it bends, five times with 5 second intervals. If no response was elicited after five applications with 5 second intervals, a filament with a higher force was used. When the rats withdrew, shook, or licked the paw, it was considered a positive response. The force of the first filament that elicits the positive response (2 out of 5 applications) was designated as the mechanical withdrawal threshold (MWT) [254].

4.2.7 Sample collection

Half of the rats were euthanised on anticipated peak severity day; day 7 for 15 Gy and day 9 for 20 Gy, and the remaining rats were euthanised on day 14 or day 16 respectively. Rats were euthanised via cardiac exsanguination and death was confirmed by snipping the heart. The small and large intestine, stomach, liver, spleen, and kidneys of each rat were collected and weighed. The tongue was excised from the base and photos were taken. Injured and ulcer-like areas on the dorsal tongue were measured via ImageJ software and calculated as described previously [283] (Fig. 5). The tissues then were cut from the tip (apex), middle (body), and base (root) of the tongue and put into cassettes for histological analysis. Tissues

were fixed for 24 h in 10% neutral buffered formalin and then processed and embedded in paraffin wax.

Total injured area = (injured surface area (*b*) ÷ irradiated tongue surface area (*a*)) × 100

Ulcer-like area = (ulcer-like area (*c*) ÷ irradiated tongue surface area (*a*)) × 100

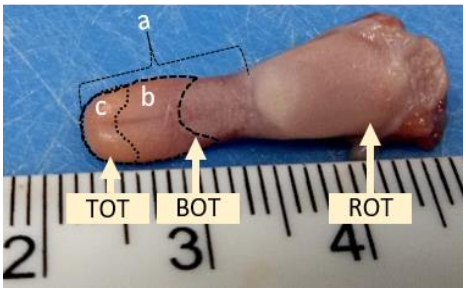

Figure 5: calculation of injured and ulcer-like area on dorsal the tongue (Nakashima et al. [283]). TOT- tip of the tongue, BOT- body of the tongue, ROT- root of the tongue.

4.2.8 Histological analysis

5 µm sections of Paraffin-embedded tissues were cut using Leica rotary microtome and stained with Hematoxylin and Eosin. Slides then were scanned using the Nanozoomer 2.0 digital slide scanner (Hamamatsu Photonics, Japan). Epithelial thickness, histological score, mucosal ulcer size, and infiltration of the immune cells were assessed using NDP.view2 Viewing software (Hamamatsu Photonics, Japan). OM histological changes were scored using a modified scoring system [321] (Table 3). The mucosal ulcer was defined as the complete loss of the epithelial layer on the dorsal tongue. The percentage of ulcer size was calculated as (length of ulcerated epithelium ÷ length of dorsal tongue epithelium) ×100. WBC infiltration was scored as follows; (0 = few WBCs, 1 = widespread infiltrate of single WBCs, 2 = widespread infiltrate and small patches of WBCs discrete from ulcerations, 3 = widespread infiltrate and large patches of WBCs discrete from ulceration).

Table 3: Modified OM histological grading scale from [321]:

Grade	Histopathologic manifestation
0	No radiation injury; normal mucosa
1	Focal or diffuse alteration of basal cell layer with nuclear atypia and ≤2 dyskeratotic squamous cells
2	Epithelial thinning (2–4 cell layer) and/or ≥3 dyskeratotic squamous cells in the epithelium
3	Loss of epithelium without a break in keratinisation or presence of atrophied eosinophilic epithelium with/without Subepithelial vesicle or bullous formation
4	Complete loss of epithelial and keratinised cell layers; ulceration

4.2.9 Statistical analysis and data presentation

Analyses were performed using GraphPad Prism 8.0. T-Test, two-way ANOVA, Kruskal-Wallis test, or mixed-effects model analysis was performed depending on the type of dataset. Data were presented as mean ± SEM. $P \leq 0.05$ was considered statistically significant. Since two separate experiments were conducted in this pilot study, with differences in initial bodyweight, radiation doses, and cull days, the results of both experiments are presented separately.

4.3 Results

4.3.1 Change in body weight

Rats were weighed daily throughout the study period. In study 1, in which the irradiated group was exposed to 15 Gy, a slight decrease in body weight was observed in both irradiated and sham groups on day 2 post-radiation. From day 3 to day 8, animals in both groups continued to gain weight and there was no statistical difference in body weight between groups (Fig. 6A). After day 7, there was no decrease in the body weight from the baseline (weight on day 1) among both groups, however, animals in the 15 Gy group gained less weight compared to the sham group (Fig. 6B). Similar to study 1, there was a slight drop in body weight of both sham and 20 Gy groups on day 2 of irradiation in study 2. Moreover, between day 8 and day 10 post-radiation, the 20 Gy group lost 7% of their weight relative to baseline (Fig. 6C). The weight then recovered from day 10 post-radiation, however, the 20 Gy rats gained less weight between days 13 and 16 compared to the sham group (Fig. 6D).

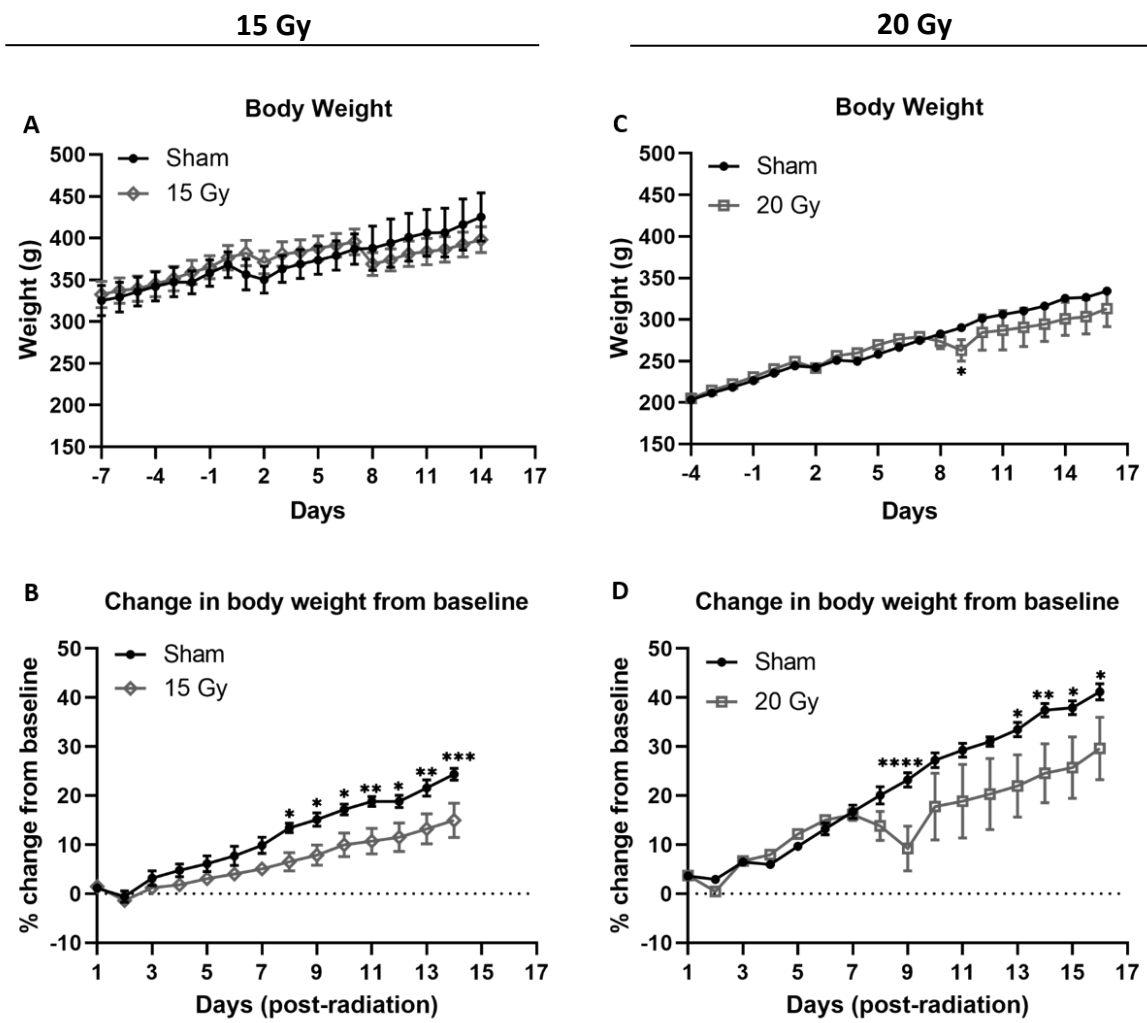

Figure 6: Change in body weight for study 1 and 2. **(A, B)** Rats exposed to 15 Gy radiation did not lose weight but tended to gain less weight compared to sham group. **(C, D)** Exposing rats to 20 Gy radiation caused a 7% reduction in body weight between days 8 and 10, relative to baseline. Following that, weight recovered but rats continued to gain less weight compared to sham group. Mixed-effects model analysis & Bonferroni's multiple comparisons test. N= 6 per group; data presented as mean ± SEM; * P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0001.

4.3.2 Change in food intake

Food intake over 24 h was assessed by measuring the weight of food pellets consumed each day throughout the experiment. Overall, there was no significant difference in the food intake between animals irradiated with 15 Gy and animals in the sham group. On day 9, there was around a 20% reduction in the 24 h food intake of the 15 Gy group from baseline. However, it recovered to baseline food intake on day 10 (Fig. 7A-B). In contrast, exposing rats to 20 Gy irradiation caused a major reduction in 24 h food intake between days 8 and 14 post-radiation. Food intake decreased from baseline by 22%, 38%, 22%, 25% and 15% on day 8, 9, 10, 11 and 12 post-radiation respectively. Moreover, only on day 15 post-radiation did food intake recover to baseline (Fig. 7C-D).

4.3.3 Change in water intake

The 24 h water intake was quantified over the experimental period for both study 1 and 2. Exposing rats to 15 Gy radiation decreased the baseline water intake between day 9 and day 14. The major reduction was observed on day 10 at 28%. However, there was no statistically significant difference in the water intake between the sham and 15 Gy group (Fig. 8A-B). Rats irradiated with 20 Gy had a significant drop in water intake compared to the sham group between day 6 and day 14 post-radiation. There was a statistical difference in the 24 h water intake on days 8, 9, and 13. After day 13, the water intake of the 20 Gy group increased and reached baseline value by day 16 post-radiation (Fig. 8C-D).

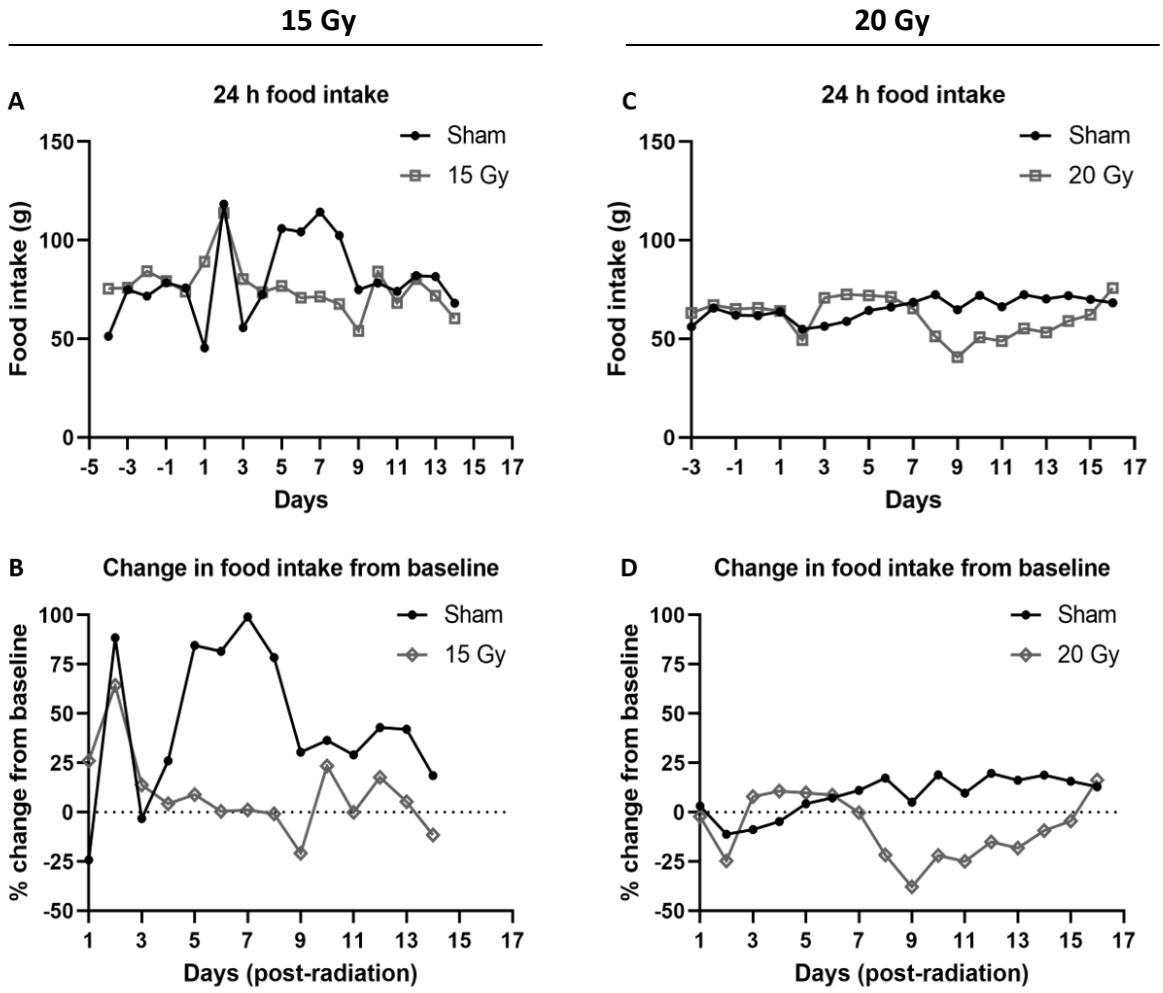

Figure 7: Change in food intake for study 1 and 2. **(A, B)** There was a slight decrease in food intake on day 9 among radiation group which was exposed to 15 Gy radiation. **(C, D)** Exposing rats to 20 Gy radiation caused a substantial reduction in food intake between day 8 and day 14 post-radiation. *N= 6* per group, Mixed-effects analysis & Bonferroni's multiple comparisons test.

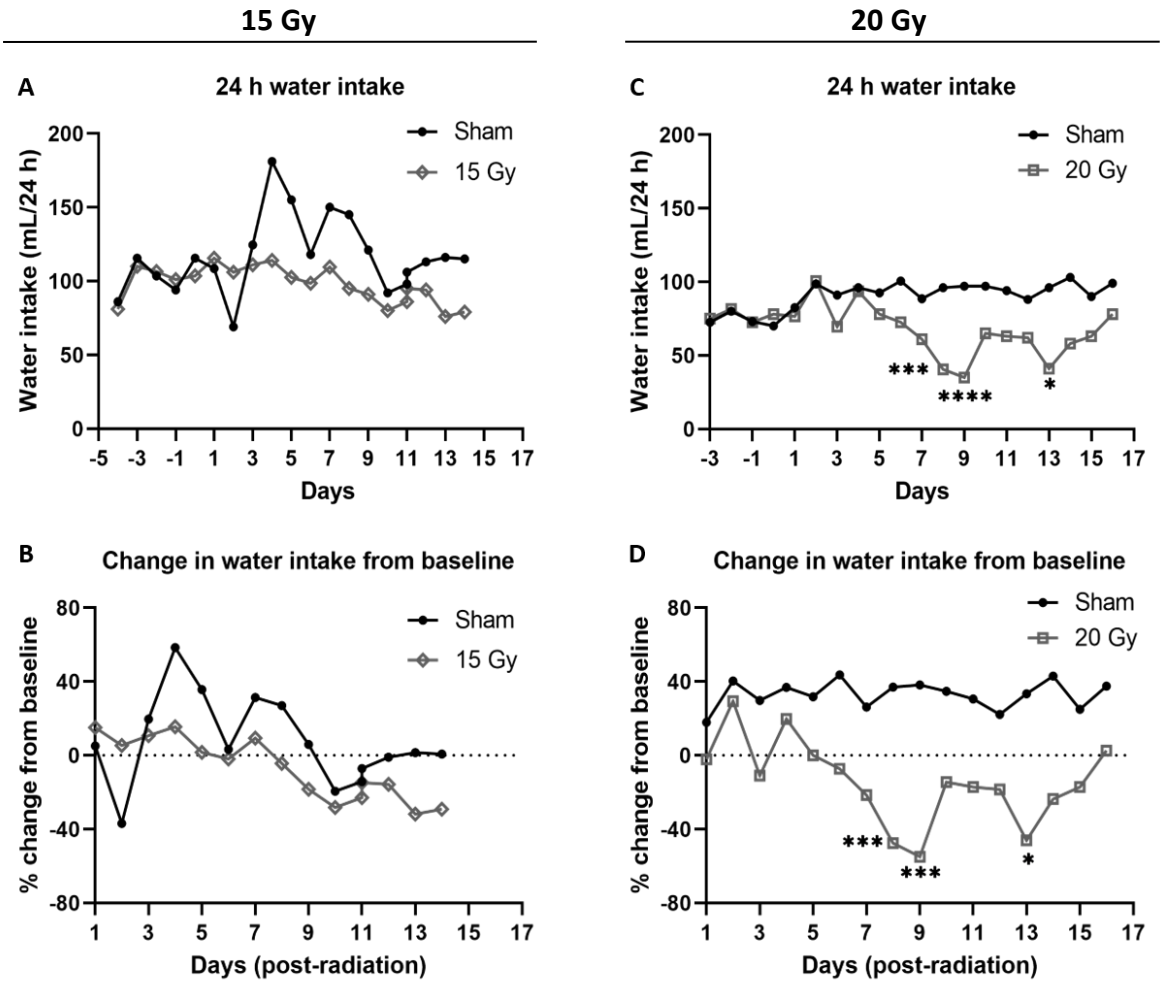

Figure 8: Change in 24 h water intake for study 1 and 2. **(A, B)** Between day 8 and day 14, there was a slight decrease in the water intake of 15 Gy group compared to sham group, however, there was no significant difference between groups. **(C, D)** Rats exposed 20 Gy showed a significant drop in water intake between day 6 and day 14. N= 6 per group; Mixed-effects analysis & Bonferroni's multiple comparisons test; Data presented as mean ± SEM; * P ≤ 0.05; ***P ≤ 0.001, **** P ≤ 0.0001.

4.3.4 Change in organ weights

The internal organs were collected and weighed on day 7 and day 14 for study 1, and day 9 and day 16 for study 2. Compared to the sham group, irradiation with 15 Gy did not cause a significant difference in the absolute organ weight for any organs on days 7 or 14. However, on day 7, the relative weight of the liver (relative to body weight) was significantly smaller among the 15 Gy group compared to the sham group (3.56 ± 0.023 vs 4.17 ± 0.19, p=0.036). In contrast, no significant difference in relative liver weight was observed between groups on day 14 (Fig. 9A-B). For animals exposed to 20 Gy, the absolute liver weight on day 9 was significantly lower compared to the sham group (8.23 ± 1.18 vs 10.54 ± 0.43, p=0.034). However, there was no significant difference in the absolute and relative weight of other organs between groups on days 9 and 16 (Fig. 9C-D).

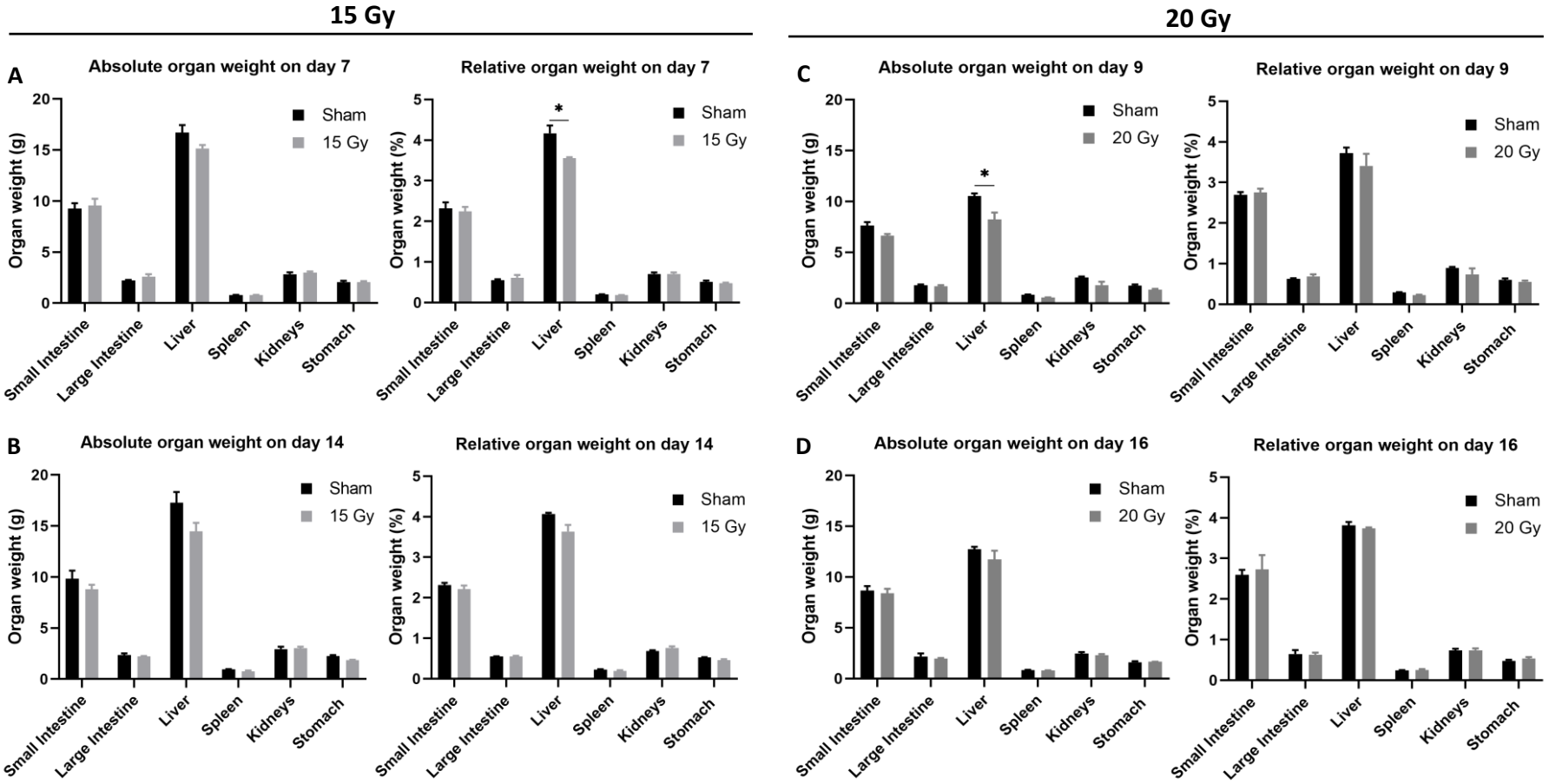

Figure 9: Absolute and relative organ weight for study 1 and 2. **(A)** Absolute and relative organ weight of rats on day 7 in study 1. There was no significant difference between absolute organs weight on day 7, however, the relative liver weight of 15 Gy group was significantly smaller compared to those of sham group. **(B)** Absolute and relative organ weight of rats on day 14 in study 1. There was no significant difference between absolute and relative organs weight on day 14. **(C)** Absolute and relative organs weight of rats on day 9 in in study 2. The absolute liver weight of 20 Gy group was significantly smaller on day 9 compared to those of sham group. There was no significant difference between relative organs weight. **(B)** Absolute and relative organs weight of rats on day 16 in study 2. There was no significant difference between absolute and relative organs weight on day 16. N= 3 per group; T-test; Data presented as mean ± SEM; * $P \leq 0.05$.

4.3.5 Oral mucositis score and tongue injury area

OM was assessed after radiation both in live animals (OM score) and on the tongue tissues that were collected on the anticipated peak severity and healing time points (% injury area). In both study 1 and 2, OM developed mainly on the anterior dorsal tongue. After 15 Gy radiation, signs of OM developed on tip of the tongue from day 6 as slight pink (day 6), swelling with slight redness (day 7-8), and swelling and severe redness (day 9-10). From day 11, OM started to resolve and completely healed by day 14 post-radiation. OM peak severity was observed on day 9 with an average OM score of 1.67 ± 0.33 (Fig. 10A). Fig. 10B shows pictures of the tongue from sham and 15 Gy groups collected on day 7 post-radiation. The tip of the tongue of two irradiated rats showed a severe red area, while the third rat developed a small slight red spot on the side of the tongue.

In rats exposed to 20 Gy radiation, signs of OM were observed from day 5 as a slight pink tip of the tongue (day 5-6), slight redness (day 7), and severe red swollen tongue covered with exudates (pseudomembrane) (day 8-11). From day 12, the OM severity started to decline and completely healed by day 16. OM reached peak severity on day 9 with an average OM score of 3.17 ± 0.98 (Fig. 10C). Fig. 10D shows rat tongues collected on day 9 from sham and 20 Gy groups. Rats developed severe OM, with severe red swollen tongues with exudates covering half of the irradiated tongue area. On tongues exposed to 20 Gy and collected on day 9, the total injured area (localised to the body of the tongue (BOT)) was 50.95 ± 5.39% and the ulcer-like area (localised to the tip of the tongue (TOT)) was 25.24 ± 2.51% of the total irradiated tongue surface area (Fig. 10E)

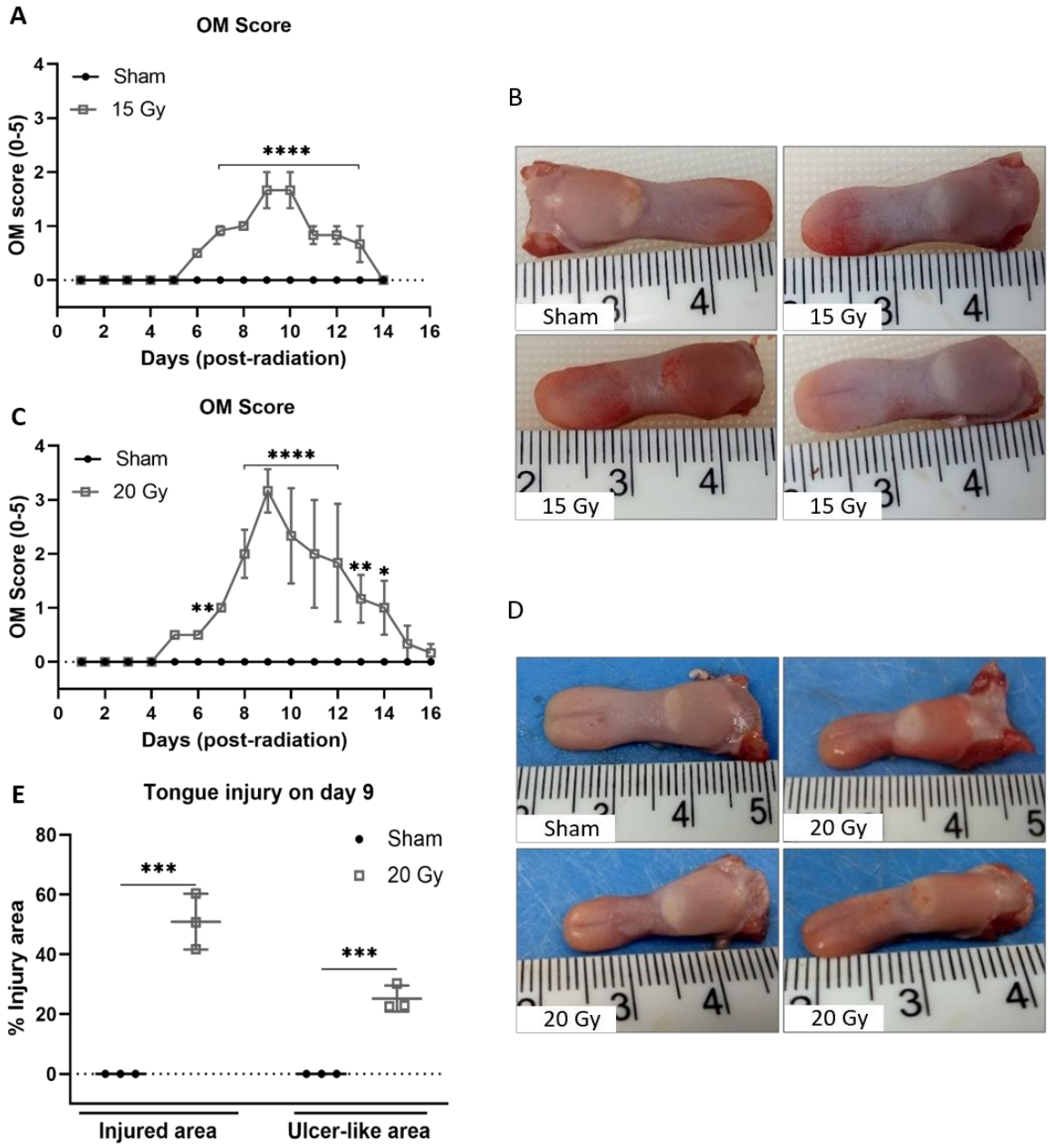

Figure 10: OM score and tongue injury for study 1 and 2. **(A)** OM score after 15 Gy irradiation. Between day 6 to 12, mild OM developed with peak severity on day 9, which completely healed by day 14. **(B)** Pictures of tongue on day 7 of rats exposed to 15 Gy. Severe red area was observed on left side of tip of the tongue of two rats and small red spot was observed on the tongue of the third rat. **(C)** OM score after 20 Gy irradiation. Signs of OM were observed from day 5, reach peak severity on day 9, and completely healed by day 16. **(D)** Pictures of tongue on day 9 of rats exposed to 20 Gy. Half of the irradiated area of the tongue was swollen, red and covered with exudates. **(E)** Tongue total injured area and ulcer-like area measured on day 9 after 20 Gy irradiation. N= 3-6 per group; Mixed-effects analysis & Bonferroni's multiple comparisons test (OM score) & Unpaired t-test (tongue injury); Data presented as mean ± SEM; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

4.3.6 Histological assessment of OM

The histological analysis was performed on the dorsal and ventral surface of the tongue on tissue sections from TOT and the BOT.

4.3.6.1 Epithelial thickness

The epithelial layer was measured to assess any change in the epithelial thickness. Exposing rats to 15 Gy radiation caused a significant thinning of the epithelial layer covering the dorsal surface of the TOT compared to those exposed to sham irradiation. However, no significant difference was found in the epithelial layer thickness on the ventral TOT or dorsal and ventral BOT (Fig. 11A-F). Exposing rats to 20 Gy caused significant thinning of the epithelial layer in the dorsal surface of the TOT but not on the ventral TOT or dorsal and ventral BOT (Fig. 12A-F).

4.3.6.2 OM histological score

Exposing rats to 15 Gy radiation caused mild histological changes on the mucosa of dorsal and ventral TOT on day 7 post-radiation. The average histological OM score of the dorsal TOT was 2 and was characterised by the epithelial thinning and presence of dyskeratotic squamous cells in the epithelium. For the ventral TOT, a diffuse alteration on the basal cell layer, nuclear atypia, and the presence of few dyskeratotic squamous cells were noted, with an overall OM grade of 1. No histological changes in the BOT were observed. On day 14 post-radiation, epithelial hyperplasia was observed on the dorsal TOT of the irradiated rats, however, other OM-related epithelial changes were completely resolved (Fig. 11A-B, G-H).

In rats irradiated with 20 Gy, histological changes were observed on both TOT and BOT on day 9 post-radiation. The OM histological scores were 3.33 ± 0.33 and 2.0 ±0.0 for dorsal and ventral TOT respectively. The dorsal TOT histological changes were characterised by

complete loss of epithelium with or without a break in the keratinised layer while a thinning of the epithelium was observed on the ventral TOT. Moreover, a thinning of the epithelium of dorsal BOT and changes in the basal layer of ventral BOT were observed. The average OM histological grades were 1.67 ± 0.33 and 1.33 ± 0.33 for dorsal and ventral BOT respectively. All histological manifestations on the TOT and BOT were resolved by day 16 post-radiation. However, similar to 15 Gy, epithelial hyperplasia on the dorsal TOT was observed in the tongue of some rats on day 16 (Fig. 12A-B, G-J).

4.3.6.3 Mucosal epithelial ulceration

Mucosal ulceration was defined as the complete loss of the epithelial layer. No ulceration was observed on dorsal and ventral surfaces of the tongue on day 7 after irradiation with 15 Gy (Fig. 11A-B). However, exposing rats to 20 Gy caused complete loss of the epithelial layer on some part of the dorsal surface of the TOT on day 9 post-radiation. The percentage of ulcer length relative to the length of the dorsal surface of TOT in these groups of animals was 33.47% ± 13.90 (Fig. 12A (b), K). The normal epithelium structure was restored by day 16. No loss of epithelial layer was observed on the ventral surface of TOT or the dorsal and ventral surfaces of the BOT (Fig. 12A-B).

4.3.6.4 Inflammatory cells infiltration

Infiltration of inflammatory cells is one of the main characteristics of OM. In this study, mild WBC infiltration was observed on day 7 after exposing rats to 15 Gy. A mixture of immune cell infiltrate was observed in the dorsal TOT only (Fig. 13B, D, E). In contrast, severe WBC infiltration was observed on day 9 following irradiation with 20 Gy. The inflammatory cells were predominantly neutrophils with some macrophages and lymphocytes (Fig. 13C, F-I). Inflammation was resolved by day 14 and day 16 for 15 Gy and 20 Gy groups respectively.

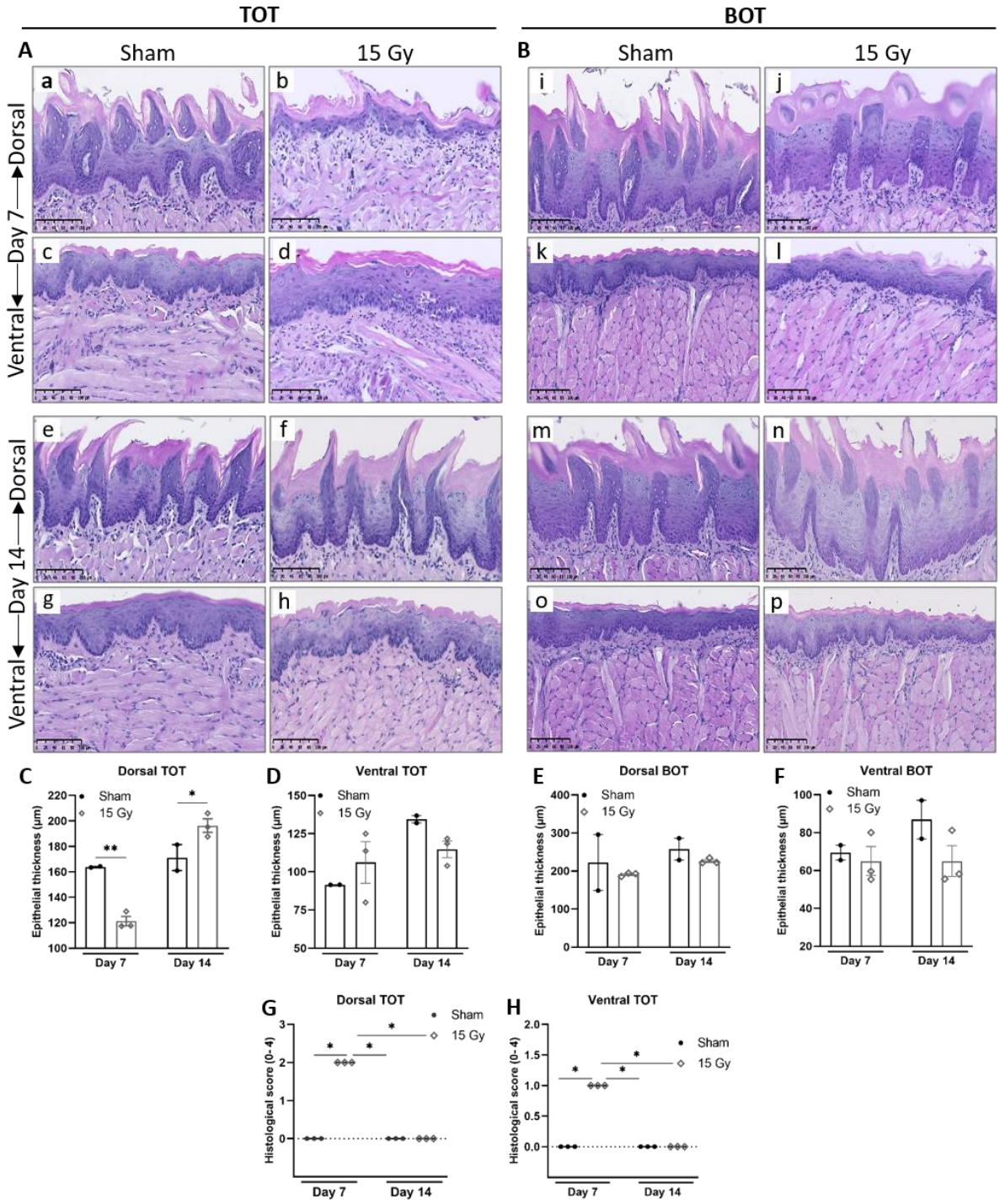

Figure 11: OM histological analysis for study 1. **(A)** The dorsal and ventral surface of TOT on day 7 (a, b, c, d) and day 14 (e, f, g, h). **(B)** The dorsal and ventral surface of BOT on day 7 (i, j, k, l) and 14 (m, n, o, p). Epithelial thickness of dorsal **(C)** and ventral **(D)** TOT and dorsal **(E)** and ventral **(F)** BOT. OM histological score of the dorsal **(G)** and ventral **(H)** TOT. N= 2-3 per group; 2-way ANOVA (epithelium thickness); Kruskal-Wallis test & Dunn's multiple comparisons test (histological score); data presented as mean ± SEM; * P ≤ 0.05; **P ≤ 0.01; Scale bar: 100 µM.

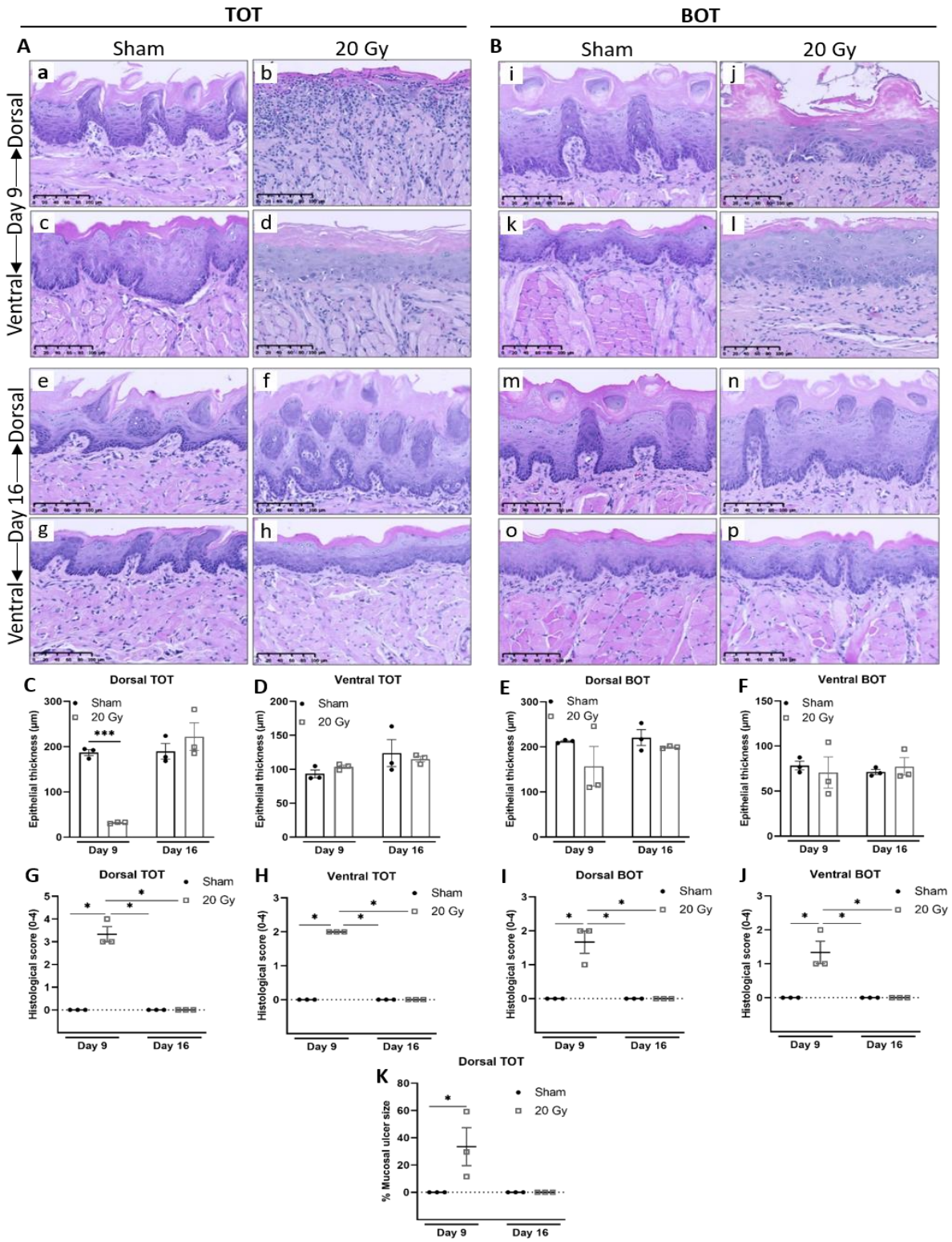

Figure 12: OM histological analysis for study 2. **(A)** The dorsal and ventral surface of TOT on day 9 (a, b, c, d) and day 16 (e, f, g, h). **(B)** The dorsal and ventral surface of BOT on day 9 (i, j, k, l) and 16 (m, n, o, p). Epithelial thickness of dorsal **(C)** and ventral **(D)** TOT, and dorsal **(E)** and ventral **(F)** BOT. OM histological score of the dorsal **(G)** and ventral **(H)** TOT, and dorsal **(I)** and ventral **(J)** BOT. Ulceration of the dorsal TOT **(K)**. N= 3 per group; 2-way ANOVA (epithelium thickness), Kruskal-Wallis test & Dunn's multiple comparisons test (histological score) & t-test (ulcer size); data presented as mean ± SEM; * P ≤ 0.05; ***P ≤ 0.001. Scale bar: 100 µM.

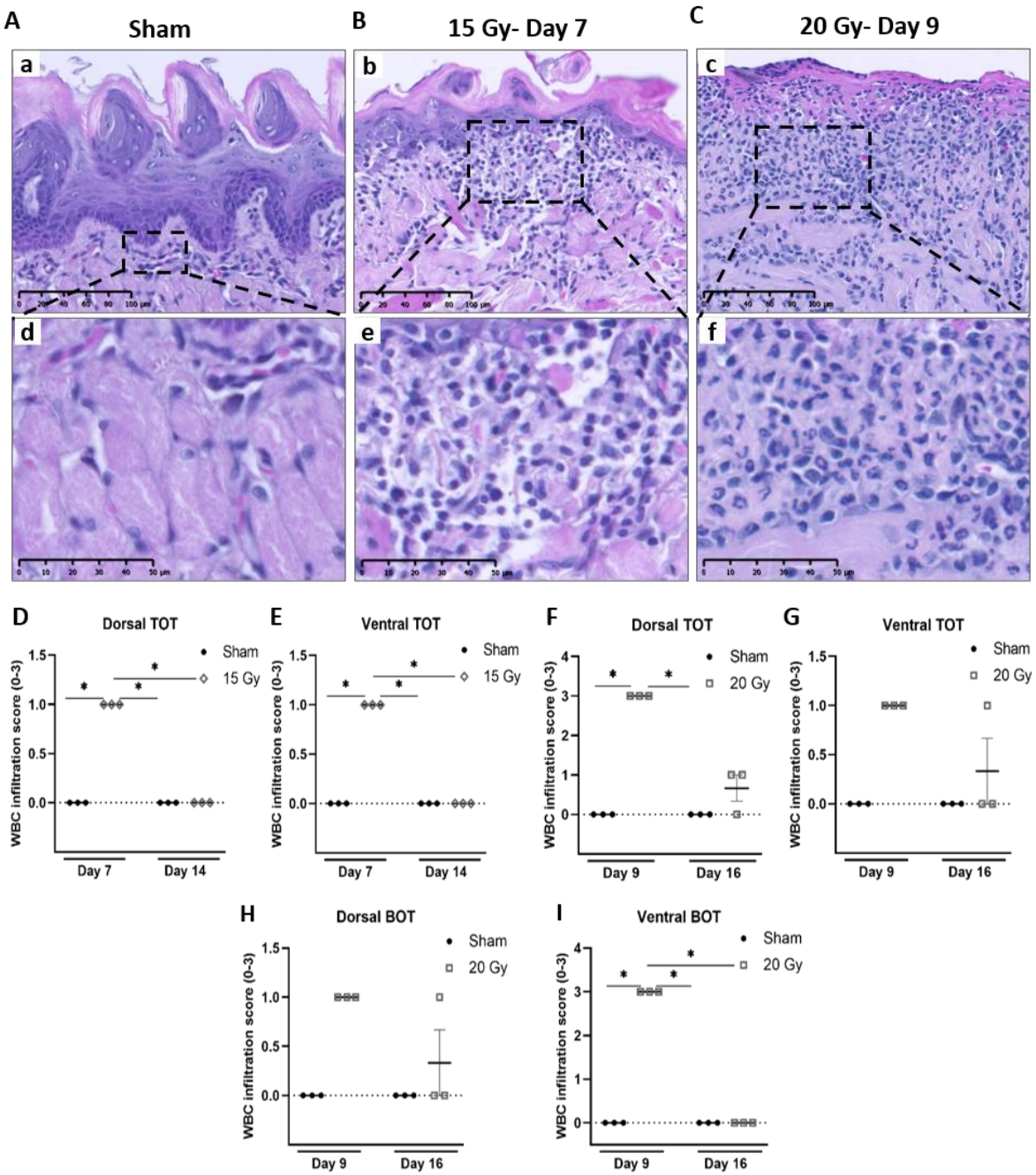

Figure 13: Inflammatory cells infiltration in tongue tissues. H&E staining of the dorsal TOT showing WBCs infiltration for sham **(A)**, 15 Gy group (on day 7 post-radiation) **(B)**, and 20 Gy group (on day 9 post-radiation) **(C)**. The WBC infiltration score for both dorsal and ventral TOT exposed to 15 Gy **(D, E)**. WBC infiltration score on dorsal and ventral TOT **(F, G)** and BOT **(H, I)** for rats exposed to 20 Gy. N= 3 per group; Kruskal-Wallis test & Dunn's multiple comparisons test; data presented as mean ± SEM. * $P \leq 0.05$. Scale bar: 100 µM (a-c) or 50 µM (d-f)

4.3.7 Assessment of pain behaviours

OM is associated with severe pain; thus, pain behaviours were assessed using RGS and VFT in this model of radiation-induced OM. Rats exposed to 15 Gy showed an increase in RGS scores from day 4. Between days 7 and 9, the average score was 0.75 ± 0 out of 2, which correlated with OM peak severity. The VFT showed that the MWT decreased after irradiation, indicating mechanical hyperalgesia, and there was a significant difference between groups on day 9 (Fig. 14A-B). Exposing rats to 20 Gy radiation was associated with increased RGS scores starting from day 5 post-radiation, with a peak RGS score on day 9 at 1.17 ± 0.23 out of 2. Moreover, the MWT decreased after 20 Gy irradiation and there was a significant difference between groups on days 8, 11, and 15 (Fig. 14C-D).

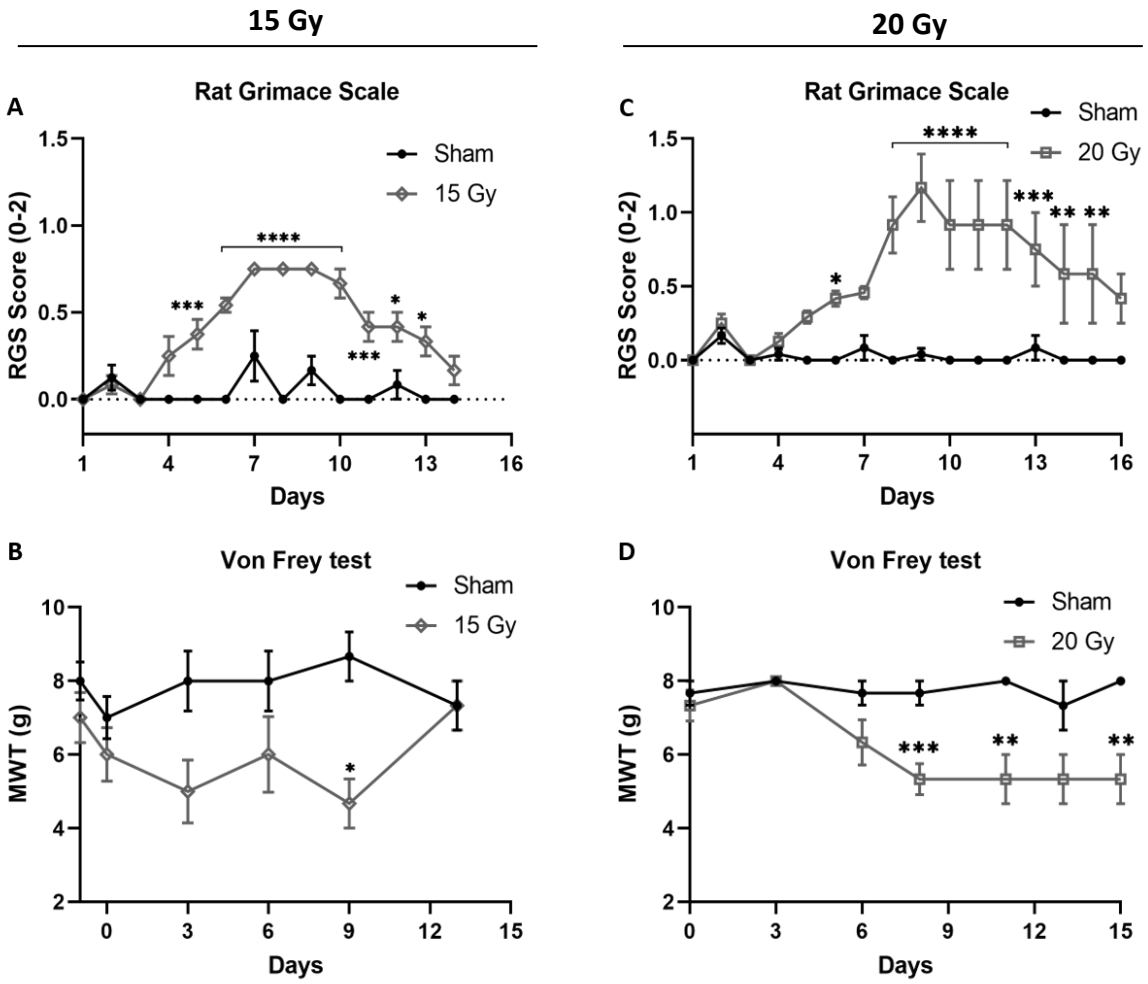

Figure 14: Assessment of pain-related behaviours via RGS and VFT. **(A)** Exposing rats to 15 Gy increased the RGS score to an average of 0.75 between day 7 and day post-radiation 9. (**B)** The mechanical withdrawal threshold (MWT) of 15 Gy group decreased significantly on day 9 post-radiation. **(C)** Rats' irradiation with 20 Gy increased RGS score to an average of 1.17 on day 9 post-radiation. **(D)** There was a significant decrease in MWT of rats in 20 Gy group on day 8, 11 and 15 post-radiation. N= 6 per group; Mixed-effects analysis & Bonferroni's multiple comparisons test; data presented as mean ± SEM. * P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; **** P ≤ 0.0001.

4.4 Discussion

Murine animal models, including rats, are a critical research tool that has been used to study pathophysiology, potential treatments, and prevention methods for pathological conditions including radiation-induced OM. However, different irradiation methodologies have been reported in different previous studies. Here, two pilot studies were performed to establish a radiation-induced OM model in SD rats using 15 or 20 Gy single-dose X-ray radiation. Single-dose radiation was used in order to reduce animal stress associated with exposure to fractionated radiation doses. Furthermore, a lead cylinder was used as a radiation shield to protect the body of rats, except for snouts, from radiation. Hence, reducing the off-target radiation-induced toxicities.

Our first study was based on previously published work by Nakashima et al. [283], in which male SD rats were exposed to 15 Gy X-ray radiation to the snout. In our study, the irradiation of rat snouts with 15 Gy led to the development of mild OM starting from day 6 with peak severity on day 9 (average OM score of 1.67 out of 5). OM was completely healed by day 14 post-radiation. The tongue injury caused by this radiation dose did not result in a significant reduction in body weight or food and water intake. Although it was associated with modest changes in pain-related behaviours including mechanical hyperalgesia (increased sensitivity to mechanically-induced pain) and RGS score. The histological analysis revealed that 15 Gy caused thinning of the epithelial layer of the dorsal TOT with mild infiltration of inflammatory cells on day 7. These observations might not be representative of the peak tissue damage caused by 15 Gy radiation as tissues were collected on day 7 while the peak OM score was observed on day 9 post-radiation. By day 14 post-radiation, all radiation-induced histological changes were resolved. Some of these findings are in contrast to those observed in Nakashima et al. study, which reported weight

loss between days 6 to 10, and reduction in food and water intake between days 6 to 14. Furthermore, the thinning of the tongue epithelium and the infiltration of inflammatory cells were more profound in their study compared to our study. By day 7 post-radiation, they reported a complete loss of squamous epithelium and significant inflammatory cell infiltration [283]. Given that they used the same rat strain, sex, and age and similar radiation dose, the variations in the findings between the two studies might be due to the fact they used a less efficient radiation shield (0.5 mm-thick lead plate), which might be not sufficient to protect the body of animals from radiation leading to earlier OM peaking and more severe tissue injury. However, we also cannot exclude the other factors such as the use of a different radiation device or the differences in animal housing and handling conditions which may affect the development of OM.

Since a radiation dose of 15 Gy did not induce normative OM and reduction in weight or oral intake, another study was undertaken using 20 Gy single-dose radiation. OM developed from day 5, peaked on day 9 (average OM score of 3.17), and resolved by day 16. Irradiation was associated with weight loss, a decrease in food and water intake, and a significant change in pain behaviour tests. Furthermore, it caused significant histological changes on both the dorsal and ventral TOT and BOT, however, the injury was more severe in dorsal TOT. Similar to our study, Jonsson et al. showed that 20 Gy induced OM in rats with peak severity on day 10, and weight loss between day 5 and day 11 [282]. Moreover, the histological observations, between day 9 and day 10, were similar to those observed in our study including loss of filiform papillae, ulceration, pseudomembrane formation, and the infiltration of WBCs [282]. However, other studies, that also used 20 Gy to establish OM in rats, reported different peak time points and survival rates. Yang et al. demonstrated that the irradiation of rat heads with 20 Gy X-ray radiation led to the development of OM

from day 6, which peaked on day 14 and recovered by day 25 [304]. Moreover, Chang et al. reported that delivering a single radiation dose of 20 Gy to the oral cavity led to the development of OM from day 7 with peak severity between days 13 and 15. However, only 2 rats survived until day 21 [284].

The key discrepancies reported in previous studies are the injury site on the tongue, OM peaking and healing time points and the survival rate. The injury site depends on the irradiated area within the head and neck region. For instance, irradiation of snouts only causes an injury on the anterior dorsal surface of the tongue, as observed in our and other studies [283, 284, 316]. OM develops on the posterior dorsal tongue when the whole head and neck area is irradiated [297, 301, 304]. The development of OM, peaking and healing times are dependent on the cellular cycle of lingual epithelial cells. The differences in irradiation settings or techniques may affect the development of OM appearance, peak severity, and healing times [283]. Furthermore, the lack of radiation shielding in studies such as Chang et al. [284] and Shin et al. [322] may have led to a decreased survival rate due to the radiation exposure to other body parts resulting in haematological toxicities and potentially subsequent infections [323]. Together, this indicates that a standardised radiation dose, irradiated site, and irradiation shielding should be used to determine a reproducible single-dose radiation-induced OM model. It also highlights the need to conduct pilot studies even when basing parameters off of previously-reported studies, since reproducibility in severity outcomes may be impacted by differences in animal care facilities, seasonal variability, radiotherapy time of day, and other factors.

Pain is one of the most common and distressing complications associated with radiotherapy-induced OM among patients with HNC [324]. To date, no study has evaluated pain in preclinical radiation-induced OM models. Orofacial pain associated with chemically-

induced oral ulceration, caused by injecting hydrochloric acid into oral mucosa, has been assessed by applying a radiant heat source or von Frey filaments to the cheek or whiskers area to test heat hyperalgesia or tactile allodynia in mice [325, 326]. However, in our study, we wanted to assess changes in pain-related behaviours or pain sensitivity (hyperalgesia) caused by the changes in systemic inflammatory responses as observed in chemotherapy-included pain [254]. Thus, we used RGS and VFT to evaluate pain in the present study. Both 15 Gy and 20 Gy radiation doses were associated with a change in pain-related behaviours in rats, however, 20 Gy caused a greater increase in RGS score and a more profound decrease in the MWT. The average baseline MWT of SD rats in our studies was 7 - 8 g, which is relatively similar to the baseline threshold (8.1 - 12.3 g) reported by Kristensen et al. [320]. Similar to our study, the researchers applied VF filaments to the plantar surface of the hind paw of male SD rats using the ascending VFT method. However, they designated the first filament that causes at least three positive responses as the MWT. In our study, the force of the filament that caused two positive responses was considered as the MWT. This may explain why the baseline MWT was slightly higher compared to our study.

In the current study, exposure to 20 Gy irradiation reduced the MWT to 5.3 g indicating increased pain sensitivity, i.e., mechanical hyperalgesia after irradiation. Previous study has shown that RGS can also detect pain, with similar patterns of response to VFT [327], however, they observed that mechanical hyperalgesia persists for a longer period after the RGS score retunes to baseline [327]. This agrees with what we observed in our study as the MWT of the 20 Gy group remained low while the RGS score returned to baseline values by day 16 post-radiation. Overall, the change in mechanical hyperalgesia and RGS score indicates that local irradiation of the snout is associated with increased pain-related behaviours. To our knowledge, this is the first study to assess changes in pain behaviours

(RGS score and mechanical hyperalgesia) in a model of radiation-induced OM. Further research is warranted to validate these findings and identify the biological mechanisms associated with OM-related pain. To assess the duration of mechanical hyperalgesia, future studies should be conducted for an extended period of time to allow for evaluating the recovery of MWT to baseline values.

4.5 Conclusion

Both 15 Gy and 20 Gy were well-tolerated doses and caused some degree of OM. 15 Gy caused mild OM but it was not sufficient to cause weight loss or reduce oral intake in rats. However, 20 Gy was associated with moderate OM, loss in body weight, and a drop in oral intake. Therefore, 20 Gy dose was determined to be the optimal dose to develop moderate, self-limiting OM in rats. Overall, in the present study, a new method of radiation shielding was developed to limit radiation to snout only. Moreover, a radiation-induced OM rat model was established using 20 Gy with OM features similar to those experienced clinically by patients with HNC including a drop in body weight and oral intake and mucosal injury. This model can be used to study OM pathogenesis and to test new potential interventions to prevent or treat radiation-induced OM.

Chapter five

This chapter describes an animal study aimed to study the impact of ablation of the gut microbiota on the development and severity of radiation-induced oral mucositis in rats. This chapter is written in a manuscript style and is intended to be submitted to the International Journal of Radiation Oncology, Biology, Physics.

Statement of Authorship

Title of Paper	Antibiotic-induced gut microbiota depletion accelerates the healing of radiation-induced oral mucositis in rats
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	This is an original research paper. It is intended to be submitted to the *International Journal of Radiation Oncology • Biology • Physics*.

Principal Author

Name of Principal Author (Candidate)	Ghanyah Al-Qadami		
Contribution to the Paper	I am the first author and was responsible for designing and conducting the study and preparing, writing, and editing the manuscript.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/06/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Joanne is the principal supervisor. Helped with study planning and execution and manuscript revision and editing.		
Signature		Date	22/06/2021

Chapter 5: Antibiotic-induced gut microbiota depletion accelerates the healing of radiation-induced oral mucositis in rats

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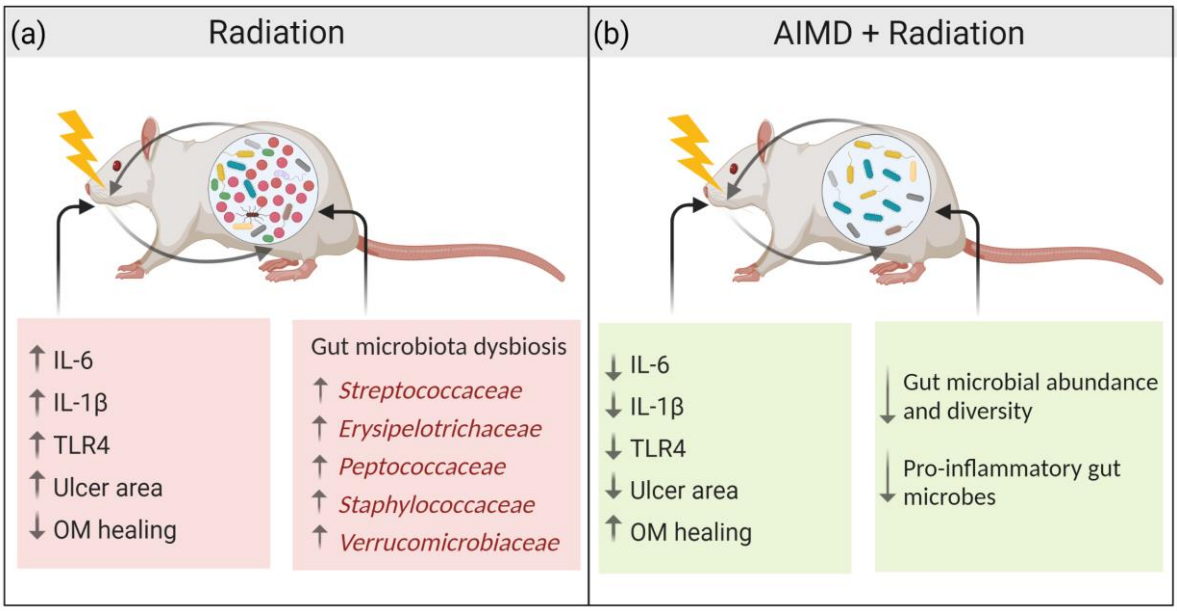
5.1 Graphical abstract



Graphical Abstract: a) Irradiation to rat snouts resulted in significant tongue injury, increased levels of lingual inflammatory markers and was associated with an increase in pro-inflammatory gut microbes. **b)** Antibiotic-induced microbiome depletion (AIMD) reduced radiation-induced ulcer area and levels of lingual inflammatory markers on the tongue, and accelerated the healing of oral mucositis, potentially through inhibiting the expansion of pro-inflammatory gut microbes. *"Image created with BioRender.com."*

Keywords: Gut microbiota, Oral mucositis, Radiotherapy, Inflammation, antibiotic depletion, supportive oncology

5.2 Abstract

Introduction: Oral mucositis (OM), frequent radiotherapy-induced toxicity, is associated with the activation of several inflammatory pathways. Due to its pivotal role in the modulation of immune and inflammatory responses, the gut microbiota has emerged as a key modulator of inflammatory conditions including cancer treatment-induced toxicity. However, it is not clear yet how it impacts radiation-induced OM. As such, this study aimed to explore the role of the gut microbiota in the development and severity of OM in a rat model. **Methods:** Male Sprague Dawley rats were treated with 20 Gy X-ray irradiation (Rx) delivered to the snout, with or without antibiotic-induced microbiota depletion (AIMD). OM severity was assessed daily throughout the experiment and lingual tissues were collected on day 9 and 15 post-radiation for the assessment of tissue injury and inflammatory markers. **Results:** The duration of severe OM (grade 4-5) was significantly shorter in AIMD+Rx rats (2±0.3 days) compared to Rx only rats (3.5±0.5 days) ($p= 0.0268$). Macroscopically, the lingual ulcer-like area was smaller in AIMD+Rx (18.9%) compared to Rx only (33.3%) ($p=0.0195$). Microscopically, a smaller percentage of mucosal ulcer size was observed in AIMD+Rx compared to Rx only (48.57% vs 21.04%, $p= 0.0045$). AIMD+Rx group had significantly lower levels of IL-6, IL-1β, and TLR4 in the lingual tissues than the Rx only group. When examining the caecal gut microbiome, an enrichment of pro-inflammatory families, *Verrucomicrobiaceae*, *Streptococcaceae*, *Peptococcaceae*, *Erysipelotrichaceae*, and *Staphylococcaceae*, were observed in Rx only group. **Conclusions:** The gut microbiota plays a role in OM pathogenesis, mainly in the healing stage, through the modulation of inflammatory cytokines. Future microbiota-targeted therapies may improve OM in clinical settings.

5.3 Introduction

Oral mucositis (OM) is one of the most common and debilitating toxicities of radiotherapy, affecting more than 90% of patients with head and neck cancer (HNC) treated with radiotherapy alone or with chemoradiotherapy [57, 59]. It negatively affects patient quality of life as it is associated with pain and difficulty in chewing, swallowing, speaking, and eating. The secondary complications of OM and their impact on quality of life can also lead to treatment breaks or interruption, therefore, impacting depth of response and ultimately prognostic outcomes [72]. While there are a number of strategies and interventions used to relieve the symptoms of OM, there is no standardised treatment or preventive measure that is broadly effective [73]. Therefore, further research is required to elucidate the best targets for intervention to prevent or minimise the severity of OM.

Exposure to radiation causes DNA damage and production of reactive oxygen species, which activates several pathways, including Nuclear factor-kappa B (NF-κB), leading to the production of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6), resulting in further tissue damage [86]. Due to the critical role inflammation plays in tissue injury processes, it has been a key therapeutic target for OM [328]. Several anti-inflammatory agents targeting pro-inflammatory cytokines, cyclooxygenase-2, or NF-κB pathways have been tested for treating OM. However, to date, most of these anti-inflammatory therapeutics have shown limited effectiveness [75]. Hence, exploring other factors that modulate the inflammatory response during OM may provide new targets for OM intervention.

In recent advances in OM aetiology and management, its relationship with the oral microbiota has received significant attention, with changes in its composition observed following anti-cancer treatment [94]. Moreover, the ulceration phase of OM is associated

with an increased bacterial load, which suggests that secondary bacterial colonisation could exacerbate OM-associated inflammatory responses, aggravating the severity of OM [329]. However, in clinical trials, elimination of the oral microbiota with antimicrobial lozenges, pastes, or mouthwash has failed to prevent or reduce OM severity [226]. While these findings were unexpected, the impact of the host microbiome, in particular the gut microbiome, remains an area of keen interest. As such, the focus has now shifted to evaluating the potential role of the gut microbiota on the development of OM.

The concept that the gut microbiota modulates events beyond its local environment reflects its profound control over the immune system and inflammatory responses [330, 331]. This is mainly mediated through the release of bacterial products such as lipopolysaccharides (LPS) [332] – a pathogen-associated danger signal – or metabolites such as short-chain fatty acids (SCFAs) [333], each of which can translocate across the mucosal barrier and affect distant sites. Of particular interest to OM, pathogenic changes in the gut microbiome, as seen after chemotherapy, can lead to intestinal barrier dysfunction (tight junction and mucus layer alterations), permitting the translocation of bacteria and/or their products resulting in activation of the systemic inflammatory response [334]. The activation of systemic inflammatory pathways interacts with pattern recognition receptors such as the Toll-like receptor (TLR) family and can aggravate inflammatory conditions in distant sites of the body [335].

Both gut microbiota and TLRs, in particular TLR4, have been linked to the pathogenesis of chemotherapy-induced gastrointestinal toxicity [336, 337]. For example, a mouse model of irinotecan-induced toxicity showed that TLR4 deletion reduced diarrhoea and mucosal tissue injury, suggesting that TLR4 may mediate the aetiology of gastrointestinal mucositis by controlling the interaction between microbes and their host [336]. In the context of

radiotherapy, a number of studies have reported that reduced microbial diversity and enrichment of certain gut microbes are associated with increased severity of radiotherapy-induced gastrointestinal mucositis [338]. Moreover, abdominal irradiation to rats caused significant intestinal injury, which was associated with an alteration of faecal microbiota diversity. Restoration of normal microbial diversity helped reduce inflammation and tissue damage [339], underscoring the contribution of the gut microbiota to gastrointestinal mucositis. What remains unclear is if and how these changes influence the development and progression of OM. Given that the administration of probiotics reduced the severity of OM in patients with HNC [340], we hypothesise that the composition of the gut microbiota is critical in dictating the severity of OM. This study, therefore, aimed to assess the impact of gut microbiota and related inflammatory pathways in the development of radiation-induced OM in a rat model.

5.4 Materials and methods

5.4.1 Animals

This project was approved by the South Australia Health and Medical Research Institute animal ethics committee (Project# SAM336). Male Sprague Dawley (SD) rats (6 weeks old, 165 –209 g) were housed in individually ventilated cages, 3 rats per cage, in a 12 h dark/light cycle at 18 – 24 °C. Standard food pellets and sterile water were provided *ad libitum*. Archived lingual and intestinal tissues and caecal content obtained from age-matched male SD rats were used as untreated controls for some parts of the data analysis.

5.4.2 Antibiotic-induced microbiota depletion

Rats were weight-matched and divided into two groups (12 per group); antibiotic-induced microbiota depletion plus radiation (AIMD+Rx) or radiation alone (Rx) group. Rats were acclimatised for five days before starting antibiotic treatment. Starting from day -21, AIMD+Rx group was provided with a water-containing antibiotic mixture (1 g/L Ampicillin sodium salt (#A9518), 1 g/L neomycin trisulfate salt hydrate (#N5285), Sigma-Aldrich, USA and 0.5 g/L vancomycin hydrochloride (#15327), Sapphire Bioscience, Australia). To assist the palatability of the antibiotics, 3 g/L of the artificial sweetener Splenda was added to the drinking water [254]. Rx group was provided with sterile water containing 3 g/L sweetener only. Water bottles were refilled daily, with an equivalent amount of antibiotic and sweetener added. Water bottles were covered with aluminium foil to protect antibiotics from light (see *supplementary materials*).

5.4.3 Irradiation

On day 1, rats were anaesthetised with ketamine (75mg/kg) and medetomidine (0.5mg/kg) via intraperitoneal injection and irradiated. A 2-mm thick lead cylinder with holes (2.5 cm diameter) to insert the rat snout was used to shield the rat body from radiation except for

the snouts (Fig. S1). Both groups were exposed to 20 Gy single-dose X-ray radiation at a dose rate of 2.13 Gy/min using a small animal irradiator (RS-2000, Radsource (USA), set at 160 kV, 13 mA for 9 min and 24 sec) (*see supplementary materials*). 20 Gy was chosen based on the results of pilot studies, described in chapter 4. After irradiation, rats were removed from the irradiator and injected subcutaneously with an anaesthetic reversal agent, Atipamezole (1 mg/kg) to reverse the sedative effect of anaesthetic drugs. All animals gained consciousness except for two rats (one from each group), which died under anaesthesia.

5.4.4 Pain assessment

The Rat Grimace Scale (RGS) and von Frey test (VFT) were used to assess changes in OM-associated pain behaviours. To determine RGS scores, photos of each rat were taken through a clear cage, in the morning at the same time of day. The photos were scored blindly and the average RGS scores were calculated as described previously [319]. The VFT platform was prepared by placing four clear mice cages upside down on the top of a mesh floor (hole size 0.65x0.65 cm). Calibrated von Frey monofilaments (Stoelting, USA) with forces of 2, 4, 6, 8, 10, and 15 g were used. Before conducting the test, rats were habituated to the test environment for 30 min for two consecutive days. On the third day, the test was conducted using the ascending stimuli method as previously described [320]. The force of the first filament that elicits the positive response (2 out of 5 applications) was designated as the mechanical withdrawal threshold (MWT) [254]. To assess the changes in pain behaviours throughout the experiment, both RGS and VFT were conducted once before antibiotic treatment, once a week leading up to radiation day, and on days 3, 6, 8, and 13 post-radiation, except for day 11, in which RGS but not VFT was performed.

5.4.5 OM assessment

Following irradiation, tongues were examined daily following irradiation by gently pulling cheeks to assess the open oral cavity. OM severity in the tongue was scored using a well-established scoring system that assesses OM based on mucosal erythema, desquamation, exudation, and ulceration [318]. Animals were euthanised on days 9 and 15 post-irradiation, correlating to OM peaking and healing time-points respectively. Photos were taken of collected tongues, and the injured and ulcer-like areas were measured using ImageJ/Fiji software and calculated as described previously [283] (Fig. S2).

5.4.6 Caecal microbial composition analysis

For animals euthanised on day 9, caecal contents were collected in sterile tubes, stored at – 80 °C. Genomic DNA extraction, microbial diversity profiling, and bioinformatic analysis were conducted at the Australian Genome Research Facility (AGRF, Queensland, Australia). Genomic DNA was extracted from 250 mg of caecal content using the Qiagen DNeasy PowerLyzer PowerSoil Kit. Microbial diversity profiling was done through 16S ribosomal RNA (16S rRNA) gene sequencing using primers targeting 341F-806R (V3-V4) region in the bacterial 16S rRNA gene (forward primer: CCTAYGGGRBGCASCAG, Reverse primer: GGACTACNNGGGTATCTAAT) (*see supplementary materials*).

5.4.7 Histological analysis

5 µm sections of paraffin-embedded lingual tissues were cut using a rotary microtome (Leica Biosystems, Germany) and stained with Hematoxylin and Eosin. Slides were scanned using a Nanozoomer 2.0 digital slide scanner (Hamamatsu Photonics, Japan). Epithelial thickness, mucosal ulcer size, white blood cells (WBC) infiltration, and histological score were assessed using NDP.view2 Viewing software. The mucosal ulcer was defined as the complete loss of the epithelial and keratinised layers of the dorsal tongue. Percentage of

mucosal ulcer size was calculated as (length of mucosal ulcer/length of dorsal epithelium) x 100. A modified histological scoring system was used to assess epithelial damage [321] (Table S1). The histological changes were evaluated in both the dorsal and ventral surface of the tip of the tongue (TOT) and body of the tongue (BOT), which represent the ulcer-like and injured area respectively.

5.4.8 Immunohistochemistry

4-5 µm lingual tissue sections were cut and mounted onto FLEX IHC microscope slides (Dako, Germany). Heat-mediated antigen retrieval in sodium citrate buffer (pH= 6.0) for IL-1β, IL-6, and TLR4, and in EDTA buffer (pH= 9.0) for TNF-α, was performed via PT Link system (Dako, Denmark). IHC was conducted using Dako EnVision Detection Systems and staining was performed via Autostainer Link 48 (Dako, Denmark). Primary antibodies (anti-IL-1β, ab9722, 1:250; anti-TNF-α, ab6671, 1:100; anti-IL-6, ab9324, 1:250 and anti-TLR4, ab22048, 1:100, Abcam, UK) were used for immunolabelling (*see supplementary materials*). Stained tissues were scanned using Nanozoomer 2.0 and evaluated using NDP.view2 software. Staining intensity was scored as follows: none= 0, weak= 1, moderate=2, strong= 3, and intense= 4.

5.4.9 Immunofluorescence double-staining of tight junction proteins

4 µm paraffin-embedded tissue sections from the ileum and colon were cut and mounted onto FLEX microscope slides. Antigen retrieval in EDTA buffer (pH= 8) was performed in the PT Link system. The staining was performed using Autostainer Link 48. Primary antibody Occludin mouse monoclonal antibody, #33-1500, 1:200, Invitrogen, US) and Zonula occludens-1 (ZO-1) (rabbit polyclonal antibody, #61-7300, 1:200, Invitrogen, US) were used for the double staining (*see supplementary materials*). Slides were imaged using an

Olympus FV3000 confocal microscope (Nikon, Japan). The percentage area of positive staining was measured using Fiji/ImageJ software.

5.4.10 Alcian Blue- Periodic acid Schiff staining

4 µm paraffin-embedded tissue sections from the colon were cut and mounted into slides. Tissues were stained in Alcian Blue-Periodic acid Schiff's stain and counterstained in Hematoxylin. Slides were scanned using Nanozoomer 2.0 and analysed via NDP.view2 software. The total number of goblet cells (GCs), acidic mucins-producing GCs, and mixed mucins-producing GCs were counted in 10 individual colonic crypts.

5.4.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0.0. For parametric data, the Student T-test, one-way and two-way ANOVA, and mixed model analysis were used. Mann–Whitney U test and Kruskal–Wallis test were used for non-parametric data. $P<0.05$ was considered statistically significant.

5.5 Results

5.5.1 Antibiotic treatment and radiation altered the gut microbiota composition

As shown in the experimental scheme (Fig. 1A), after 3 weeks of antibiotic treatment, animals in both groups were exposed to single-dose radiation. The assessment of the caecal microbiome (day 9) revealed that antibiotic treatment significantly reduced the number of observed operational taxonomic units (OTUs) (Fig. 1B) and Shannon diversity index at the genus level (Fig. 1C). Moreover, the Principal Coordinates Analysis (PCoA) revealed that antibiotic treatment shifted the gut microbiota, and created a distinct microbial composition compared to that of the Rx group (Fig. 1D). The relative abundance graph shows that AIMD method successfully depleted the majority of the bacterial taxa of the AIMD+Rx group except for one taxon belonging to the Proteobacteria phylum (*Enterobacteriaceae* family), which was relatively enriched after antibiotics (Fig. 1E).

When comparing Rx to a control group (unirradiated), results showed that irradiation of the snout did not impact the overall diversity of gut microbiota (Fig. S3). However, a decrease in the Bacteroidetes and an increase in Verrucomicrobia phyla were observed in the Rx group (Fig. 1F). Due to the reduction in Bacteroidetes, the Firmicutes/Bacteroidetes (F/B) ratio was increased after irradiation (Fig. 1G). The linear discriminant analysis Effect Size (LEfSe) showed that *Verrucomicrobiaceae*, *Streptococcaceae*, *Peptococcaceae, Erysipelotrichaceae,* and *Staphylococcaceae* families were differentially increased whereas *rikenellaceae*, *prevotellaceae* and *odoribacteraceae* families were differentially decreased in the Rx group compared to controls (Fig. 1I). The most noticeable alteration was the increase in *Verrucomicrobiaceae,* particularly *Akkermansia muciniphila* (*A. muciniphila*) in the Rx group (Fig. 1J-K).

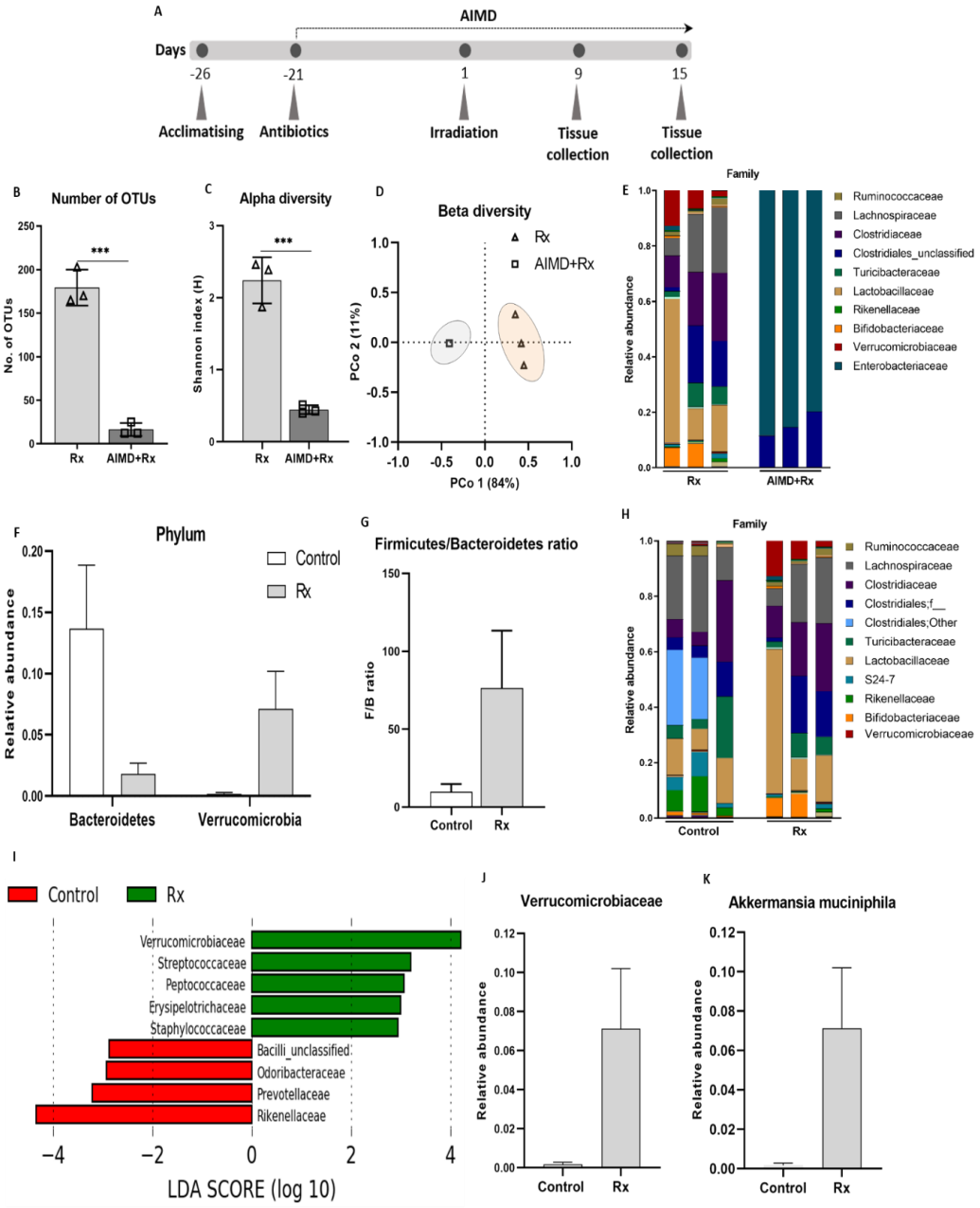


Figure 1: Microbial analysis after AIMD and radiation. **A)** Experimental design scheme**. B**) Antibiotic treatment significantly reduced the number of positive OUTs among the AIMD+Rx group (T-test)**. C)** Shannon index, calculated at the genus level, was significantly lower among the AIMD+Rx group (T-test). **D)** AIMD+Rx group has distinctive microbial composition compared to the Rx group. **E)** Relative abundance at the family level for Rx and AIMD+Rx groups showing the enrichment of *Enterobacteriaceae* family in AIMD+Rx group. **F-G)** An increase in Verrucomicrobia and decrease in Bacteroidetes and F/B ratio was observed in Rx compared to control group. **H)** Relative abundance at the family level for control and Rx groups showing an altered composition following radiation. **I)** LEfSe plot showing an increase in five bacterial families and a decrease in three Bacteroidetes families after irradiation. **J-K)** The relative abundance of Verrucomicrobiaceae (*A. muciniphila*) increased among the Rx group compared to the control. Data presented as mean ± SEM; *N= 3* per group; ***P ≤ 0.001.

5.5.2 AIMD did not affect weight loss or oral intake but improved pain-associated behaviours

Rats in both groups lost weight between day 8 and 11 post-radiation with major weight loss observed on day 9 (5% weight loss) (Fig. 2A). After day 11, AIMD+Rx gained more weight compared to the Rx group, however, the difference between groups was not statistically significant (Fig. 2A, S4A). In both groups, there was a reduction in food (Fig. 2B, S4B) and water intake (Fig. 2C, S4C) between day 7 and day 11 post-radiation, but the difference between groups was not significant.

To assess OM-associated pain, VFT, and RGS were performed. From day 6 post-irradiation, the MWT, assessed by VFT, decreased in both groups indicating mechanical hyperalgesia (Fig. 2D). The RGS scores, a validated measure of pain behaviours, increased from day 8 in both groups and started to recover by day 13 (Fig. 2E). While there were no differences between the groups in the MWT (Fig. 2D), the RGS scores were significantly lower among animals in AIMD+Rx compared to the Rx group on day 8, 11, and 13 (Fig. 2E).

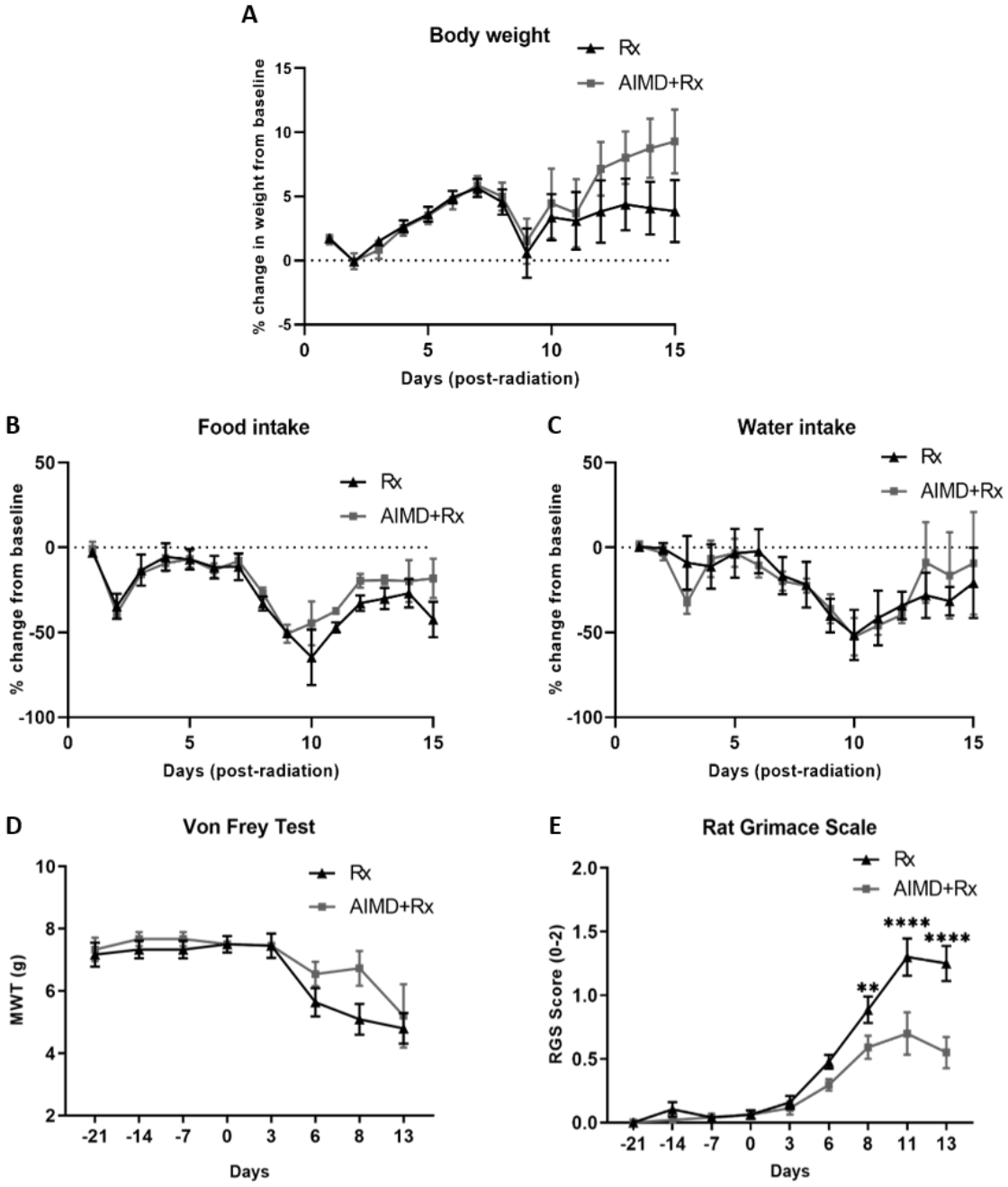


Figure 2: Clinical observations. **A)** Change in body weight throughout the study. Rats in both groups lost weight between day 8 and day 11 and recovered after day 11. **B)** Change in food intake over the study period. There was a similar decline in food intake of both groups between day 8 and day 11, which recovered by day 12 post-radiation. **C)** Change in water intake. There was a similar decline in water intake of both groups between day 8 and day 12, which recovered by day 13 post-radiation. **D)** Change in the MWT measured by VFT. The MWT decreased in both groups from day 6 post-radiation. **E)** Change in RGS score. Rats in the Rx group had significantly higher RGS scores after day 8 post-radiation. Mixed-effects analysis followed by Bonferroni's multiple comparisons tests; Data presented as mean ± SEM; *N= 11* per group; **P ≤ 0.01, ****P ≤ 0.0001.

5.5.3 AIMD reduced ulcer-like area and accelerated the healing of OM

Tissue injury was mainly observed on the anterior dorsal surface of the tongue. Signs of OM appeared from day 5, peaked on day 9, and started healing by day 12 post-radiation (Fig. S5). As shown in Fig. 3A, the OM score of the AIMD+Rx group was significantly lower during the healing phase (day 11-13) compared to the Rx group. Moreover, the area under the OM score curve of AIMD+Rx was significantly smaller for animals that were culled on day 15 post-radiation, indicating a shorter duration of higher OM score (Fig. 3B). AIMD+Rx group also had a significantly shorter duration (2±0.3 days) of severe OM (grade 4-5) than the Rx group (3.5±0.5 days) (Fig. 3C). The tongue injured area (red area in the BOT) was similar between groups. However, the ulcer-like area (red, shiny, and swollen localised to the TOT) was significantly smaller in the AIMD+Rx (18.9%) compared to the Rx group (33.3%) (Fig. 3D-F).

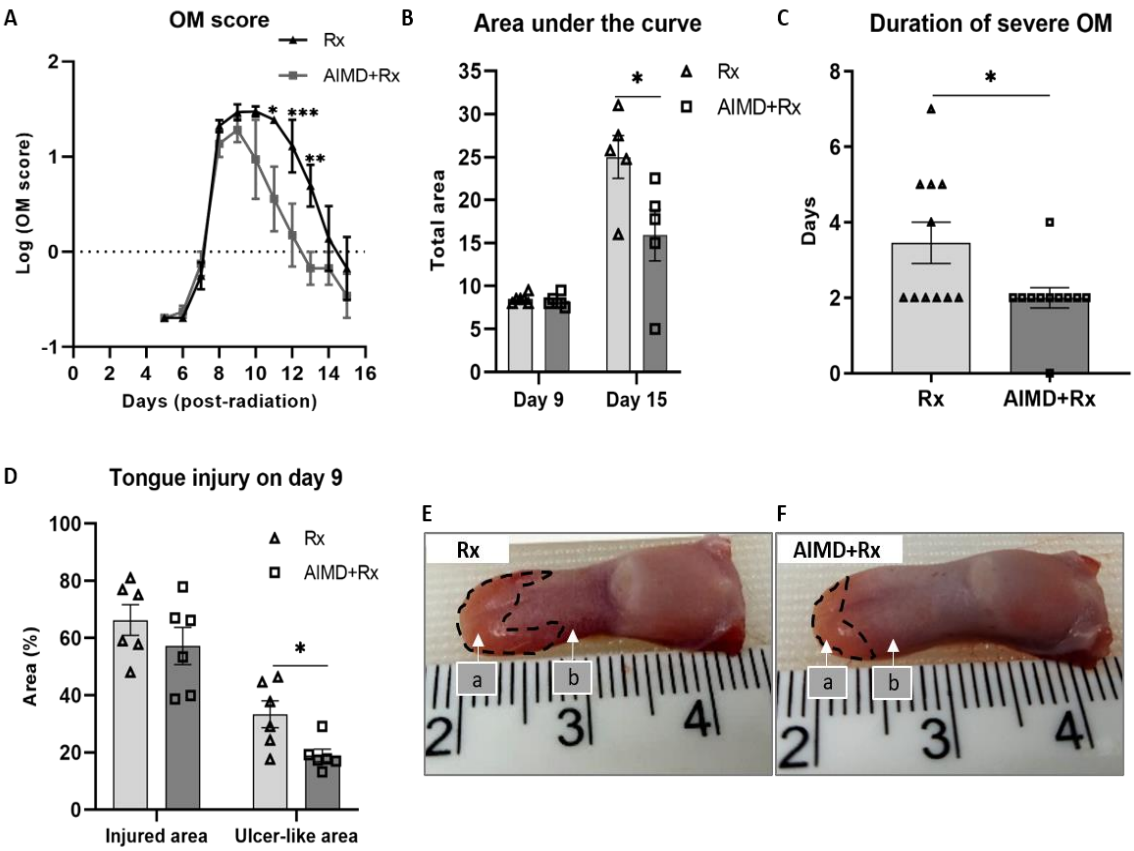


Figure 3: Macroscopic assessment of OM. **A)** Log of OM score post-radiation. Rats in the Rx group had significantly higher OM scores between day 11 and day 13) (Log transformation of non-parametric data, Mixed-effects analysis followed by Bonferroni's multiple comparisons test). **B)** Area under the curve of OM score for animals that were culled on day 9 and day 15. For rats that were monitored until day 15, the AIMD+Rx group had a significantly smaller area under the OM score curve than Rx group (Unpaired t-test). **C)** Duration of severe OM (grade 4-5). Rats in the AIMD+Rx group had a significantly shorter duration of severe OM (Mann Whitney test)**. D)** Tongue injury on day 9 post-radiation. Both groups had a similar injured area in BOT, however, the AIMD+Rx group had a significantly smaller ulcer-like area in TOT (Unpaired t-test). Image of the tongue collected on day 9 for Rx **(E)** and AIMD+Rx group **(F)** showing the ulcer-like area **(a)** and injured area **(b)**. Data presented as mean ± SEM; *N= 11* per group (day 9= 6; day 15= 5); *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

5.5.4 AIMD reduced the mucosal ulceration in the dorsal tongue

The histological changes of the injured area (located at the BOT) and ulcer-like area (located at the TOT) were assessed on day 9 and day 15 post-radiation. On day 9, the dorsal TOT epithelium of both groups was completely damaged. This was accompanied by the infiltration of inflammatory cells, mainly neutrophils, to the ulceration area. By day 15, complete healing of the TOT epithelial layer was observed in 80% of the AIMD+Rx group and 40% of the Rx group. Hyperplasia was observed in all irradiated animals compared to the control group (Fig. 4A). The epithelial thickness of both Rx and AIMD+Rx was significantly less compared to untreated controls on day 9. Conversely, on day 15, the epithelium of the irradiated groups was thicker than the control group, indicating hyperplasia (Fig. 4B). The percentage mucosal ulcer size in the dorsal tongue was significantly smaller among the AIMD+Rx group (Fig. 4C), however, there was no significant difference in the histological WBC infiltration score between Rx and AIMD+Rx groups (Fig. 4D-E).

For the dorsal BOT, radiation caused moderate histological changes in the epithelial layer with mild inflammatory infiltration compared to the control group (Fig. 4F). There was no significant difference in the epithelial thickness between the control and AIMD+Rx groups, however, Rx had a significantly thinner epithelial layer compared to the control group (Fig. 4G). Similar to dorsal TOT, there was no difference between irradiated groups in the histological and WBC infiltration scores (Fig. 4H-I). Both areas of interest on the ventral tongue showed minimal histological changes after irradiation compared to the control group and there was no difference between Rx and AIMD+Rx groups (Fig. S6).

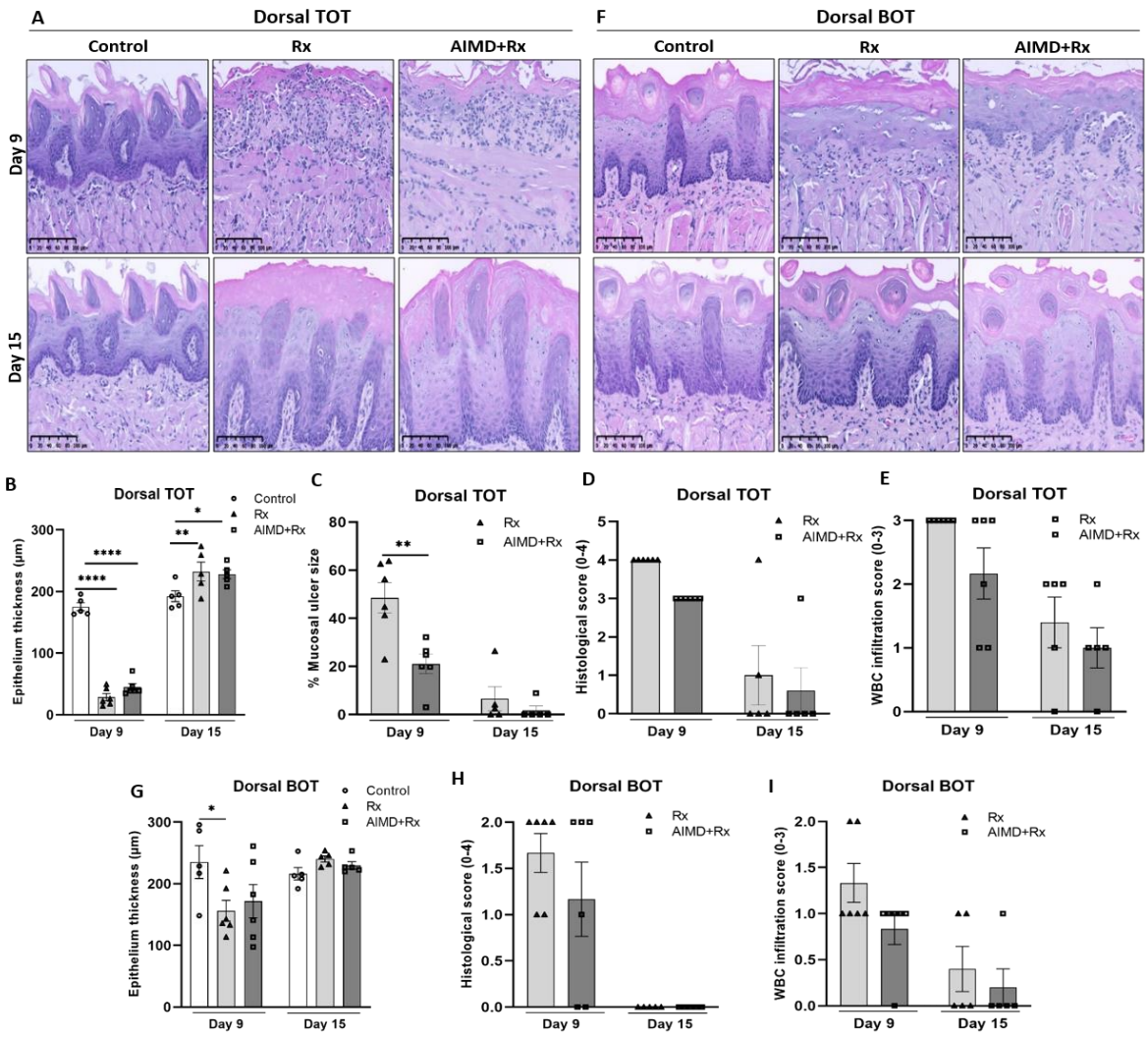


Figure 4: Histological assessment of OM in the dorsal tongue. **A)** H&E staining showing epithelial damage and inflammatory cell infiltration in TOT on day 9 and 15. **B)** 20 Gy radiation caused a significant reduction in the epithelial thickness on day 9 for both Rx and AIMD+Rx groups, while epithelial hyperplasia was observed in irradiated rats on day 15 (Mixed-effects analysis/Bonferroni's multiple comparisons test)**. C)** Mucosal ulceration was significantly smaller among the AIMD+Rx group compared to the Rx group (Unpaired t-test). **D)** There was no significant difference in the histological score between groups on both day 9 and day 15 **(**Kruskal-Wallis test)**. E)** There was no significant difference in the WBC infiltration score between groups on both day 9 and day 15 (Kruskal-Wallis test). **F)** H&E staining showing the epithelial damage and the infiltration of inflammatory cells for BOT on day 9 and 15**. G)** BOT epithelial thickness of the Rx group was significantly lower than the control groups, however, there was no significant difference between Rx and AIMD+Rx groups (Mixed-effects analysis/Bonferroni's multiple comparisons test)**. H)** There was no significant difference in BOT histological score between groups on both day 9 and 15 (Kruskal-Wallis test)**. I)** There was no significant difference in BOT WBC infiltration score between groups on both day 9 and 15 (Kruskal-Wallis test). Data presented as mean ± SEM; *N= 5-6* per group; *P ≤ 0.05, **P ≤ 0.01, ****P ≤ 0.0001; scale bar=100 µM.

5.5.5 AIMD reduced the expression of inflammatory markers in the tongue

The levels of the pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were assessed in both dorsal and ventral TOT and BOT. Overall, there was an increase in the three cytokines after irradiation compared to unirradiated control. On day 9 post-irradiation, there was a significant increase in IL-1β (localised to the nucleus of the epithelial and immune cells) in both dorsal and ventral TOT and dorsal BOT among irradiated groups compared to the control group. Compared to Rx, the AIMD+Rx group had significantly lower IL-1β in the dorsal and ventral TOT on day 9. On day 15, IL-1β on the TOT decreased to normal levels in the AIMD+Rx group, however, it remained significantly higher in the Rx group (Fig. 5A-D; S7A-D). IL-6, mainly found in the mucosal epithelium and submucosa, was the most abundant cytokine observed post-radiation. On day 9, IL-6 in dorsal TOT significantly increased after irradiation compared to unirradiated control. Moreover, it was significantly lower among the AIMD+Rx group compared to the Rx group in both dorsal TOT and BOT. On day 15, IL-6 remained higher in the Rx group compared to controls in the dorsal TOT (Fig. 5E-H; S7E-H). TNF-α increased in the dorsal TOT of the Rx group compared to the control group on day 9, however it was similar between groups on day 15 (Fig. S8).

The level of TLR4, a key inflammatory response mediator, was assessed in the lingual tissues. As expected, the TLR4 significantly increased following radiation in both TOT and BOT. On day 9, TLR4 in the TOT of both Rx and AIMD+Rx was significantly higher than the control group. There was no difference in TLR4 between Rx and AIMD+Rx in TOT, however, AIMD+Rx had lower TLR4 in the dorsal and ventral BOT. On day 15, TLR4 in the TOT of the Rx group remained higher than control and AIMD+Rx groups (Fig. 5I-L; S7I-L).

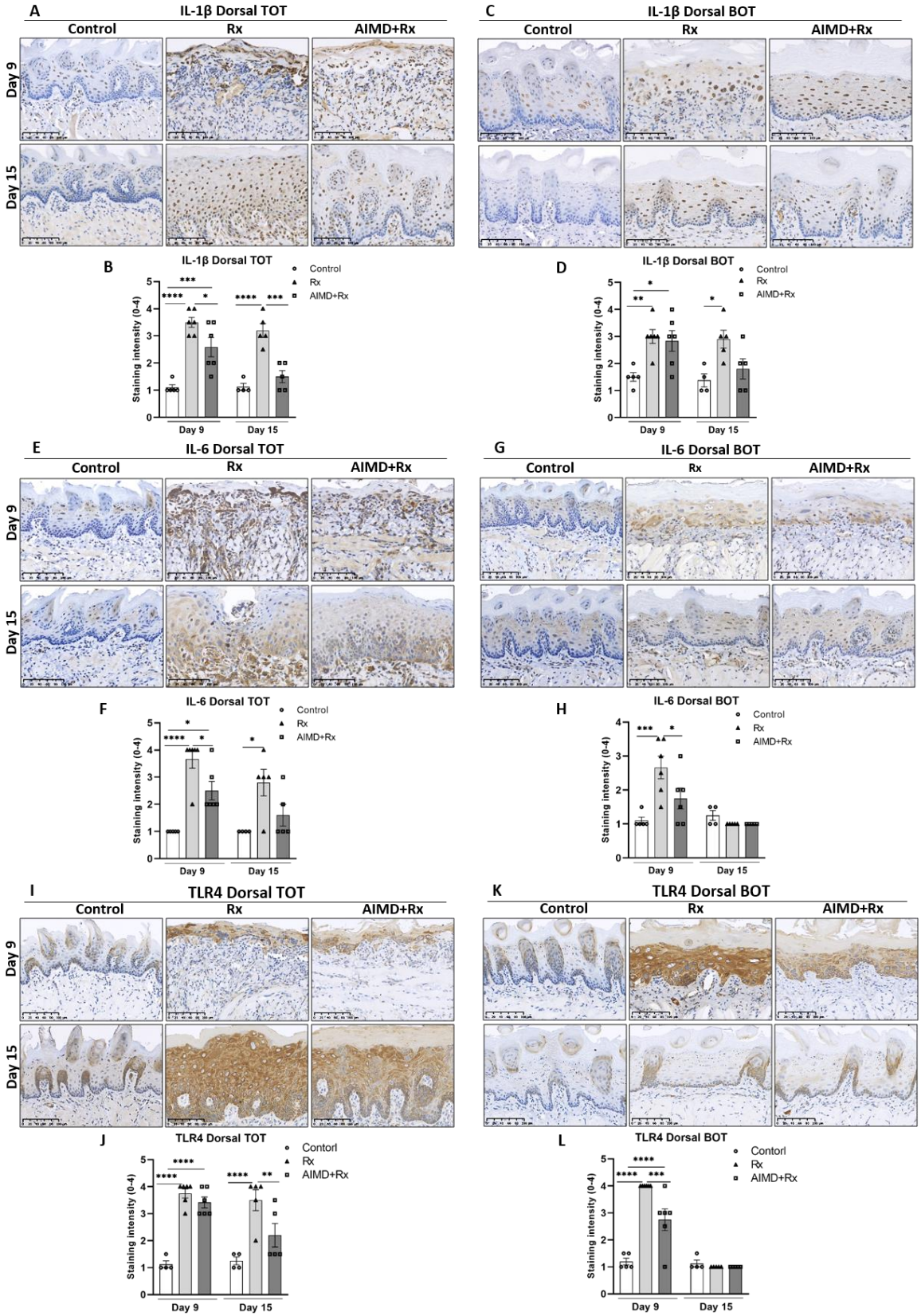

A
IL-1β Dorsal TOT
Control
Rx
AIMD+Rx
Day 9
Day 15
C
IL-1β Dorsal BOT
B
IL-1β Dorsal TOT
Staining intensity (0-4)
Control
Rx
AIMD+Rx
Day 9
Day 15
D
IL-1β Dorsal BOT
E
IL-6 Dorsal TOT
G
IL-6 Dorsal BOT
F
IL-6 Dorsal TOT
H
IL-6 Dorsal BOT
I
TLR4 Dorsal TOT
K
TLR4 Dorsal BOT
J
TLR4 Dorsal TOT
Contorl
L
TLR4 Dorsal BOT

Figure 5: Changes in the levels of pro-inflammatory cytokines and TLR4 in the dorsal tongue. **A-D)** Immunohistochemical staining of IL-1β in the dorsal TOT **(A)** and BOT **(C).** IL-1β staining intensity significantly increased on day 9 in both TOT **(B)** and BOT **(D)**, however, it was lower among the AIMD+Rx group compared to the Rx group. IL-1β of Rx remained higher than control and AIMD+Rx by day 15. **E-H)** Immunohistochemical staining of IL-6 in the dorsal TOT **(E)** and BOT **(G)**. IL-6 levels in dorsal TOT **(F)** and BOT **(H)** of the irradiated group were significantly higher than the control group on day 9. AIMD+Rx group had lower levels of IL-6 in both TOT and BOT on day 9. **I-L)** Immunohistochemical staining of TLR4 in the dorsal TOT **(I)** and BOT **(K).** TLR4 significantly increased on day 9 in both TOT **(J)** and BOT **(L)**. TLR4 was similar between irradiated groups in the TOT, however, the AIMD+Rx group had lower TLR4 in BOT compared to the Rx group. On day 15, TLR4 in dorsal TOT of Rx remained higher than control and AIMD+Rx. Mixed-effects analysis/Bonferroni's multiple comparisons test. Data presented as mean ± SEM; N= 4-6 per group *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. Scale bar: 100 μm.

5.5.6 AIMD and snout irradiation did not disrupt the intestinal tight junction proteins but altered the characteristics of colonic GCs

Occludin and ZO-1 tight junction proteins were double-stained in both the ileum and colon. Intestinal tight junctions were assessed rather than lingual tight junctions because we wanted to investigate whether snout irradiation with or without antibiotics alters intestinal barriers, hence, facilitating bacterial translocation into the systemic circulation. Overall, there were no changes in the expression of either occludin or ZO-1 between groups in the colon and ileum (Fig. 6A-D). To assess the change in mucin composition, another intestinal barrier, colonic GCs were stained with Alcian blue stain with GCs containing acidic mucins and mixed mucins (neutral and acidic) stained blue and purple respectively [341]. There was no change between groups in the total number of colonic GCs, however, the irradiated groups (Rx & AIMD+Rx) had a significantly lower number of acidic mucin-producing GCs compared to the control (Fig. 6E-F).

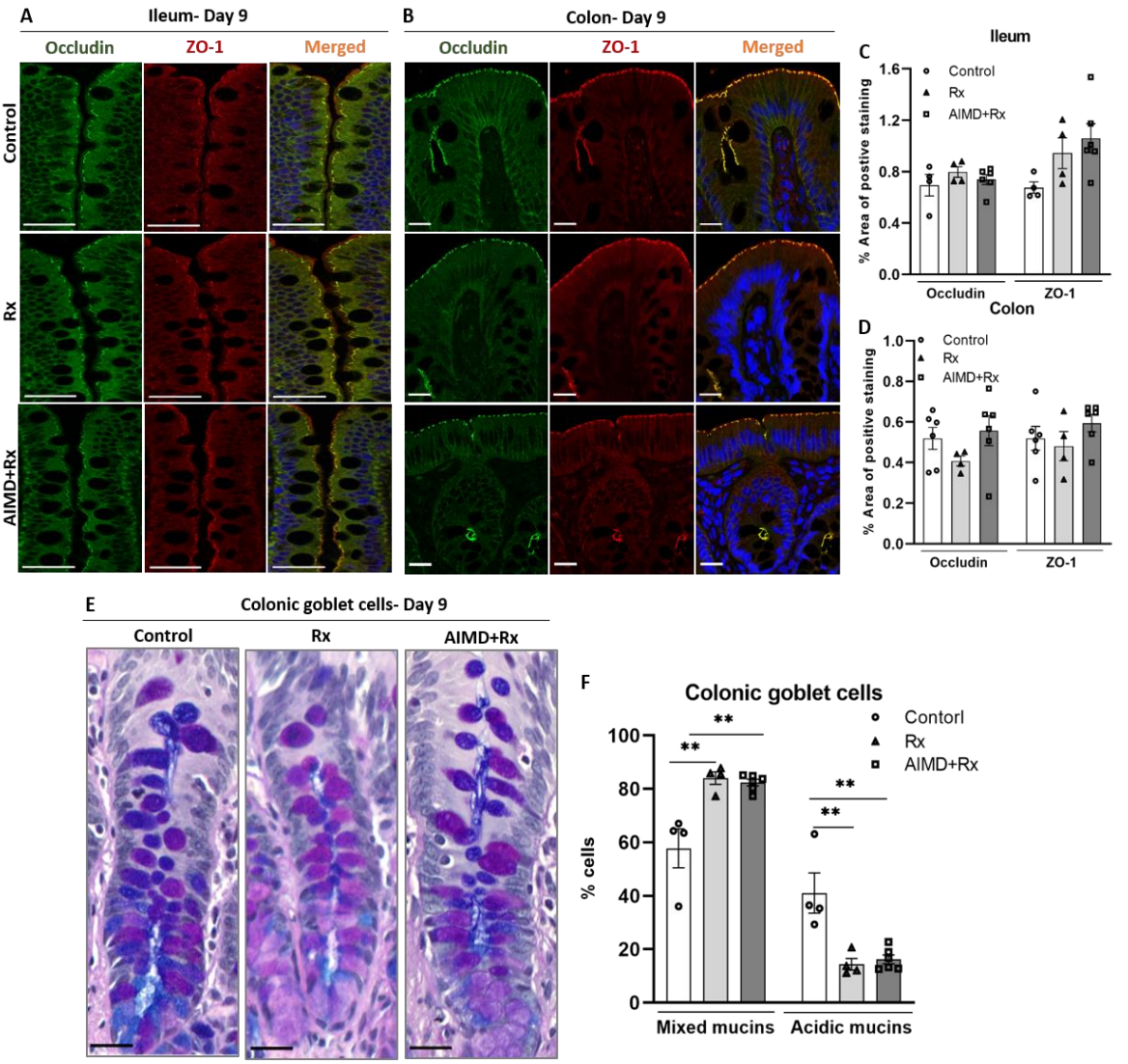


Figure 6: Changes in intestinal barriers. **A-D)** Immunofluorescence staining of occludin (green) and ZO-1 (red) in the epithelium of the ileum **(A)** and colon **(B)**. There was no difference between groups in the expression of occludin and ZO-1 in the ileum **(C)** and colon tissues **(D)** collected on day 9. **(E)** GCs staining in the colon crypts. **(F)** There was a lower number of acidic mucins-producing GCs (blue) and a higher number of mixed mucins-producing GCs (purple) in irradiated groups compared to the control group. One-way ANOVA; Data presented as mean ± SEM; *N= 4-6* per group; **P ≤ 0.01; Scale bar: 20 µm.

5.6 Discussion

Radiation-induced OM is common dose-limiting toxicity, accompanied by weight loss, reduction in oral intake, and severe pain [72]. Since the gut microbiota is a key modulator of systemic inflammatory responses, and radiation treatment is associated with gut microbiome dysbiosis [338], this study aimed to assess whether the gut microbiota has an impact on the inflammatory process associated with radiation-induced OM.

The microbial analysis of the caecal contents showed that the antibiotic cocktail successfully reduced the abundance and diversity of gut microbiota as expected. Antibiotic treatment ablated all major bacterial taxa except for the Proteobacteria taxon, which was enriched among the AIMD+Rx group. The enrichment of this bacterial taxon after antibiotic treatment has been observed in several previous studies [239, 248, 254]. It has been suggested that Proteobacteria may be introduced to the gut from autoclaved food. Since antibiotic treatment depleted the majority of gut microbes, this bacterial taxon is the only one detected during sequencing [239]. Although Proteobacteria, a phylum of gram-negative bacteria that can release the danger signal LPS was enriched in the AIMD+Rx group, this group of animals had overall better OM outcomes in the current study. It has been previously reported that, despite the significant enrichment of Proteobacteria in AIMD mice, there was a reduction in systemic LPS suggesting that LPS level in the circulation is more related to the overall bacterial load rather than the load of Proteobacteria phylum [248, 254]. In the present study, the overall bacterial richness was significantly reduced as represented by the reduction in the total number of observed OTUs among the AIMD+Rx group. Therefore, Proteobacteria enrichment alone might not have a significant influence on OM in the AIMD+Rx group. Together, these findings indicate that AIMD methods used

in the study successfully depleted the gut microbiota in this rat model which allows for studying OM development in the absence of the major bacterial taxa.

When comparing the caecal microbial composition of the Rx group to unirradiated controls, there was no significant change in the overall alpha and beta diversity. However, snout irradiation was associated with a decrease in three major Bacteroidetes families, *Rikenellaceae, Prevotellaceae,* and *Odoribacteriaceae*, and an increase in the F/B ratio, which is an indicator of microbial dysbiosis. *Rikenellaceae* and *Prevotellaceae* families are key SCFA-producing bacteria, mainly the anti-inflammatory propionate [342], therefore, they may play a role in modulating systemic inflammation. Conversely, there was an increase in the abundance of five bacterial families, *Verrucomicrobiaceae*, *Streptococcaceae, Peptococcaceae, Staphylococcaceae,* and *Erysipelotrichaceae*, all of which have been found to increase following the exposure to total body irradiation (TBI) [343] and have been implicated in inflammatory conditions [344-346]. A notable observation in the current study is the significant increase in the *Verrucomicrobiaceae* family, particularly the *A. muciniphila* species. *A. muciniphila* is a gram-negative, mucin-degrading bacteria [347]. Casero et al. reported an increase in this species in response to TBI in mice and suggested that this increase might be a protective or compensatory mechanism in the gut to harmful stimuli [348]. However, its exact role in radiotherapy-induced tissue injury is still not clear. *A. muciniphila* has been linked to colitis due to its mucolytic nature, which reduces the mucus layer thickness and therefore increases intestinal permeability and bacterial translocation [349]. However, the oral administration of *A. muciniphila* supplements restores and increases mucus layer thickness in aging mouse models through the stimulation of mucus production [350]. Moreover, evidence suggests that this bacterium mediates the abscopal effect and antitumour activity of radiotherapy

[351]. Thus, the exact role of *A. muciniphila* in radiotherapy-induced immunogenic cell death and radiotherapy-associated toxicities requires further investigation. Collectively, these findings suggest that local irradiation of the oral cavity alters the gut microbiota composition by diminishing propionate-producing Bacteroidetes and enrichment of pro-inflammatory microorganisms and this may influence the severity of radiation-induced OM.

To assess the impact of AIMD on radiation-induced OM development and severity, we evaluated the OM-associated complications including change in body weight, oral intake, and pain in both groups. Consistent with previous findings, rats in the current study had a reduction in body weight and oral intake following irradiation [282-284]. Overall, there was no difference in AIMD+Rx or Rx groups in weight loss or oral intake, however, rats in the AIMD+Rx group gained more weight during the healing phase (day 12-15). Although there was no significant difference in mechanical hyperalgesia (MWT) between groups, the AIMD+Rx group had a significantly lower RGS score. This suggests that microbiota ablation may influence radiation-induced OM pain by reducing the extent of oral tissue injury and inflammation [352] rather than preventing the centrally-mediated mechanical hyperalgesia as observed previously in chemotherapy-induced pain in mice [254]. In that previous study, eradication of mice gut microbiota with antibiotics before receiving oxaliplatin prevented the development of mechanical hyperalgesia and was associated with lower LPS, IL-6, and TNF-α in dorsal root ganglia (DRG) suggesting that the ablation of the gut microbiota reduces inflammation in DRG after exposure to oxaliplatin, hence preventing hyperalgesia [254]. Here, we did not observe any difference in mechanical hyperalgesia between groups. This might be due to the fact that oxaliplatin causes more intestinal damage and release of LPS than the snout radiation resulting in increased inflammatory responses in the DRG.

Therefore, a more profound impact of microbiota depletion in the chemotherapy-induced pain model would be expected compared to our local radiotherapy model.

In the present study, signs of OM developed on day 5, peaked on day 9, and predominantly resolved by day 15 post-radiation. Tissue injury was limited to the anterior dorsal tongue. This observation was similar to previous studies, in which percutaneous snout irradiation caused OM to the dorsal tongue of rats [283, 284]. There was no difference between groups in the OM score between day 1 and 10, however, AIMD+Rx had lower OM scores during the healing phase (day 11-13). AIMD+Rx group also had a smaller OM area under the curve, shorter duration of severe OM, and smaller ulcer-like area than the Rx group, suggesting that AIMD accelerated OM healing. These findings indicate that AIMD did not impact the radiation-induced initial tissue damage, instead, it might impact the inflammatory process at later stages. As mentioned above, snout irradiation was associated with microbial dysbiosis characterised by an increase in several pro-inflammatory microbes. Hence, AIMD might prevent the expansion of these pro-inflammatory microbes resulting in a less inflammatory response and hence a faster recovery of mucosal tissue injury.

The impact of the dysbiotic microbes on OM-associated inflammation could be mediated by the modulation of pro-inflammatory cytokines. In the current study, radiation caused a significant increase in both IL-6 and IL-1β and a slight increase in TNF-α at the peak injury of OM in the Rx group. These results support findings from Nakashima et al., which showed a significant increase in the lingual mRNA expression of these cytokines following snout irradiation, with IL-6 and IL-1β as the most abundant cytokines during the peak severity time-point [283]. Interestingly, AIMD reduced the levels of both IL-6 and IL-1β and maintained normal levels of TNF-α level after irradiation. The increase in inflammatory cytokines observed in Rx compared to the AIMD+Rx group is potentially mediated by the

abovementioned alterations in the gut microbiota composition following irradiation. The reduction in Bacteroidetes and increase in the F/B ratio have been linked to increased systemic inflammation [353]. Furthermore, the decrease in *Rikenellaceae* and *Prevotellaceae* families [342] results in reduced propionate production, hence, less anti-inflammatory effect and exacerbated radiation injury. The role of propionate in alleviating radiation injury has been confirmed by Guo et al., who demonstrated that the administration of propionate improved animal survival, intestinal crypt length, and mucus thickness in mice exposed to 8- 8.2 Gy of TBI [343]. Conversely, radiation was associated with an increase in bacterial families that have been associated with inflammation and the production of pro-inflammatory cytokines [344-346]. In the context of radiation injury, Guo et al. demonstrated that lower abundance of Erysipelotrichales, the order to which *Erysipelotrichaceae* belong, and the abundance of *Streptococcaceae, Peptococcaceae, Staphylococcaceae,* and *Verrucomicrobiaceae* families was associated with better survival and clinical score in mice that received TBI [343]. The results of the present study indicate that exposure to snout irradiation shifts the gut microbiota to a more pro-inflammatory microbial composition. This also has been observed in the context of rectal irradiation. Gerassy-Vainberg et al. showed that rectal irradiation with four fractions of 5.5 Gy (total of 22 Gy) caused microbial dysbiosis in mice. The transfer of post-radiation gut microbiota into GF mice increased susceptibility to radiation-induced damage and enhanced the secretion of IL-1β [354]. Overall, the enrichment of pro-inflammatory gut microbes in the Rx group correlates to higher levels of pro-inflammatory cytokines in that group.

In contrast, antibiotic treatment could prevent the increase in pro-inflammatory microbes resulting in lower levels of pro-inflammatory cytokines in the AIMD+Rx group. Previous studies have reported that the ablation of gut microbiota with antibiotics reduces pro-

inflammatory cytokines in tissues, such as the intestine [355, 356] or in circulation [258]. We believe that the better OM outcomes observed in the AIMD group are due to the reduction in the pro-inflammatory cytokines mediated by the gut microbiota ablation. This is supported by the findings of a study by Gupta et al., which reported that chemotherapy-induced OM in germ-free mice was associated with lower pro-inflammatory cytokines (IL-1β and TNF-α) in tongue tissues compared to specific pathogen-free mice [357]. Since lower levels of pro-inflammatory cytokines were observed after antibiotic treatment on both day 9 (IL-6, IL-1β) and day 15 (IL-1β), AIMD is likely to reduce tongue injury and enhance healing through the suppression of inflammatory signals. Together, the current results suggest that the protective effects of antibiotics are microbiome-dependent, however, further research to assess whether the antibiotics have a direct impact on the capabilities of immune cells to release pro-inflammatory cytokine is needed. In addition, further studies are warranted to investigate the impact of the gut microbiota on the signalling pathways associated with OM healing in particular.

One of the microbiota-immune pathways involved in the regulation of inflammatory responses is the TLR4 signalling pathway. TLR4 can recognise both microbial-associated molecular patterns such as LPS and damage-associated molecular patterns like high mobility group box protein 1 (HMGB1) leading to the activation of the NF-κB signalling pathway and production of pro-inflammatory cytokines [358]. Therefore, we assessed TLR4 in the tongue of irradiated and control animals. Our results showed that TLR4 significantly increased in tongue tissues following irradiation while AIMD decreased TLR4 levels, particularly in the injured tongue area. This could be due to the reduction in LPS due to the elimination of both oral and gut microbes by antibiotic treatment. TLR4 has been linked to the pathophysiology of chemotherapy-induced gastrointestinal mucositis [336], and given

the ubiquitous pathophysiology, it could play a similar role in radiation-induced OM. In one study, the inhibition of HMGB1 significantly reduced the severity of chemoradiotherapy-induced OM indicating that TLR4-mediated activation of NF-kB is critical in OM pathogenesis [359]. The current study shows that TLR4 is increased in radiotherapy-induced OM and AIMD reduces its levels in lingual tissues, suggesting that microbial activation of TLR4 contributes to OM-associated inflammation.

Recent studies have demonstrated that local irradiation of specific body parts such as the oral cavity can cause out-of-field effects in other organs, particularly in rapid turnover intestinal tissues [360]. Therefore, we assessed whether snout irradiation had an impact on the intestinal barriers including the expression of tight junction proteins in both ileum and colon and count of colonic GCs day 9 post-radiation. In our study, there was no significant difference in the levels of both occludin and ZO-1 between irradiated and control groups. In contrast, Fernández-Gil et al. showed that irradiation of the rat mouth with a total of 37.5 Gy in 5 fractions over 5 consecutive days led to a reduction in ZO-1 in the small intestine [360]. Fractionated radiation and higher radiation dose cause more oxidative stress, resulting in more disruption in the intestinal barriers, which may explain why no changes to tight junction proteins were noted in our study. In the present study, we also found no change in the levels of tight junctions among AIMD+Rx groups. A previous study has also demonstrated that oral gavage of individual antibiotics (Amoxicillin, Cefotaxime, Vancomycin, or Metronidazole) for 10-11 days did not affect the expression of rat tight junction proteins including ZO-1 and occludin [266]. It is important to note however that we did not perform any functional analysis of the intestinal barrier (e.g., FITC-fluxes or Ussing Chamber studies). As such, while tight junction protein expression was not directly affected, we cannot assume the same for the function of the barrier. We did however

assess the number and characteristics of GCs in the colon of irradiated and control rats. Similar to what has been reported before, the colon of control SD rats contain both neutral and acidic mucins with more predominant mixed mucins [361]. Our results showed that there was no change in the total GCs count, however, irradiated animals had a reduced number of acidic mucin-producing GCs. A decrease in the number of acidic mucin-producing GCs also has been observed after chemotherapy treatment [362]. Acidic mucins are less degradable by microbial and host proteases; hence, it has been suggested that they can protect against bacterial translocation [341]. Since the change in the mucin dynamics in the colon was observed in both AIMD+Rx and Rx groups, it could be mediated by irradiation or antibiotics. Interestingly, in the present study, we noticed a significant increase in the abundance of the mucin-degrading *A. muciniphila* in the Rx group, which may contribute to the alteration of the mucin composition following radiation exposure. Furthermore, since the gut microbiota plays a major role in the regulation of the intestinal mucus layer, AIMD will certainly alter the GCs characteristics but a more in-depth analysis would be required to determine those effects. Cumulatively, while no change was observed in the expression of tight junction proteins, GC characteristics were altered in irradiated groups compared to untreated controls. Further research to assess the change in gut mucus composition after oral irradiation is required to confirm the impact of reduced acidic mucin-producing GCs in intestinal barrier integrity.

One of the limitations of this study is that we cannot rule out the impact of oral microbiota on radiation-induced OM in this model. The administration of oral antibiotics will impact both oral and gut microbial composition with a more profound effect on the gut microbiome. While we believe the effects observed in our study are more related to the gut microbiota ablation, changes in the oral microbiota cannot be discounted as a potential

protective mechanism and future research should sample both oral and gut microbiome concurrently. However, given that previous clinical studies showed that the use of topical antimicrobial agents is ineffective in reducing OM [226], the causative effects of oral microbiota need further exploration. The lack of benefit seen in these studies could be due to the use of selective, non-targeted, narrow-spectrum antibiotics or the low bioavailability of topically-administrated antibiotics. Nevertheless, the use of antibiotics in the present study was experimental, to study OM development in the absence of major gut bacterial taxa, and was not intended as an intervention. The use of broad-spectrum antibiotics cannot be translated clinically as intact and normal gut microbiome composition is essential for optimal cancer treatment anti-tumour response [363]. In the current study, the impact of antibiotics on cellular response to radiation was not investigated and as such, this needs to be investigated in tumour-bearing models. Given this, methods other than antibiotics should be explored to manipulate the gut microbiome in clinical practice.

5.7 Conclusion

In conclusion, our study shows that ablation of the gut microbiota reduces lingual ulcer size and accelerates healing of OM by reducing levels of pro-inflammatory cytokines and TLR4 in the lingual tissues. It also resulted in lower OM-associated pain represented by reduced RGS scores, however, this needs to be validated using more precise pain assessment methods. The current results suggest the gut microbial composition plays an important role in OM pathogenesis, particularly during the healing phase, through the modulation of inflammatory cytokines. Studies to characterise the gut microbiome of patients with HNC and its association with OM severity are needed to confirm these findings clinically. Upon the validation of these results clinically, the gut microbiota can be manipulated, using probiotics, for example, to improve radiotherapy-induced OM outcomes. The administration of ingested probiotics has been found to significantly improve the severity of OM among patients with locally advanced nasopharyngeal carcinoma treated with chemoradiotherapy [364]. Better results might be achieved by specifically targeting those microbes implicated in OM severity.

Supplementary materials

Methods:

Antibiotic-induced microbiota depletion (AIMD):

An antibiotic cocktail containing ampicillin, vancomycin, and neomycin was used for AIMD. Previous studies have used ampicillin, vancomycin, neomycin, and metronidazole (ANVM) antibiotic cocktail, however, some have reported that rodents restrain from drinking antibiotic-containing water, despite the addition of sweetener, and this might be due to the bitter taste of metronidazole [239, 240]. Therefore, in the present study, metronidazole was excluded from the antibiotic cocktail. Based on the findings of a pilot study we conducted, the ANV cocktail successfully depleted the gut microbiota of rats and led to changes in the gut microbiota similar to those reported using the ANVM cocktail.

Food and water intake assessment:

Since 3 rats were housed per cage, the water and food intake were assessed for each cage. Rats in each cage were provided with 200 ml of sterile water. Water bottles were weighed twice daily and 24 h water consumption was calculated for each cage. Rats were provided also provided with 750 g per cage of standard food pellets. The food hopper in each cage was weighed daily and the 24 h food intake was calculated.

Animal irradiation:

A lead cylinder with holes on its sides was used as a radiation shield. The size of each hole was 2.5 cm, which fits the rats' snout (Fig. S1). To irradiate animals, they were first anaesthetised with ketamine and medetomidine. While deeply anaesthetised, animals were positioned on their right side, and snouts were placed through the holes of the lead cylinder and irradiated using a small animal irradiator.

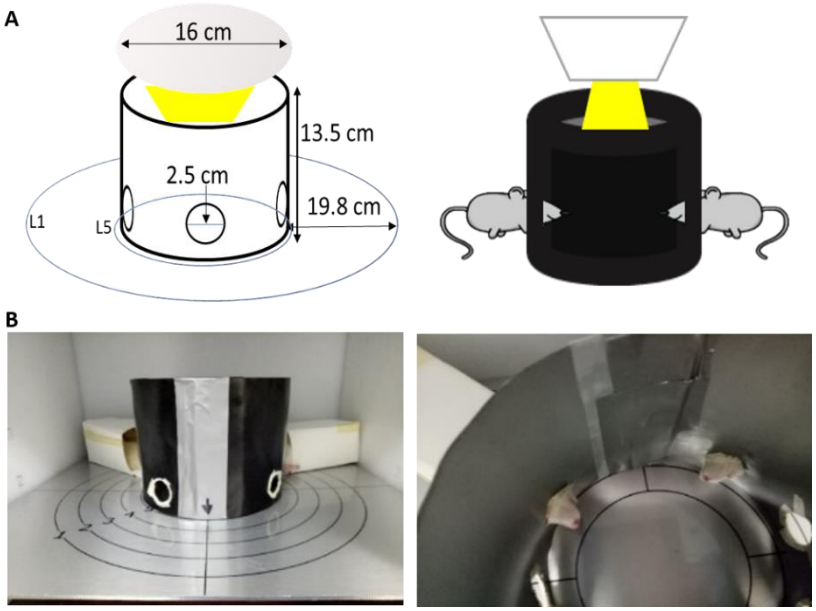


Figure S1: Dimensions and the design of the lead cylinder used as a radiation shielding.

Injured area = injured surface area (b)/ tongue surface area (a) x 100

Ulcer-like area = ulcer-like area (c) / tongue surface area (a) x 100

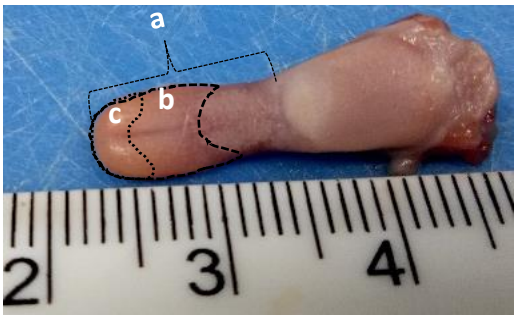


Figure S2: formula used to calculate injured and ulcer-like areas in the tongue.

Macroscopic assessment of OM:

Table S1: A modified OM histological scoring system [321]:

Grade	Histopathologic manifestation
0	No radiation injury; normal mucosa
1	Focal or diffuse alteration of basal cell layer with nuclear atypia and ≤2 dyskeratotic squamous cells
2	Epithelial thinning (2–4 cell layer) and/or ≥3 dyskeratotic squamous cells in the epithelium
3	Loss of epithelium without a break in keratinisation or presence of atrophied eosinophilic epithelium with/without Subepithelial vesicle or bullous formation
4	Complete loss of epithelial and keratinised cell layers; ulceration

Microbial composition analysis:

The bioinformatic analysis of microbial sequencing data was performed by the Australian Genome Research Facility (AGRF). It was done using PEAR (version 0.9.5), QIIME 1.8.4, USEARCH (version 8.0.1623), and UPARSE software. Paired-end reads were assembled using PEAR. After trimming sequences, they were processed and filtered using QIIME 1.8.4, USEARCH, and UPARSE software. Then, reads were mapped back to operational taxonomic units (OTUs) with a minimum identity of 97% using Greengenes (v13.8) as a reference database. For microbial diversity analysis within and between groups, the Shannon index was calculated to assess alpha diversity while Principal Coordinates Analysis (generalised UniFrac distance) was calculated using QIAGEN CLC Genomics Workbench 21.0.3 to assess beta diversity. Linear discriminant analysis effect size (LEfSe) analysis was done using Galaxy online tool using default settings (http://huttenhower.sph.harvard.edu/galaxy/)[365].

Immunohistochemistry:

Tissues were dewaxed in histolene and dehydrated in graded ethanol (100 %, 90%, and 70%). Heat-mediated antigen retrieval in sodium citrate buffer (pH = 6.0) for IL-1β, IL-6, and TLR4, and in EDTA buffer (pH = 9.0) for TNF-α was performed using PT Link system (Dako, Denmark). IHC was performed using Dako EnVision Detection Systems via Autostainer Link 48 (Dako, Denmark). Briefly, endogenous peroxidase activity was blocked by incubating tissues in peroxidase block (K4011, Dako) for 5 min. To block unspecific binding, tissues were incubated with protein block (X0909, Dako) for 30 min. Then, primary antibodies, diluted in antibody diluent (K8006, Dako), were incubated for 1 h at room temperature. HRP polymer-conjugated secondary antibody (Anti-rabbit, (K4003) or Anti-mouse (K4001), Dako) was added and incubated for 30 min followed with DAB substrate (K3468, Dako) for 10 min. Tissues then were counterstained in hematoxylin.

Immunofluorescence double staining:

Tissues were dewaxed in histolene and rehydrated in graded ethanol. The immunofluorescence double staining was performed using Autostainer Link 48. First, tissues were incubated with 10% normal horse serum (NHS) for 1 h. Then, the primary antibody mixture was prepared by diluting occludin mouse monoclonal antibody, 1:200 (#33-1500, Invitrogen, USA) and ZO-1 rabbit polyclonal antibody, 1:200 (#61-7300, Invitrogen, USA) in 5% NHS. Primary antibodies were added to the tissues and incubated for 1 h at room temperature. The secondary antibody mixture was prepared by adding 0.8 µg/mL AlexaFluor 488-conjugated donkey anti-mouse antibody (#A-21202, Invitrogen, USA), and 0.8 µg/ml Alexa Fluor 568- conjugated donkey anti-Rabbit antibody (#A10042, Invitrogen, USA), in secondary antibody diluent (1% bovine serum albumin plus 1% fetal bovine serum in 1x phosphate-buffered saline). The secondary antibody mixture was added and incubated for 1 h. Then, tissues were incubated in DAPI (#D9542, Sigma-Aldrich, USA)

for 15 min. Tissues then were mounted with fluoroshield mounting medium (#F6182, Sigma-Aldrich, USA) and kept in dark at 4 °C.

Results:

Microbial diversity of control group compared to Rx group:

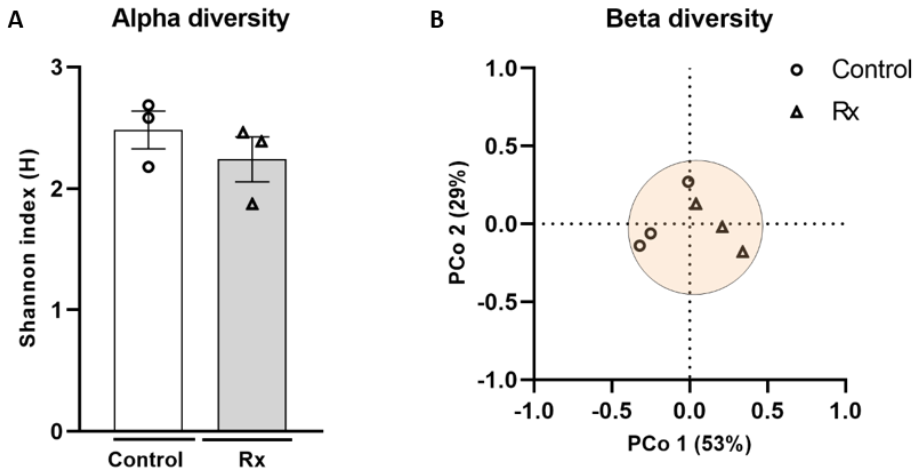


Figure S3: Microbial diversity of control and Rx groups. **A)** There was no difference in Alpha diversity between groups. **B)** Both groups had similar beta diversity.

Change in body weight and oral intake over the study period:

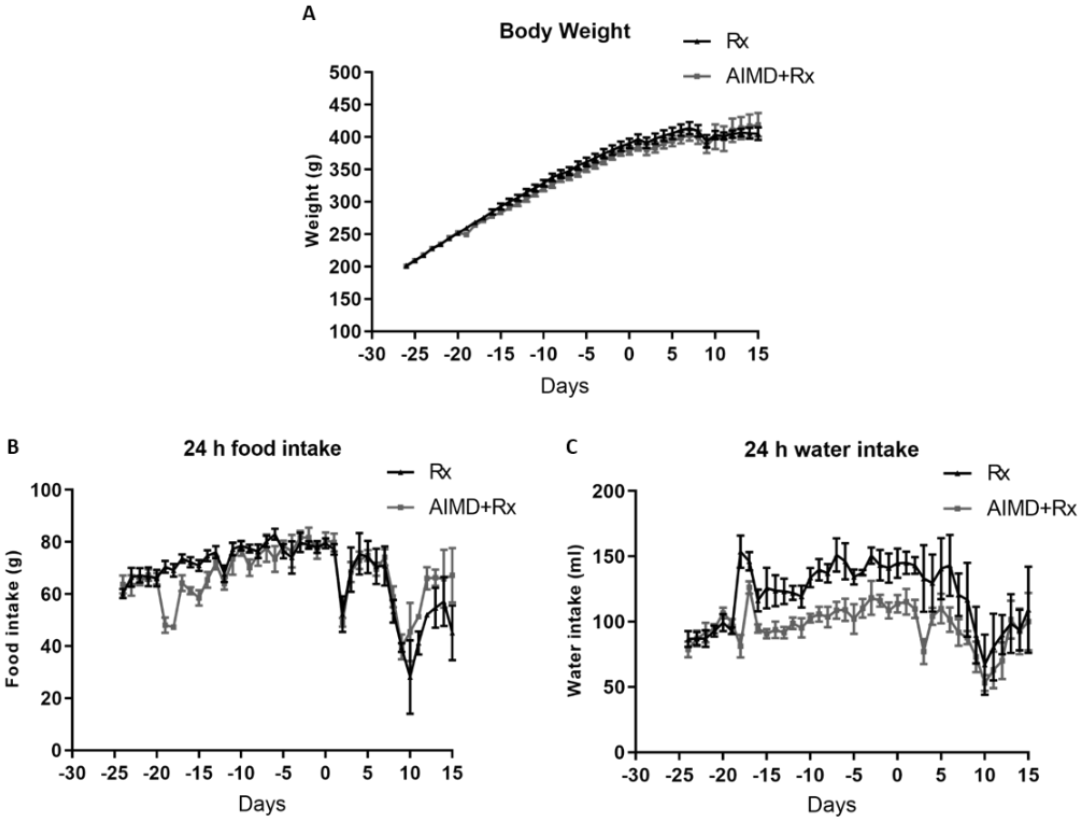


Figure S4: Body weight and oral intake throughout the study period. **A)** Change in body weight throughout the experiment. **B)** Change in 24 h food intake throughout the experiment. **C)** Change 24 h water intake throughout the experiment.

OM severity score over the study period:

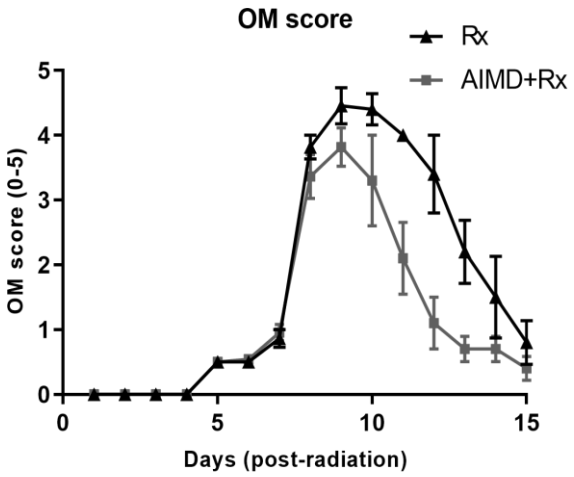


Figure S5: OM severity score following radiation.

Histological changes in the ventral surface of the tongue:

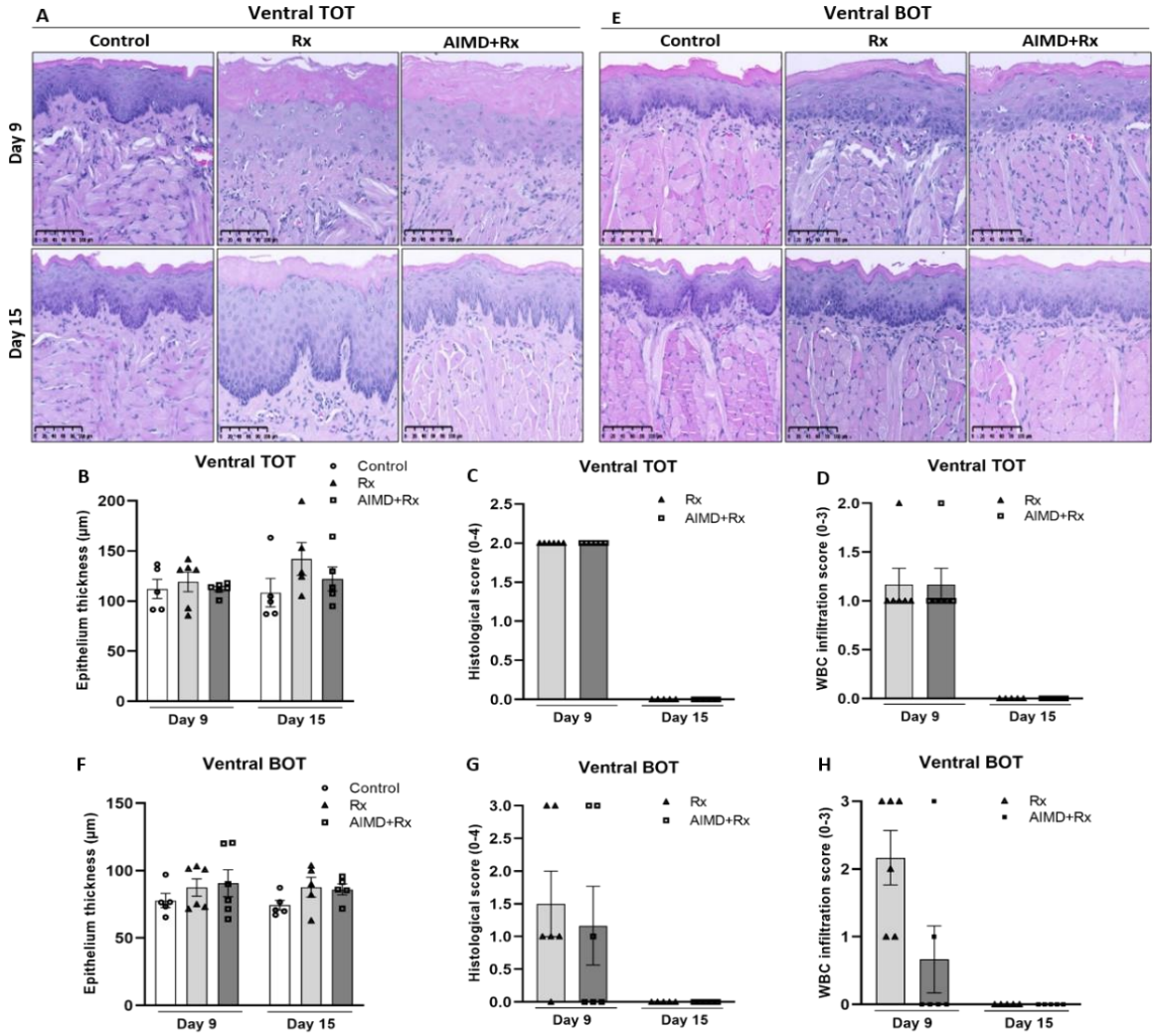


Figure S6: Histological assessment of OM in the ventral tongue. **A)** H&E staining showing the epithelial damage and the infiltration of inflammatory cells for TOT on day 9 and day 15. There was no significant difference between groups for epithelial thickness (Mixed-effects analysis) (**B**), histological score (**C**), and WBC infiltration score (**D**) on both day 9 and day 15 **(**Kruskal-Wallis test). **E)** H&E staining showing the epithelial damage and the infiltration of inflammatory cells for BOT on day 9 and day 15**.** There was no significant difference between groups for epithelial thickness (Mixed-effects analysis) (**F**), histological score (**G**), and WBC infiltration score (**H**) on both day 9 and day 15 **(**Kruskal-Wallis test). Data presented as mean ± SEM; *N= 5-6* per group; Scale bar: 100 µm.

Inflammatory markers in the ventral surface of the tongue:

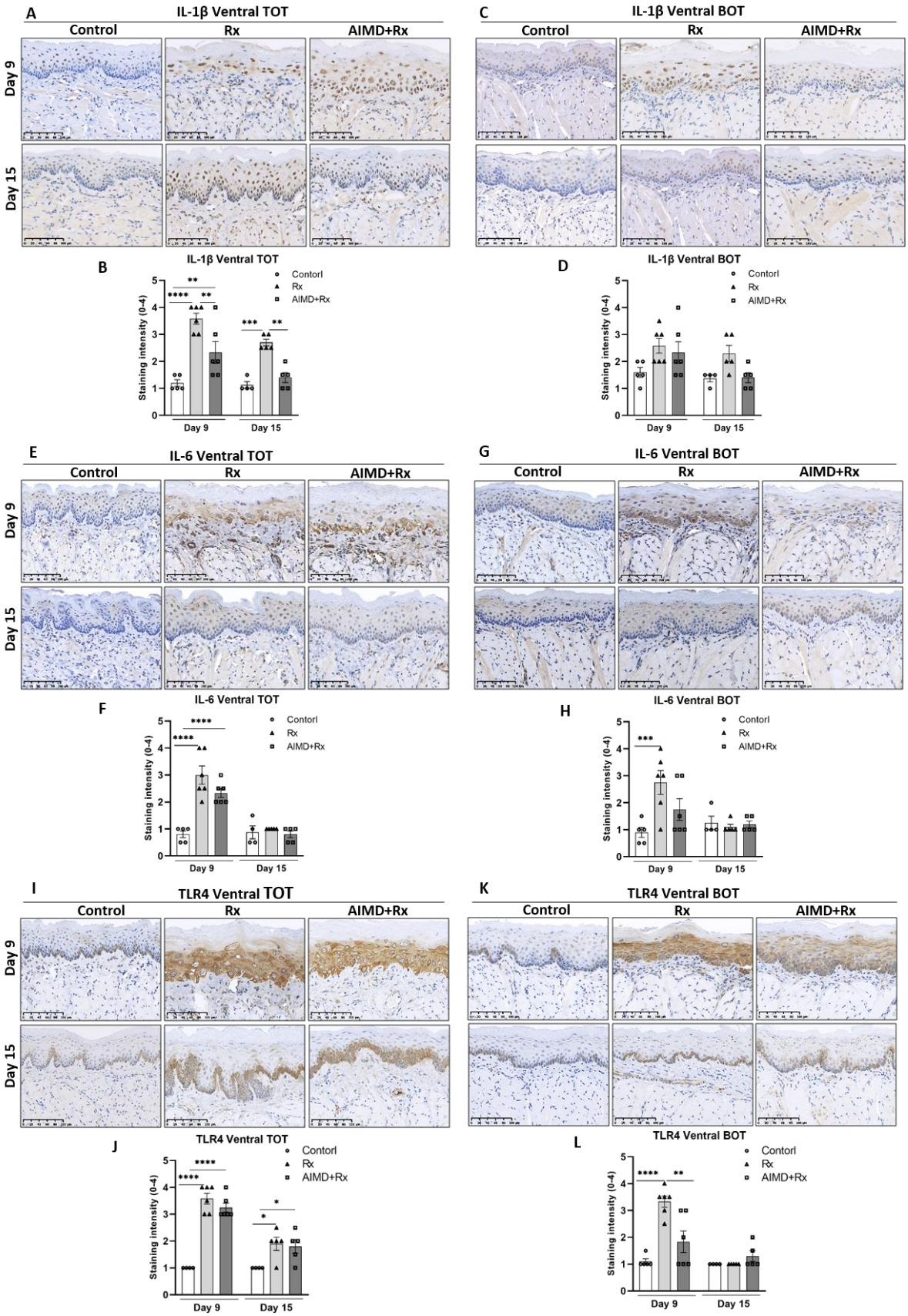


Figure S7: Changes in the levels of pro-inflammatory cytokines and TLR4 in the ventral tongue. Immunohistochemical staining of IL-1β in the ventral TOT **(A)** and BOT **(C)** for Control, Rx, and AIMD+Rx groups on day 9 and day 15. IL-1β staining intensity significantly increased on day 9 in TOT **(B)** but not BOT **(D)** and it was lower among the AIMD+Rx group compared to the Rx group. IL-1β of Rx remained higher than Control and AIMD+Rx by day 15. Immunohistochemical staining of IL-6 in the ventral TOT **(E)** and BOT **(G)** for Control, Rx, and AIMD+Rx groups on day 9 and day 15. IL-6 levels in ventral TOT **(F)** and BOT **(H)** of the Rx group were significantly higher than the Control group on day 9. Immunohistochemical staining of TLR4 in the ventral TOT **(I)** and BOT **(K)** for Control, Rx, and AIMD+Rx groups on day 9 and day 15. TLR4 significantly increased on day 9 in both TOT **(J)** and BOT **(L)**. TLR4 was similar between irradiated groups in the TOT, however, the AIMD+Rx group had lower TLR4 in BOT compared to the Rx group. On day 15, TLR4 in ventral TOT of irradiated groups remained higher than Control and AIMD+Rx. Mixed-effects analysis followed by Bonferroni's multiple comparisons test. Data presented as mean ± SEM; *N= 4-6* per group**,** **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001; Scale bar: 100 µm.

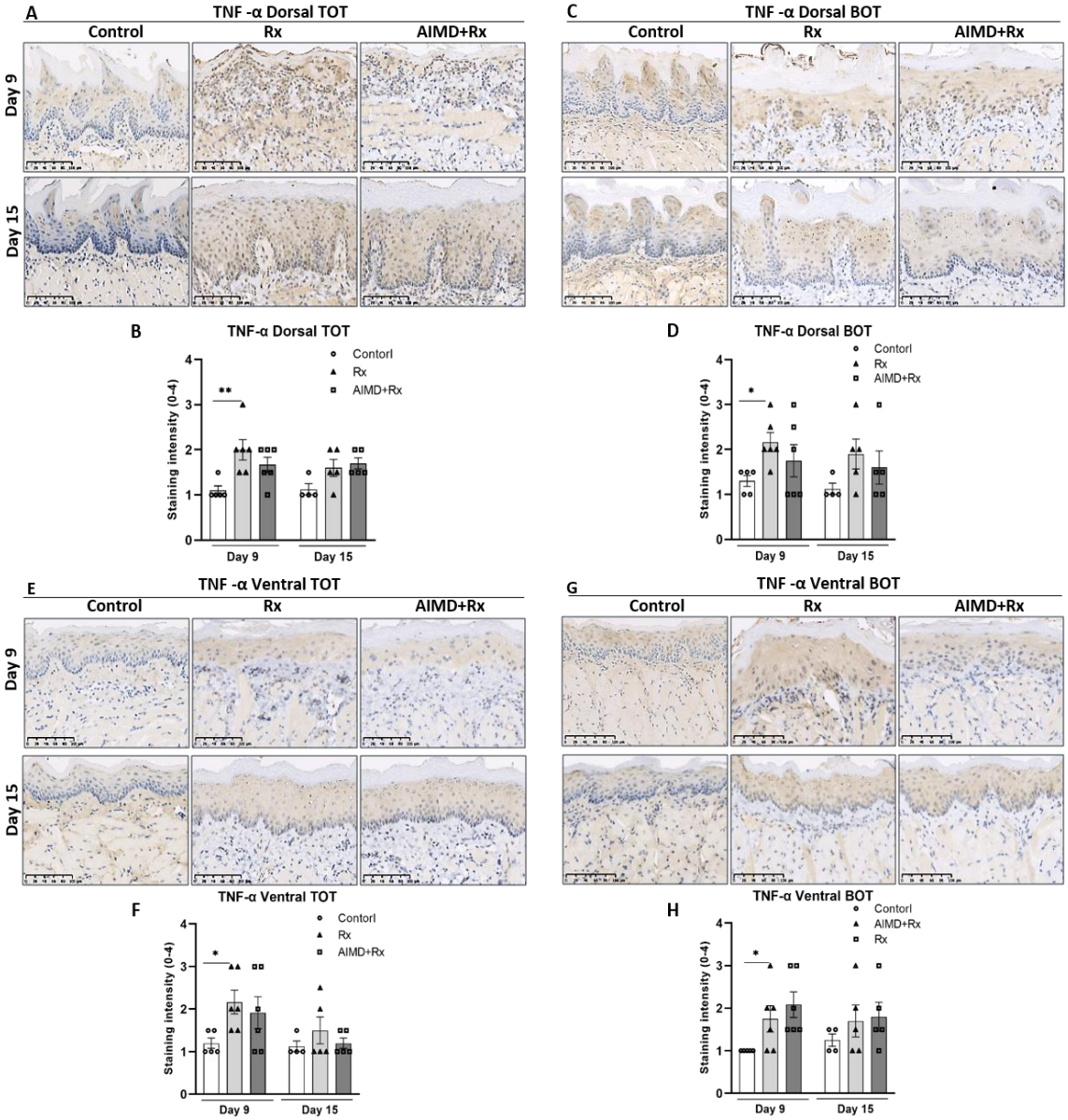


Figure S8: Changes in the levels of TNF-α in the dorsal and ventral tongue. Immunohistochemical staining of TNF-α in the dorsal TOT **(A)** and BOT **(C)** for Control, Rx, and AIMD+Rx groups on day 9 and day 15. TNF-α staining intensity of Rx on day 9 in TOT **(B)** and BOT **(D)** was higher in the AIMD+Rx and Control groups. TNF-α of the Rx group was reduced to normal levels by day 15. Immunohistochemical staining of TNF-α in the ventral TOT **(E)** and BOT **(G)** for Control, Rx, and AIMD+Rx groups on day 9 and day 15. TNF-α levels in ventral TOT **(F)** and BOT **(H)** of the Rx group were significantly higher than the Control group on day 9. Mixed-effects analysis followed by Bonferroni's multiple comparisons test. Data presented as mean ± SEM; *N= 4-6* per group**,** *P ≤ 0.05, **P ≤ 0.01; Scale bar: 100 µm.

Chapter Six

This chapter describes a clinical observational study aimed to assess the impact of gut microbiome composition on radiotherapy response and severity of radiotherapy-induced oral mucositis in head and neck cancer. This chapter is written in a manuscript style and is intended to be submitted to the journal of Radiotherapy and Oncology

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Statement of Authorship

Title of Paper	Association between Pre-Treatment Gut Microbiome and Radiotherapy Response and radiotherapy-induced oral mucositis in Patients with Head and Neck Cancer: A pilot study
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Publication Details	This is an original research paper. It is intended to be submitted to the journal of Radiotherapy & Oncology.

Principal Author

Name of Principal Author (Candidate)	Ghanyah Al-Qadami		
Contribution to the Paper	I am the first author and was responsible for designing and conducting the study and preparing, writing, and editing the manuscript.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/06/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Ysabella is a co-supervisor and helped with editing and revising the manuscript.		
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Contribution to the Paper	Kate helped with laboratory experiments, data analysis, editing, and revising the manuscript.		
Signature		Date	04/06/2021

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Contribution to the Paper	Hannah is PI for the PREDiCT Study, securing relevant ethical approvals and seed funding. She also helped with data analysis and editing and revising the manuscript.		
Signature		Date	22/06/2021

Name of Co-Author	Hien Le		
Contribution to the Paper	Hien is a co-supervisor and he helped with facilitating patient recruitment, data extraction, and editing and revising the manuscript.		
Signature		Date	22/06/2021

Chapter 6: Association between Pre-Treatment Gut Microbiome and Radiotherapy Response and radiotherapy-induced oral mucositis in Patients with Head and Neck Cancer: A pilot study

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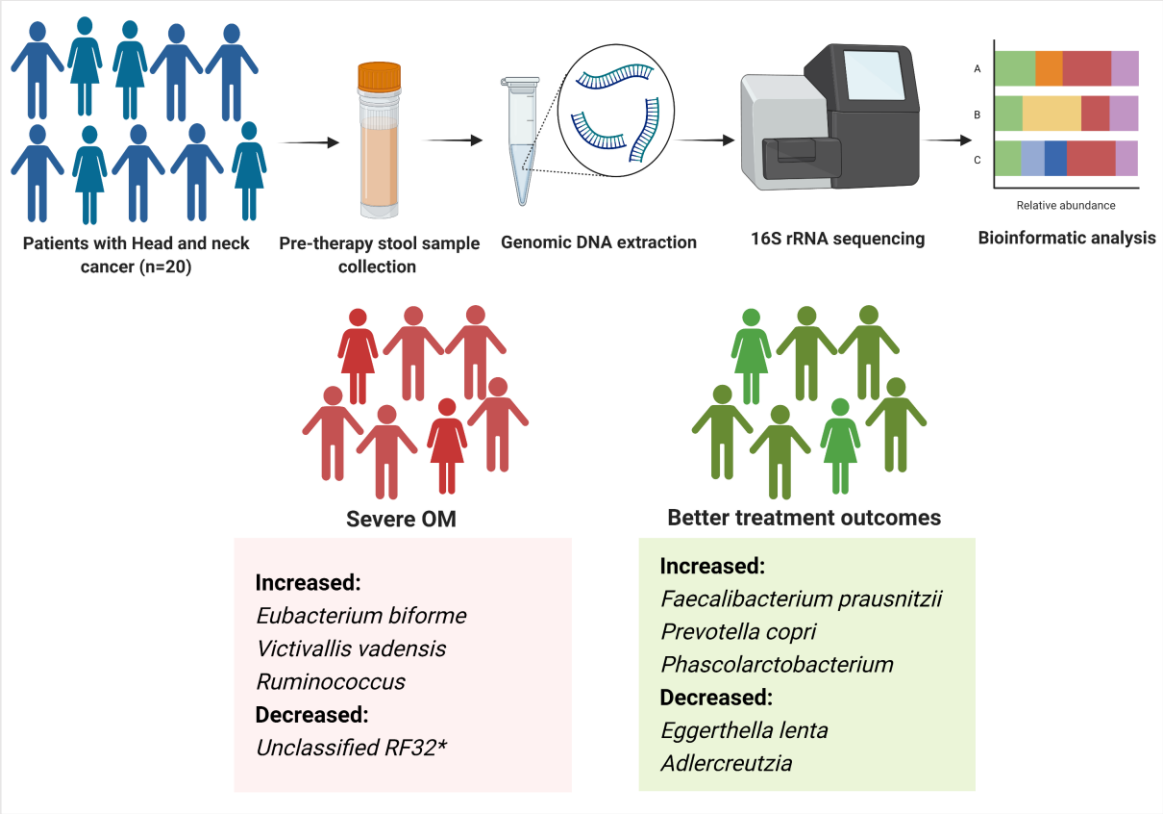
6.1 Graphical abstract



Graphical Abstract: Stool sample from 20 patients with head and neck cancer were collected before commencing treatment. Genomic DNA was extracted from stool samples. This was followed by bacterial 16S rRNA gene sequencing and bioinformatic analysis to determine the microbiome composition of each patient. Results showed that genera *Eubacterium* (*E. biforme* species), *Victivallis* (*V. Vadensis* species), *Ruminococcus* were enriched while unclassified *RF32* were decreased in severe (grade 3-4) oral mucositis. Favourable treatment outcome was associated with an increase in *Faecalibacterium prausnitzii*, *Prevotella copri*, and *Phascolarctobacterium* and decrease in the abundance of *Eggerthella lenta* and *Adlercreutzia*. *"Image created with BioRender.com."*

Keywords: Radiotherapy, Head and neck cancer, Oral mucositis, Inflammation, anti-tumour immune response, gut microbiome

6.2 Abstract

Introduction: Emerging evidence suggests that an individual's gut microbiota contributes to radiotherapy efficacy and adverse toxicities through the modulation of immune signalling and inflammatory responses. However, little is known about its role in the context of head and neck cancer (HNC). This study, therefore, aimed to assess the association between an individual's pre-therapy gut microbiome and i) severity of radiotherapy-induced oral mucositis (OM), and ii) response/recurrence outcomes in patients with HNC. **Methods:** In a prospective pilot study, 20 patients scheduled to receive radiotherapy or chemoradiotherapy were recruited from a single site. Pre-therapy stool samples were collected with microbial composition analysed using 16S rRNA gene sequencing. OM severity grades and treatment outcomes (tumour response and recurrence) were obtained from medical records and correlated to gut microbial composition. **Results:** Of the 20 participants, 80% were male with a median age of 66 years. Tumours were located in different primary sites including oral cavity (4), nasal cavity (2), oropharyngeal (5), salivary gland (6) and HN skin (3). Taxonomic analysis revealed that severe OM (Grade 3-4) was associated with a higher relative abundance of three bacterial genera, *Eubacterium*, *Victivallis*, and *Ruminococcus*. Furthermore, three bacterial genera with immunomodulatory properties, *Faecalibacterium*, *Prevotella*, and *Phascolarctobacterium*, were associated with favourable radiotherapy treatment outcomes. **Conclusions:** These pilot data suggest that a patient's unique pre-therapy gut microbiome composition influences OM severity and radiotherapy efficacy. Hence, it has the potential to be used as a biomarker to help predict how an individual may respond to radiotherapy, enabling personalised care through targeted supportive care or microbial modulation.

6.3 Introduction

Head and neck cancer (HNC) refers to a group of different tumour types that arise within the head and neck region [1]. Collectively, these cancers represent the sixth most common type of cancer with around 930,000 new cases, and more than 460,000 deaths reported worldwide annually [3]. Radiotherapy is a crucial treatment modality for the management of both early- and advanced-stage HNC [366]. Furthermore, it can be used for curative or palliative purposes either alone or in combination with other cancer treatments such as surgery and chemotherapy [35].

Currently, one of the key challenges of HNC radiotherapy is the heterogeneity in patient responses to treatment, tumour recurrence, and severity of impactful toxicities. Radiotherapy-induced oral mucositis (OM), characterised by inflammation and ulceration of the oral mucosa following radiotherapy, is one of the most common dose-limiting side effects in patients with HNC [72]. Curiously, the incidence and severity of OM vary greatly between patients [102], impacting decision-making and the provision of optimal supportive care. As such, it is critical we identify methods to predict patients at the greatest risk. Currently, patient-related factors (genetics, sex, age, smoking, and oral care), tumour-related factors (size, stage, and volume), and treatment-related factors (radiation dose, fractionation, site, delivery method, and combined therapies), while associated with OM, do not fully explain variation in OM severity and have limited effectiveness in predicting those at high risk of developing OM [367]. The same challenge impacts the anti-tumour response to radiotherapy, with the incidence of recurrence still largely unexplained [97, 368, 369]. Although several studies have investigated the use of a number of biomarkers, such as epidermal growth factor receptor, p53 mutations, and hypoxia markers, to predict radiotherapy prognosis in HNC, only human papillomavirus (HPV) has been incorporated in

clinical practice [101]. Generally, the current risk factors do not sufficiently explain the variation in tumour response and normal tissue toxicities. Therefore, there is a need for robust predictive biomarkers that can help identify those who will respond well to radiotherapy and those at higher risk of severe toxicities and recurrence in order to personalise treatment and optimise outcomes.

Recent years have witnessed a growing interest in the role of the gut microbiome (the collective genome of the vast array of microbes that colonise the gastrointestinal tract) in the efficacy and toxicity of cancer chemotherapy and immunotherapy [125, 363]. Although only a few studies have been conducted, the current evidence also suggests that the gut microbiome can influence radiotherapy anti-tumour response and severity of gastrointestinal toxicities [338, 351]. Based on the same biological underpinnings for radiotherapy, chemotherapy, and immunotherapy responses, it is, therefore, plausible to suggest that an individual's microbiome composition may influence response to radiotherapy. This is also supported by data that show the gut microbiome is implicated in the pathogenesis of radiotherapy-associated gastrointestinal mucositis [351]. Since specific gut microbiome signatures have been linked to certain disease states, recent research has been focusing on exploiting the baseline gut microbiome as a predictive marker for different conditions including cancer treatment efficacy and toxicities [370].

Currently, there is limited evidence on the role of the gut microbiome on radiotherapy response and toxicities in the context of HNC. It has been found that the use of antibiotics is associated with poor treatment outcomes among patients with locally advanced HNC treated with chemoradiotherapy [371]. Moreover, the consumption of probiotics has shown promising results in reducing the severity of OM among patients with HNC [340]. However, no study has investigated the association between the pre-therapy gut

microbiome and radiotherapy-induced OM severity or treatment outcomes in HNC. Nevertheless, a few studies have investigated the oral microbiome as a predictive marker for the risk of OM among patients with HNC [92, 93]. For instance, oral microbiota, obtained from mucosal swabs, from patients with nasopharyngeal carcinoma treated with radiotherapy, demonstrated that oral microbiome signatures post-treatment, but not pre-treatment, were associated with OM progression [93]. This indicates that while the oral microbiome may be impacted by or involved in OM, it was not a useful predictive marker.

Several challenges prevent the use of oral microbiome to predict the risk of OM. First, due to the presence of soft and hard surfaces, different sites of the oral cavity have unique microbial compositions and diversity [372], which impose sampling difficulty. Moreover, the presence of the tumour within the oral cavity could alter baseline microbial composition, thus, patients with different tumour primary sites will have different oral microbiome patterns, which will impact the consistency of the results. The gut microbiome is more stable than the oral microbiome and is not directly affected by HNC tumour sites. Moreover, the gut microbiome has been found to play a profound role in modulating the systemic inflammatory and immune response, and dysbiotic gut microbiome composition has been implicated in many extraintestinal inflammatory conditions at sites far from the gut [373]. Together, the gut microbiome could offer a better marker to predict OM severity and tumour response than the oral microbiome. This study, therefore, aimed to explore the association between the gut microbiome and the severity of radiotherapy-induced OM and treatment outcomes among patients with HNC.

6.4 Methods

6.4.1 Human research 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.

6.4.2 Patient recruitment and biospecimen collection

Patients were recruited from the Department of Radiation Oncology at the Royal Adelaide Hospital between October 2018 and December 2019. Patients who were adults (> 18 years), diagnosed with HNC and were scheduled to receive radiotherapy (alone or in combination with other therapies) were included in the study. Patients who had a past medical history of any chronic gastrointestinal disorder (inflammatory bowel disease or irritable bowel syndrome), intestinal symptoms (unrelated to cancer or treatment) at the time of recruitment, or had previous colonic surgery were excluded. Pre-therapy stool samples were collected by patients in Zymo research DNA/RNA Shield Faecal Collection Tubes (Zymo, USA) following kit instructions. Samples were collected prior to commencing radiotherapy and stored at −80°C.

6.4.3 Clinical data collection

Patients were provided with an induction survey, which collected patient information regarding demographics (age, sex, height, and weight), smoking status, alcohol intake, and medical history. Clinical data regarding tumour characteristics, treatment plan and delivery, radiotherapy toxicity, and response 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) functional scoring system (version 5.0)

[68], which grades OM as follows: 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. Tumour response was assessed by an experienced radiation oncologist using the Response Evaluation Criteria in Solid Tumours (RECIST 1.1) [374], which describes tumour response as either complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Patients were also followed for 12 months to assess tumour recurrence within 12 months after treatment completion.

6.4.4 Genomic DNA extraction

Frozen stool samples were allowed to thaw at room temperature and mixed by brief vortexing. To extract genomic DNA, 2 mL of Zymo tube content 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 separate tube (not discarded) while the faecal 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 faecal pellet and mixed by brief vortexing. To lyse bacteria cells, the faecal 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. Extracted DNA samples were eluted in 50 µl nuclease-free water. To increase the purity of extracted DNA,

samples were precipitated using ethanol and sodium chloride and resuspended in nuclease-free water, and stored at -20°C.

6.4.5 16S rRNA gene sequencing

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

- Forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
- Reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

The bioinformatics analysis of Illumina output was performed using Qiagen CLC Genomics Workbench 21.0.3. Briefly, pair-end reads were trimmed using the “Trim read” tool and filtered based on the number of reads. All samples had sufficient coverage (number of reads); hence, they were included in the Operational taxonomic units (OTUs) clustering analysis. Trimmed and filtered reads were mapped back to OTUs and taxonomy was assigned using Greengenes reference database (v13.8, 2013) with a minimum identity of 97%. To determine alpha diversity (within sample diversity), OTUs were aligned using MUSCLE. Then, phylogeny trees were reconstructed and the Shannon index was calculated. The beta diversity (between sample diversity) was assessed by performing the principal coordinates analysis (PCoA) on generalised UniFrac distances. Following that, PERMANOVA analysis was used to measure effect size and significance on 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/). The LDA score threshold for discriminative features was set as >2 and the pairwise Wilcoxon test Alpha value was set as 0.05.

6.4.6 Statistical analysis

The statistical analysis was performed using GraphPad prism 9. For quantitative data including age, body mass index (BMI), radiotherapy cumulative dose, number of fractions, and treatment period, unpaired T-test or Mann–Whitney test were used depending on the Gaussian distribution of the dataset. Fisher's exact test was used to analyse categorical data such as sex, smoking status, alcohol intake, antibiotic use, tumour site and stage, treatment type and intent, hospitalisation, and feeding tube insertion. A P-value of <0.05 was considered statistically significant.

6.5 Results

6.5.1 Patients' characteristics

A total of 20 patients with HNC were recruited during the study period. As shown in Table 1, the median age of patients was 65.5 years, and 80% of the patients in this cohort were males. 50% of patients had a BMI within the normal range [range 18.5-24.9], and 45% were overweight or obese. In this study, 80% of patients were born vaginally. 75% of patients were either smokers or ex-smokers, however, 85% reported drinking less than 10 drinks per week, which is within the recommended weekly alcohol intake in Australia. The major comorbidities were arthritis (35%), hypertension (30%), and gastroesophageal reflux (30%), and 80% of patients were on regular medications for different conditions. Finally, 40% reported receiving antibiotics within 2 weeks before radiotherapy treatment.

Patients presented with tumours in different sites within the HN region, including oral cavity (20%), oropharynx (25%), nasal cavity (10%), salivary glands (30%), and HN skin (15%). All salivary gland cancers were located in the parotid glands. The majority of the tumours were squamous cell carcinoma (SCC) with 20% HPV-positive. Half of the patients had early-stage disease (I/II) and the other half had late-stage disease (III/IV). Patients received different treatment regimens depending on tumour type, site, and stage. All patients completed the planned radiotherapy course except for one who discontinued treatment after completing two fractions of radiotherapy only, hence, they were not included in treatment-related analysis. The remaining 19 patients were treated with radiotherapy alone (31.6%), surgery followed by radiotherapy (47.4%), chemoradiotherapy (15.8%), or surgery followed by 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. Around 79% of the patients were treated for curative intent.

Among these 19 patients, two received palliative treatment (36 Gy; 6 Gy/F) over 2 weeks. Due to the low exposure, they were excluded from OM severity and treatment response analyses. All of the 17 patients included in the analysis developed some degree of OM. Based on the NCI-CTCAE functional OM scoring system, 17.7%, 35.3%, 29.4%, and 17.7%, had G1, G2, G3, and G4 OM respectively. Six patients (35.3%) had feeding tube insertion either before or during treatment. Moreover, 29.4% of patients required hospitalisation and 23.5% had treatment breaks or dose modification. This included a delay in the chemotherapy cycle due to thrombocytopenia, a break in radiotherapy due to gastroenteritis, early termination of radiotherapy due to discomfort during radiation or OM complications. The tumour response was assessed by post-therapy radiology scans. Since 2 patients did not have post-therapy scans, there were excluded from tumour response analysis. Among the 15 patients who had post-therapy scans, 13 patients (86.7%) had CR and 2 patients (13.3%) had PD. To assess tumour recurrence, we followed all 17 patients for 12 months after treatment completion. A total of 3 patients (17.6%) had tumour recurrence (including the 2 who had PD).

Table 1: Patients' characteristics and medical history

Basic characteristics	n	%
Age (median= 65.5)		
<55	5	25
55-65	5	25
>65	10	50
Sex		
Male	16	80
Female	4	20
BMI		
18.5-24.9	10	50
25-29.9	4	20
>30	5	25
Missing	1	5
Birth		
Caesarean	1	5
Vaginal	16	80
Unknown	3	15
Tobacco smoking status		
Non-smoker	5	25
Ex-smoker	12	60
Smoker	3	15
Alcohol use (standard drinks/week)		
≤10	17	85
>10	3	15
Comorbidities		
Diabetes mellitus (type 2)	1	5
Hypertension	6	30
Emphysema	1	5
Osteoarthritis	7	35
COPD	2	10
Gastroesophageal Reflux Disease	6	30
Mental health conditions	3	15
HIV	1	5
Previous cancer diagnosis	6	30
Antibiotics (2 weeks BT)		
Yes	8	40
No	12	60
Regular medications	16	80
Complementary medications	4	20
Had diarrhoea (2 weeks BT)	0	0
Had constipation (2 weeks BT)	5	25
Experienced nausea/vomiting (2 weeks BT)	1	5
Had a blood transfusion (4 weeks BT)	1	5
Had a vaccination (4 weeks BT)	1	5

Tumour site		
Oral cavity	4	20
Oropharynx	5	25
Nasal cavity	2	10
Salivary gland (Parotid gland)	6	30
HN skin	3	15
Tumour type		
SCC	19	90
BCC/SCC	1	5
ACC	1	5
Tumour stage		
I	7	35
II	3	15
III	2	10
IV	8	40
HPV+	4	20
Treatment type (n= 19)		
Radiotherapy alone	6	31.6
Postoperative radiotherapy	9	47.4
Chemoradiotherapy	3	15.8
Postoperative chemoradiotherapy	1	5.3
Cumulative dose (Gy; Mean ± SD)	58.62 ± 8.78	-
Fraction/dose (Mean ± SD)	2.53 ± 1.21	-
Treatment period (week; Mean ± SD)	5.53 ± 1.46	-
Radiotherapy delivery methods		
Volumetric modulated arc therapy	18	94.7
Electron beam therapy	1	5.3
Treatment intent		
Curative	15	78.9
Palliative	4	21.1
Oral mucositis (n= 17)		
G1	3	17.7
G2	6	35.3
G3	5	29.4
G4	3	17.7
Feeding tube	6	35.3
Hospitalisation	5	29.4
Mucositis/Worsening dysphagia/ feeding tube insertion	3	60
Respiratory tract infection	1	20
Gastroenteritis	1	20
Treatment gaps/breaks	4	23.5
Response (n=15)		
Complete response	13	86.7
Progressive disease	2	13.3
Recurrence within 12 months (n= 17)	3	17.6

SD, standard deviation; BT, Before treatment; SCC, squamous cell carcinoma*; BCC,* basal cell carcinoma*; ACC, Adenoid cystic carcinoma*

6.5.2 Association between the gut microbiome and patient characteristics

To characterise the gut microbiome of patients with HNC and assess the variation in composition based on different patient and tumour-related variables, we analysed the gut microbiome of all 20 patients recruited. Overall, at the genus level, the gut microbiome predominantly consists of eight microbial genera including *Bacteroides* (39.9%), unclassified *Ruminococcaceae* (7.4%), *Faecalibacterium* (6.8%), *Parabacteroides* (5.6%), unclassified *Lachnospiraceae* (4.8%), unclassified *Clostridiales* (4.6%), *Prevotella* (3.6%) and *Oscillospira* (2.6%) (Fig. 1A) The average number of positive OTUs was 603.9 [229 - 864 range] and the average Shannon diversity index value was 3.2 [1.3 - 4.1 range] (Fig. S1A & S2A).

To determine the effect of patient-related variables on gut microbiome composition, we compared the microbial richness, diversity, and differential features between patients based on sex, age, BMI, tobacco smoking, alcohol intake, comorbidities, antibiotic use, previous cancer, tumour site, and stage (Fig. 1B-H, S1A-M, S2A-N & S3A-H). Of these variables, 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 represented by Shannon diversity index (p= 0.0289). Moreover, male and female patients form distinctive gut microbiome clusters represented by generalised UniFrac distances (p= 0.0052) (Fig. 1B-D). LEfSe analysis showed that five bacterial genera mainly *Prevotella* and *Phascolarctobacterium* were enriched in men while unclassified lactobacillales and *P-75-a5* genera were differentially increased in women (Fig. 1E).

Although there was no significant difference in the OTUs richness, alpha, and beta diversity between patients when divided based on patient-related factors other than sex, certain

bacterial genera were found to be enriched in a specific subgroup of patients. For instance, some genera were enriched in different age groups; <50 (*Faecalibacterium*, *Anaerococcus*), 55-65 (*Paraprevotella*), and >65 (*Ruminococcus-2*, *Akkermansia*, *Adlercreutzia*) (Fig. 1F). Furthermore, patients with cutaneous tumours had significantly increased abundance of unclassified RF32 while *SMB35* genus was found to be increased in abundance among patients with salivary gland tumours (Fig. 1G). In terms of tumour stage, *Phascolarctobacterium* was differentially 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 the pre-therapy microbiota of 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 BMI, smoking, alcohol intake, antibiotic use, previous cancer diagnosis, and comorbidities (hypertension, arthritis, and constipation) were also observed (Fig. S3A-H).

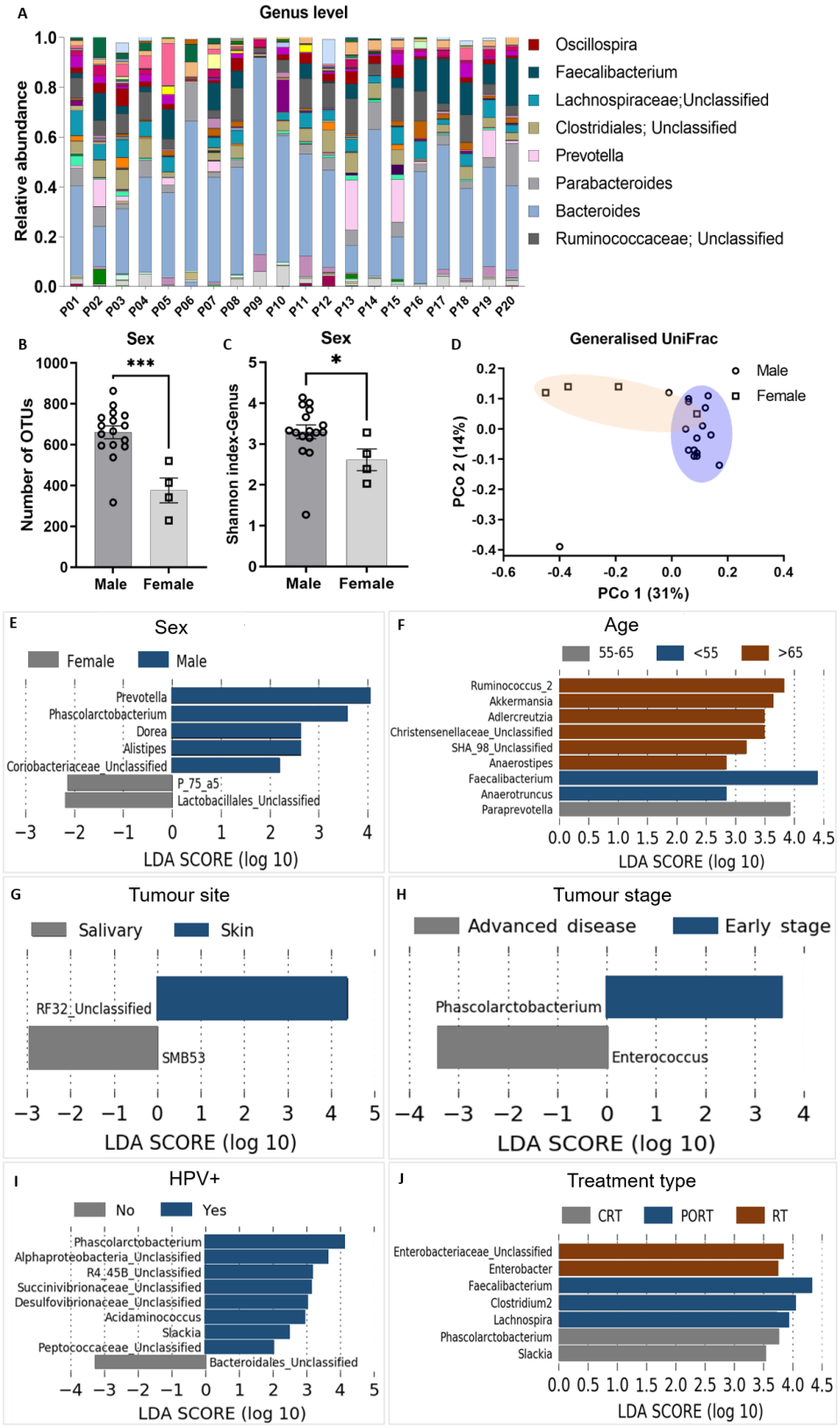


Figure 1: Overview of the gut microbiome composition of patients with HNC. **A)** The relative abundance of gut microbiome of all patients at genus level. **B-C)** Male patients had significantly higher number of OTUs (Unpaired t-test) and higher alpha diversity (Mann Whitney test) than female patients. **D)** Female patients have distinctive microbial pattern compared to males. The differential change in microbial genera based on 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.

6.5.3 Demographic factors impacting radiotherapy-induced oral toxicities

Three patients were excluded from OM severity analysis (1 patient completed 2/30 fractions and 2 patients received low dose palliative radiotherapy for two weeks). We first assessed the impact of patient and treatment-related factors (age, sex, BMI, tobacco, alcohol intake, antibiotic use, tumour site and treatment type, cumulative dose, and period) on the severity of OM. Patients were divided into two groups based on the severity of OM; mild/moderate (G1-2) or severe OM (G3-4). As shown in Table 2, the average age of patients with G1-2 OM was 67.89 years compared to 62.13 years for patients with G3-4 OM and the difference in age was not statistically significant between groups. Moreover, there was no statistical difference between the groups in BMI with an average BMI of 28.62 and 25.54 for the G1-2 and G3-4 groups respectively. In this cohort, there was no effect of sex, tobacco, or alcohol intake, or antibiotics on OM severity. Tumours located in the oral cavity or oropharynx have been associated with more severe OM. In this study, 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. Radiotherapy cumulative dose and treatment period did not have a significant impact on OM severity, 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 2: Risk factors of OM severity among patients

	G1-2 (Mild/Moderate) (n=9)	G3-4 (Severe) (n=8)	**P value**
Age (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 (Mean ± SD)	59.89 ± 4.26	62.84 ± 4.47	0.184
Treatment period (Mean ± SD)	5.78 ± 0.67	6.13 ± 1.13	0.445

*SD, standard deviation; B/D, Before or during radiotherapy; Unpaired T-test; Fisher's exact test; * p< 0.05*

6.5.4 Gut microbiome impact on radiotherapy-induced oral mucositis

There were variable links observed between OM and gut microbiome composition in patients. As shown in Fig. 2A, the eight most abundant genera among patients with G1-2 OM were *Bacteroides* (40%), *Parabacteroides* (7.8%), *Faecalibacterium* (6.9%), *unclassified Ruminococcaceae* (6.8%), unclassified *Clostridiales* (4.7%), unclassified *Lachnospiraceae* (4.1%), unclassified RF32 (3.4%) and *Prevotella* (2.8%). Whereas, in the G3-4 OM group the most abundant genera were *Bacteroides* (41.9%), *Faecalibacterium* (7.9%), unclassified *Ruminococcaceae* (7.2%), *Prevotella* (5.5%), unclassified *Lachnospiraceae* (4.2%), unclassified *Clostridiales* (3.9%), *Parabacteroides* (3.6%) and unclassified *Barnesiellaceae* (3.2%) (Tabel S1). Although there was no significant difference in the OTUs richness (p= 0.475) (Fig. S4A), alpha diversity (p= 0.781) (Fig. S4B), and beta diversity (p= 0.712) (Fig. S4C) between G1-2 and G3-2 groups, LEfSe analysis demonstrated that six bacterial genera (*Eubacterium*, *Victivallis*, *Ruminococcus*, *Oxalobacter*, unclassified *Victivallaceae,* and unclassified *desulfovibrionaceae*) were differentially increased among those patients with G3-4 OM. Furthermore, three genera (unclassified RF32, *Alistipes,* and unclassified ML615J-28) were increased in the G1-2 OM group (Fig. 2B) (Table S4, S5).

Among the six genera enriched in the G3-4 OM group, *Eubacterium*, *Victivallis,* and *Ruminococcus* showed the most significant association with OM severity. 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. I-J).

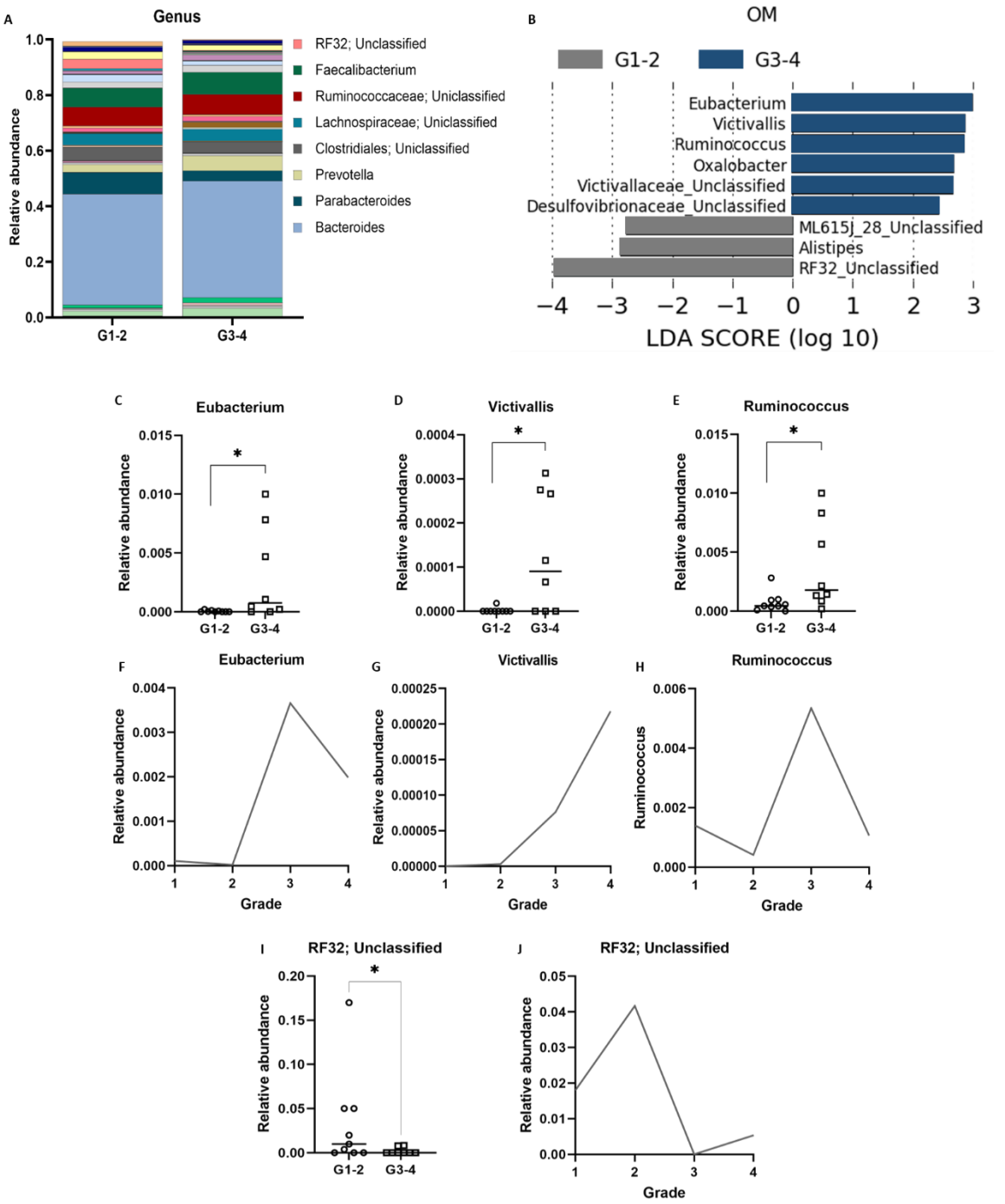


Figure 2: Association between gut microbiome composition and OM severity. **A)** The relative abundance of gut microbiome of G1-2 and G3-4 OM groups at genus level. **B)** The differential change in microbial genera based on OM grade. The relative abundance of *Eubacterium* (**C**), *Victivallis* (**D**), and *Ruminococcus* (**E**) was significantly higher in G3-4 group compared to G1-2 group. **F-H)** Change in average relative abundance of *Eubacterium* (**F**)*, Victivallis* (**G**) *and Ruminococcus* (**H**) according to change in OM severity grade. **I)** The relative abundance of *unclassified RF32* was significantly higher in G1-2 group compared to G3-4 group. **J)** Change in average relative abundance of *unclassified RF32* according to change in OM severity grade. LDA; Linear discriminant analysis. *P ≤ 0.05. Mann Whitney test; line in C, D, E, and J represents the median.

6.5.5 Demographic factors associated with treatment outcomes

15 patients were included in treatment response analysis while 5 patients were excluded (one completed 2/30 fractions, two received low palliative radiation doses and two did not have radiology scans to assess response). Among the 15 patients, 13 patients had CR while 2 patients had PD. No patient was reported to have PR or SD. In this cohort, there was no significant association between any of the patient-related factors and treatment response (Table 3). Both patients with PD were >65 years old. Moreover, 1 out of the 2 female patients and 1 out of the 13 male patients had PD. There was no effect of alcohol intake, smoking, or antibiotic use in response outcomes. Among the two patients who developed PD, one had oropharyngeal cancer and one had salivary gland cancer. Patients with PD had advanced-stage disease. They were treated with radiotherapy only and received similar radiotherapy doses and fractions over a similar treatment period compared to the CR group. Both patients who developed PD experienced unplanned treatment interruption compared to 2 out of the 13 patients who had CR, but this difference was not statistically significant ($p= 0.057$).

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

Table 3: Patient and treatment-related factors impacting treatment response

	CR (n=13)	PD (n=2)	P value	No REC (n=14)	REC (n=3)	P value
Age (Year; mean ± SD)	64.31 ± 10.70	69.50 ± 1.12	0.519	63.57 ± 10.65	72.67 ± 5.69	0.178
Sex, n (%)						
Male	11 (91.7)	1 (8.3)	0.371	12 (92.3)	1 (7.7)	0.121
Female	2 (66.7)	1 (33.3)		2 (50)	2 (50)	
BMI (Mean ± SD)	28.55 ± 4.75	20.76 ± 0.00	0.140	28.09 ± 4.88	21.56 ± 1.12	0.088
Smoking, n (%)						
Non-smoker	3 (75.0)	1 (25.0)	0.476	3 (75.0)	1 (25.0)	>0.999
Ex-smoker/Smoker	10 (90.9)	1 (9.1)		11 (84.6)	2 (15.4)	
Alcohol (# drinks/week), n (%)						
≤10	11 (84.6)	2 (15.4)	>0.999	11 (78.6)	3 (21.4)	>0.999
>10	2 (100)	0		3 (100)	0	
Antibiotics (B/D radiotherapy), n (%)						
Yes	8 (100)	0	0.200	8 (100)	0	0.206
No	5 (71.4)	2 (28.6)		6 (66.7)	3 (33.3)	
Tumour site, n (%)						
Oral cavity	2 (100)	0	-	3 (100)	0	-
Oropharynx	4 (80)	1 (20)		4 (80)	1 (20)	
Nasal cavity	1 (100)	0		1 (50)	1 (50)	

Salivary gland	4 (80)	1 (20)		4 (80)	1 (20)	
HN skin	2 (100)	0		2 (100)	0	
Tumour stage, n (%)						
Early stage (I/ II)	7 (100)	0	0.467	8 (88.9)	1 (11.1)	0.577
Advanced disease (III/ IV)	6 (75)	2 (25)		6 (75)	2 (25)	
HPV+, n (%)	4 (100)	0	-	4 (100)	0	-
Treatment type, n (%)						
Radiotherapy	9 (81.8)	2 (18.2)	>0.999	10 (76.9)	3 (23.1)	0.541
Chemoradiotherapy	4 (100)	0		4 (100)	0	
Cumulative dose (Gy; mean± SD)	61.98 ± 3.68	58.00 ± 11.31	0.288	61.41 ± 3.39	60.67 ± 9.24	0.665
Dose/Fraction (Gy/F)	2.14 ± 0.24	2.10 ± 0.14	>0.999	2.11 ± 0.23	2.13 ± 0.12	0.337
Treatment period (week; mean ± SD)	5.92 ±0.76	5.50 ± 2.12	0.657	6.00 ± 0.78	5.67 ± 1.51	0.941
Treatment intent, n (%)						
Curative	12 (92.3)	1 (7.7)	0.257	13 (86.7)	2 (13.3)	0.331
Palliative	1 (50)	1 (50)		1 (50)	1 (50)	
Treatment gaps/breaks, n (%)						
Yes	2 (50)	2 (50)	0.057	2 (50)	2 (50)	0.121
No	11 (100)	0		12 (92.3)	1 (7.7)	

CR, complete response; PD, progressive disease, REC: recurrence; SD, standard deviation; B/D, Before or during; Unpaired T-test; Fisher's exact test

6.5.6 Gut microbiome impact on radiotherapy outcomes

To assess the association between the gut microbiome and radiotherapy outcomes in terms of tumour response, the microbial composition of patients was compared between those patients who had CR (n=13) or PD (n=2). As shown in Fig. 3A, the most abundant genera in the CR group were *Bacteroides* (40.6%), *Faecalibacterium* (7.9%), *unclassified Ruminococcaceae* (6.9%), *Parabacteroides* (6.6%), *Prevotella* (4.5%), *unclassified Lachnospiraceae* (4.1%), unclassified *Clostridiales* (4%) and *Sutterella* (2.7%) (Table S2). While the most abundant genera in PD group were *Bacteroides* (45.4%), *unclassified Ruminococcaceae* (7.1%), *Blautia* (6.7%), *unclassified Clostridiales* (6.6%), *Akkermansia* (5.2%), *unclassified Barnesiellaceae* (4.4%), *unclassified Lachnospiraceae* (3.0%) and *Parabacteroides* (3%) (Fig. 3A) (Table S3). There was no significant difference in the number of OTUs (p= 0.948), alpha (p= 0.994), and beta diversity (p= 0.160) between CR and PD groups (Fig. S5A-B). The PCoA analysis showed that PD microbial composition clusters formed a distinctive microbial pattern, however, this pattern was not prominent due to the small sample size in this group of patients (Fig. 3B). The LEfSe analysis identified four genera (*Megasphaera*, *p-75-a5*, unclassified SHA-98*,* and *Desulfitobacter*) to be differentially increased in the PD group compared to the CR group (Fig. 3C) (Table S4, S5). However, the relative abundance of *unclassified SHA-98* only was significantly higher in PD compared to the CR group (p= 0.038) (Fig. 3D). There was no significant difference in the relative abundance of other genera (*Megasphaera*, *p-75-a5*, and *Desulfitobacter*) between CR and PD groups (Fig. S5C-E).

Then we compared the microbiome composition between patients who did not develop recurrence (n=14) and those who had recurrence (n=3) within 12 months following treatment completion. Fig. 3E shows that the most abundant genera among patients with

no recurrence were *Bacteroides* (39%), *Faecalibacterium* (8.9%), *unclassified Ruminococcaceae* (7.2%), *Parabacteroides* (5.9%), *Prevotella* (4.9%), *unclassified Lachnospiraceae* (4.4%), *unclassified Clostridiales* (3.9%) and *Sutterella* (2.4%). While those who developed recurrence had a microbial composition dominated by *Bacteroides* (50%), *unclassified Clostridiales* (6.4%), unclassified *Ruminococcaceae* (6.0%), Parabacteroides (5.7%), *Blautia* (4.5%), unclassified *Barnesiellaceae* (3.9%), *Akkermansia* (3.5%) and unclassified *Lachnospiraceae* (3%) (Fig. 3E). Similar to tumour response, there was no significant difference in the number of positive OTUs (p= 0.454), alpha (p= 0.511), and beta diversity (p=0.056) between recurrence and no recurrence groups (Fig. S5F-G). The microbial composition of the recurrence group tends to cluster together as determined by the generalised UniFrac distances plot (Fig. 3F). Three bacterial genera (*Faecalibacterium*, *Prevotella,* and *Phascolarctobacterium*) were found to be differentially increased in patients who did not develop recurrence. Whereas six bacterial genera (*Adlercreutzia*, *Pseudoramibacter_Eubacterium*, *Desulfitobacter*, *Eggerthella*, *Megasphaera,* and *p-75-a5*) were differentially increased in patients who developed recurrence (Fig. 3G) (Table S4, S5). The relative abundance of *Faecalibacterium* (p= 0.029), *Prevotella* (p= 0.031), *Phascolarctobacterium* (p= 0.019) were significantly higher in the no recurrence group compared to those in the recurrence group (Fig. 3H-J). Furthermore, the *Prevotella to Bacteroides* (P/B) ratio was significantly higher in those patients with no recurrence (n= 0.047) (Fig. 3K). Conversely, the relative abundance of *Adlercreutzia* (p= 0.006) and *Eggerthella* (p= 0.006) genera were significantly higher in the recurrence group than the no recurrence group (Fig. 3L-M). There was no significant difference between recurrence and no recurrence groups in the relative abundance of other genera (Fig. S5H-K).

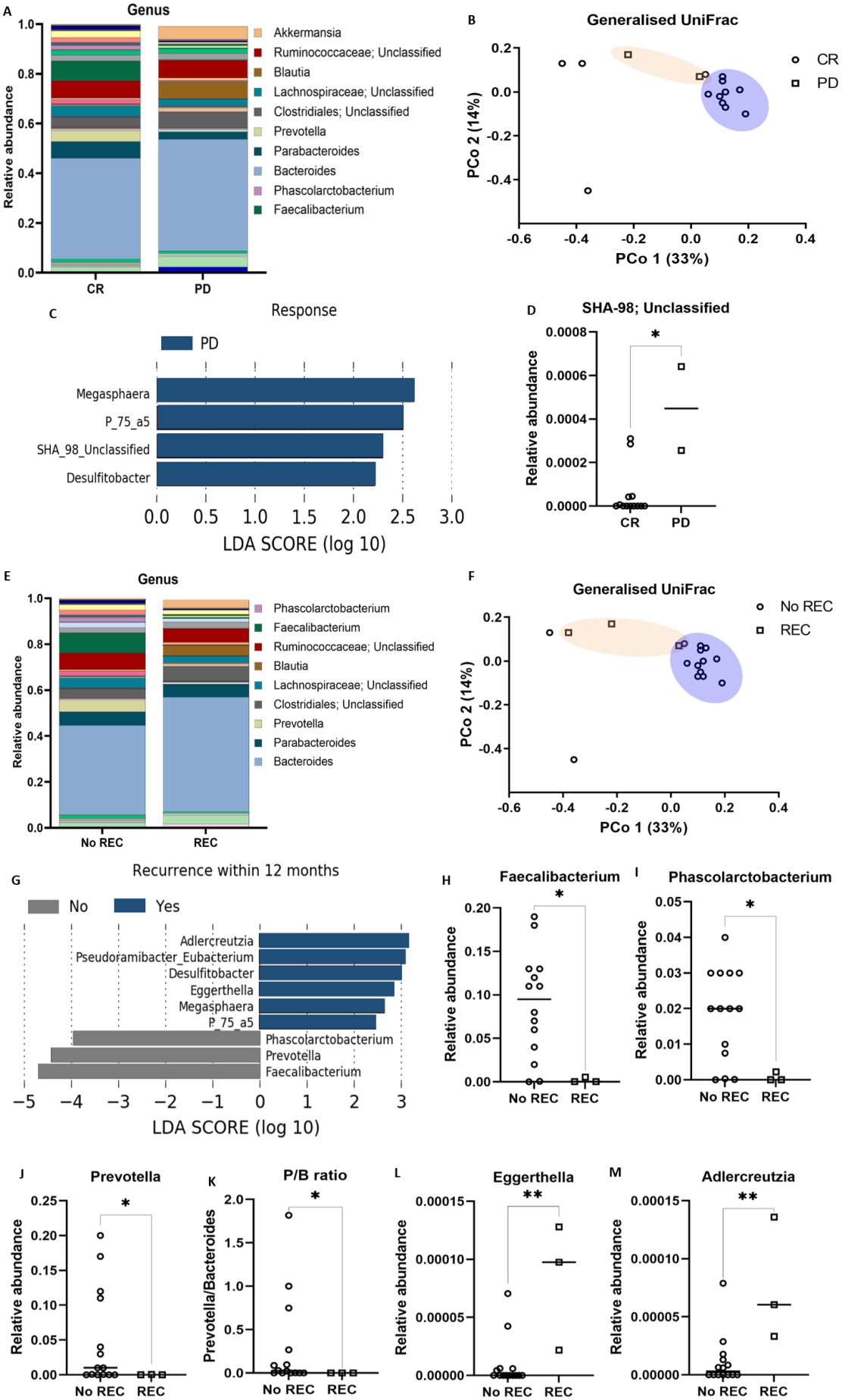

A
Genus
Relative abundance
CR
PD
Akkermansia
Ruminococcaceae; Unclassified
Blautia
Lachnospiraceae; Unclassified
Clostridiales; Unclassified
Prevotella
Parabacteroides
Bacteroides
Phascolarctobacterium
Faecalibacterium
B
Generalised UniFrac
PCo 2 (14%)
PCo 1 (33%)
CR
PD
C
Response
PD
Megasphaera
P_75_a5
SHA_98_Unclassified
Desulfitobacter
LDA SCORE (log 10)
D
SHA-98; Unclassified
Relative abundance
CR
PD
E
Genus
Relative abundance
No REC
REC
Phascolarctobacterium
Faecalibacterium
Ruminococcaceae; Unclassified
Blautia
Lachnospiraceae; Unclassified
Clostridiales; Unclassified
Prevotella
Parabacteroides
Bacteroides
F
Generalised UniFrac
PCo 2 (14%)
PCo 1 (33%)
No REC
REC
G
Recurrence within 12 months
No
Yes
Adlercreutzia
Pseudoramibacter_Eubacterium
Desulfitobacter
Eggerthella
Megasphaera
P_75_a5
Phascolarctobacterium
Prevotella
Faecalibacterium
LDA SCORE (log 10)
H
Faecalibacterium
Relative abundance
No REC
REC
I
Phascolarctobacterium
Relative abundance
No REC
REC
J
Prevotella
Relative abundance
No REC
REC
K
P/B ratio
Prevotella/Bacteroides
No REC
REC
L
Eggerthella
Relative abundance
No REC
REC
M
Adlercreutzia
Relative abundance
No REC
REC

Figure 3: Association between gut microbiome composition and treatment outcomes. **A)** The relative abundance of gut microbiome of CR and PD groups at genus level. **B)** The PCoA (generalise UniFrac distance) of CR and PD groups. **C)** The LEfSe analysis showing the differential genera increased in PD group. **D)** The relative abundance of unclassified *SHA-98* was significantly higher in PD group compared to CR group. **E)** The relative abundance of gut microbiome of No REC and REC groups at genus level. **F)** The PCoA (generalise UniFrac distance) of No REC and REC groups. **G)** The LEFSE analysis showing the differential genera increased in No REC and REC groups. **H-K)** The relative abundance of *Faecalibacterium* (**H**), *Phascolarctobacterium* (**I**), *Prevotella* (**J**), and P/B ratio (**K**) was significantly higher in the No REC group compared to REC group. **L-M)** The relative abundance of *Eggerthella* (**L**) and *Adlercreutzia* (**M)** was significantly higher REC group compared to No REC group. LDA; Linear discriminant analysis. *P ≤ 0.05; **P ≤ 0.001; Mann Whitney test; line in D, H, I, J, K, L and M represents the median. CR, complete response; PD, progressive disease; REC, recurrence.

6.6 Discussion

Radiotherapy is a critical part of HNC management. Despite the technological advances in radiotherapy in recent years, variability in radiotherapy outcomes and severity of radiotherapy-induced toxicities, mainly OM, remains a key challenge. Identifying patients who will benefit most from radiotherapy and the ability to predict patients at high risk of severe toxicities is essential to personalise and tailor treatment for each patient to achieve optimal treatment outcomes. Since the baseline gut microbiome has been linked to cancer chemotherapy and immunotherapy efficacy and toxicities, it also may provide a new tool for predicting radiotherapy response and toxicities. This study, therefore, aimed to assess whether the baseline gut microbiome can be used as a predictive marker of radiotherapy-induced OM or radiotherapy outcomes in HNC.

We first characterised the gut microbiome of all patients. Overall, 92% of the gut microbiome belongs to Bacteroidetes (56.4%) and Firmicutes (35.6%) phyla. At the genus level, *Bacteroides* was the most abundant genus across all patients. These results are relatively comparable to what has been reported previously. A Canadian study by Oliva et al. analysed the gut microbiome of 22 patients with HPV+ oropharyngeal cancer and found that the Bacteroidetes phylum and *Bacteroides* genus dominated patient gut microbiomes [375]. Similarly, Bai et al. analysed the gut microbiome of 13 patients with HNC in the USA and found that the gut microbiome was predominantly composed of Firmicutes (43.1%) and Bacteroidetes (43.8%) with *Bacteroides* the most abundant genus [376]. However, the abundance of Bacteroidetes was higher and Firmicutes was lower in our study compared to Bai et al. study. The characteristics of the patients in both of these studies were relatively similar to our study as the majority of them were male, with an age ranging between 47-76 years, and had a smoking history [375, 376]. The variation in the percentage of the two

most abundant phyla between reported in our and Bai et al. study could be due to the sample collection and storage kit or other features characteristic of the local region (including race, diet, environmental exposures). For instance, Olive et al. used the same collection kit as we used in our study, which yields more similar results to the present study. The overall microbial composition of this cohort of HNC patients is similar to healthy individuals [377]. Bacteroidetes and Firmicutes represent 90% of the gut microbiome in healthy people, with *Bacteroides* and *Faecalibacterium* representing the most abundant genera in the Bacteroidetes and Firmicutes phylum, respectively [377]. In the current study, we did not include a healthy control cohort so there might be a compositional difference between healthy controls and patients with HNC that were not identified. Therefore, future studies should compare the composition of the gut microbiome of patients with HNC to those of age and gender-matched healthy controls. Utilising available 16s rRNA gene sequence data from the Human Microbiome Project could provide a potential approach for the preliminary analysis of patients' gut microbiome compared to healthy individuals. However, considerations should be given to confounding factors such as age, sex, race, geographical location, diet, and other factors that may affect the gut microbiome.

To assess if there is a difference in the gut microbiome based on patient demographics, we assessed the number of observed OTUs, alpha and beta diversity, and differential microbes between groups according to the different demographic variables. Interestingly, sex was the strongest factor associated with gut microbiome composition, with male patients having higher OTUs richness and alpha diversity. The difference in microbial richness, diversity, and composition between males and females has been reported previously [378]. In addition to compositional differences, women have generally been reported to have higher microbial diversity than men [378]. However, in the present study, the female

subjects had an older age (average of 72 years), therefore, the reduction in the microbial richness and diversity might be due to postmenopausal hormonal changes [379]. It has been reported that postmenopausal women have lower microbial diversity and different composition changes due to oestrogen deficiency [379]. The other factors did not affect the richness and diversity of the gut microbiome, however, some compositional differences in the abundance of specific bacteria were associated with factors such as age, tumour site, and stage, antibiotic use, HPV status, and treatment type. Interestingly, although 40% reported receiving antibiotics two weeks before sample collection, there was no significant difference in their microbial diversity compared to those who did not receive antibiotics. We did however notice that the patient with the lowest alpha diversity had received intravenous antibiotics to treat a neck lump before cancer diagnosis. Unfortunately, data regarding the type, dosage, or duration of antibiotics was not available in this cohort, and all these factors influence changes in the gut microbiome after antibiotics. Overall, the present findings suggest that, in this cohort, sex can impact the microbial diversity and richness while other demographics are only associated with compositional changes in the relative abundance of certain types of microbes.

OM is one of the most frequent and troubling acute toxicities of radiotherapy in HNC. In line with previous studies, all patients treated with radiotherapy developed a varying degree of OM with around half of them progressing to severe (G3-4) OM before the end of treatment. In this study, patient and treatment-related risk factors of OM showed no significant impact on its severity, except for treatment type. Patients treated with concurrent chemoradiotherapy had significantly more severe OM than those treated with radiotherapy only. An increase in OM incidence and severity in patients treated with chemoradiotherapy has been reported consistently in previous studies [72]. Some other

factors such as female sex, low BMI, and smoking history have been associated with increased risk of OM [367]. Although this association was not observed in the current study, it is likely due to the small sample size. Overall, among the existing patient and treatment-related risk factors, only treatment type was associated with the severity of OM in the current cohort. This indicates that these other factors have limited ability to predict the severity of OM and thus there is a need for more efficient predictive markers for OM risk and severity.

To assess whether the pre-treatment gut microbiome can be used to predict the severity of OM, we assessed the association between the pre-therapy gut microbiome and OM severity grades. Overall, there was no difference between patients with mild/moderate or severe OM in both the microbial richness, represented by the number of observed OTUs and alpha diversity represented by the Shannon index. However, LEFSe analysis showed enrichment of six bacterial genera in the severe OM group and three in the mild/moderate OM group. Among microbes enriched in the severe OM group, *Eubacterium*, *Victivallis*, and *Ruminococcus* genera were the most significantly increased. *Eubacterium* is a phylogenetically diverse genus of gram-positive anaerobic bacteria belonging to different bacterial families within the Firmicutes phylum. The genus of interest in this study is the *Eubacterium* of the *Erysipelotrichaceae* family. *Eubacterium biforme (E. biforme)* is one of the main species within *Eubacterium* [380]. *Eubacterium* genus was reclassified in 2014 as *Holdemanella* and *E. biforme* as *Holdemanella biformis (H. biformis)* [381]. Since we used the Greengenes database (v13.8) released in 2013, we refer to it here as *E. biforme.* In the present study, the relative abundance of the *Eubacterium* genus *(E. biforme* species*)* was significantly increased in patients with severe OM. Both beneficial and detrimental effects of the enrichment of this bacterial genus have been reported. On one hand, it has been

associated with reduced dextran sulfate sodium-induced colitis in a mouse model [382]. Pujo et al. showed that *E. biforme* can produce significant levels of C18-3OH, a free long-chain fatty acid with potential anti-inflammatory properties. The oral administration of C18-3OH reduced colitis severity in mice [382]. However, other studies have reported that an increase in this genus is associated with severe cystic fibrosis [383], nonalcoholic steatohepatitis [384], worse clinical outcomes of traumatic injury [385], irritable bowel syndrome [386], and HIV infection [387]. Moreover, in vitro studies, incubating 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 (IL-10) ratio as compared to incubating cells with lipopolysaccharides or three other bacterial species, suggesting a pro-inflammatory property of this species [387].

Another genus associated with severe OM was *Victivallis. Victivallis* genus is a gram-negative anaerobic bacterium belonging to the *Victivallaceae* family of the *Lentisphaerae* phylum. It is the only genus of the *Victivallaceae* family and includes one well-characterised species, *Victivallis vadensis* [388]. 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 some inflammatory and immune-related pathological conditions such as colorectal cancer [389], Hashimoto's thyroiditis [390] and cerebral ischemic stroke [391]. Although this bacterium is present in a low abundance (less than 0.0003 of the relative abundance proportion), the detection rate (OTUs>0) was 62.5% (5/8 patients) in G3-4 compared to only 11.1% (1/9 patients) in the G1-2 group. This suggests that *Victivallis* may contribute to OM severity despite its low abundance and warrants further investigation.

Ruminococcus genus was also increased in the severe OM group. It is a polyphyletic genus of strictly anaerobic gram-positive cocci belonging to the *Ruminococcaceae* and *Lachnospiraceae families* [392]. *Ruminococcus genus of interest in this study belongs to the Lachnospiraceae family.* Recently. it has been proposed to reclassify it as *Mediterraneibacter* to distinguish it from *the Ruminococcus genus of the Ruminococcaceae* family [393]. It comprises five species mainly *R. gnavus*, *R. torques,* and *R. gauvreauii* [393]. Both *R. gnavus* and *R. torques* are mucolytic species that have been linked to the pathogenesis of chronic inflammatory conditions such as inflammatory bowel disease [394]. *R. gnavus* can also secrete a pro-inflammatory polysaccharide, which induces the production of TNF-α cytokine through the toll-like receptor (TLR4) dependent pathway; hence, contributing to the pathogenesis of Crohn's disease [395]. The role of *R. gauvreauii* in inflammatory responses is yet to be investigated, however, one study has demonstrated that this species is beneficial in the context of cardiovascular diseases [396]. Cumulatively, the current evidence suggests that mucolytic and pro-inflammatory species within the *Ruminococcus* genus could contribute to radiotherapy-induced OM potentially through reduction of the mucus layer and enhancing systemic inflammation. The main common features between the three genera enriched in the severe OM group are that they are strictly anaerobic gram-positive cocci (except for *Victivallis* which is gram-negative). Moreover, species within these genera have been reported to have pro-inflammatory properties. Further studies are needed to validate the association between these bacterial genera and OM severity and to determine the mechanism by which these microbes may influence OM pathogenesis.

Among the compositional changes observed is the differential increase of three bacterial genera in patients with mild/moderate OM. Unclassified RF32 was the most abundant

genus in the mild/moderate OM group. Since this genus was increased in patients with tumour primary site in HN skin, and all developed mild/moderate OM, we believe that this genus may actually be associated with tumour site, not OM severity. Although the LEFSe analysis revealed the *Alistipes*, and unclassified ML615J-28 were enriched in the G1-2 compared to G3-4 OM, the comparison of relative abundance did not yield a significant difference between groups. Therefore, in this study, we did not identify any bacterial taxa to be associated specifically with mild/moderate OM.

The gut microbiome may play a role in the tumour response to cancer treatments including radiotherapy. Therefore, we evaluated the association between the gut microbiome and treatment outcomes in terms of tumour response and recurrence. First, we assessed the effect of patient- and treatment-related factors known to influence treatment outcomes. All of these factors did not have a significant impact on tumour response or recurrence. Among patients included in the analysis, only 2 patients had PD and 3 patients had recurrence within 1 year after treatment completion. No patients were classified as PR or SD. Then, we correlated the gut microbiome and treatment outcomes. There was no difference in microbial richness and diversity between CR and PD groups. However, four low abundance bacterial genera were found to be differentially increased in the PD group. Among these is unclassified SHA-98 (Firmicutes phylum), which was detected in 100% (2/2) of PD and 38.5% (5/13) of the CR group. Given that only two patients had PD, these results might not be representative of the larger population. Moreover, here, we analysed the response of 15 patients out of 17 patients who received the planned radiotherapy dose. Excluding these patients may alter the differential features associated with tumour response. Therefore, the impact of the gut microbiome in tumour response should be investigated further in a larger cohort of patients.

For tumour recurrence outcomes, the microbial diversity and richness were similar between groups, however, patients who did not develop recurrence, had a significantly higher abundance of *Faecalibacterium, Prevotella,* and *Phascolarctobacterium* genera. Additional differential abundance analysis at species level identified that *Faecalibacterium Prausnitzii* (*F. Prausnitzii*) *and Prevotella Copri* (*P. Copri*) were enriched in patients with no recurrence. In general, 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. A meta-analysis study that included patients with metastatic melanoma revealed that *Faecalibacterium* was enriched in responders treated with immunotherapy [397]. Furthermore, Gopalakrishnan et al. analysed the faecal microbiome of patients with melanoma treated with anti-PD-1 immunotherapy and reported that responders had higher microbial diversity and increased abundance of *Faecalibacterium* and *Phascolarctobacterium,* with *Faecalibacterium* also associated with prolonged progression-free survival [398]. In a cohort of patients with Non-small cell lung cancer treated with anti–PD-1 immunotherapy, an increase in *P. Copri* (Bacteroidetes phylum) was associated with a preferred response to treatment [399]. We also noticed that the P/B ratio was significantly higher in the no recurrence group. An increase in P/B ratio is an enterotype associated with a favourable response to anti–PD-1/PD-L1 immunotherapy in patients with gastrointestinal cancers [400]. Evidence from the abovementioned studies suggests that the gut microbiome modulates immunotherapy anti-tumour response through enhancing $CD8^+$ T cell expansion and function [398, 399]. This may also be the case in the context of radiotherapy as anti-tumour immune response also plays a key role in radiotherapy-induced tumour control [351]. In a preclinical study, Uribe-Herranz et al. reported that the gut microbiota can impact radiotherapy efficacy [401]. Targeting gram-positive bacteria with vancomycin enhanced radiotherapy anti-tumour activity against both tumours in the

irradiated site and distant sites (abscopal effect) by enhancing tumour-associated antigen presentation to $CD8^+$ T cells [401]. Conversely, two genera *Adlercreutzia* and *Eggerthella (Eggerthella Lenta species)* were increased in those who developed recurrence. Both of these genera belong to the same family, *Eggerthellaceae*, of the Actinobacteria phylum. Previous studies have reported that these genera are enriched in non-responders treated with immunotherapy for metastatic melanoma [397]. Together, the current results suggest that certain gut microbes can positively or negatively influence risk of recurrence in HNC patients and could be exploited to predict radiotherapy outcomes.

Interestingly, genera that were associated with better outcomes, *Faecalibacterium* and *Phascolarctobacterium,* were associated with younger age, and early-stage disease. Younger age is associated with better anti-tumour immune response [402] and early-stage disease is an indicator of better tumour prognosis [1]. However, *Adlercreutzia*, enriched in patients with recurrence, was increased in older patients who are known to have an impaired anti-tumour immune response. *Prevotella* and *Phascolarctobacterium* genera were reduced in females compared to male patients. Additionally, *Phascolarctobacterium* was increased in patients with HPV+ tumours, which has been found to have a better prognosis [101]. This suggests that these genera could influence tumour prognosis and treatment outcomes and may in fact be the biological underpinnings of demographic risk factors previously documented to impact prognosis.

While our data provide a compelling evidence base to suggest that the gut microbiome is equally important to radiotherapy outcomes as it is other treatment modalities, it is not without its limitations. We recognise the small sample size of our cohort and the presence of different confounding factors at baseline, and as such, emphasise our results must be interpreted with caution. First, the small sample size did not allow us to avoid biases in the

data analysis related to smoking status, type of treatment, and tumour site which all are confounding risk factors of OM. Furthermore, the small cohort affects the analysis of treatment outcomes. For instance, only two patients developed PD and three patients developed tumour recurrence in this cohort, and this limits the interpretation of the results. Therefore, current findings need to be validated in a larger cohort of patients. Moreover, in this study, patients had many baseline confounding factors including heterogeneity in tumour primary sites and treatment types, which impact both OM severity and treatment outcomes. Variation in tumours site means that different radiation doses are delivered directly into the oral mucosa resulting in a varying degree of OM. For example, in patients with parotid gland cancer, the oral/oropharyngeal mucosa is exposed to lower radiation doses as compared to those with oral and oropharyngeal tumours and hence lower risk of severe OM. In addition, the inclusion of patients who received chemoradiotherapy results in analysis bias as this group is known to experience more severe OM compared to those treated with radiotherapy alone. Therefore, futures studies should be designed to minimise the variation in the baseline confounding factors. For example, the effect of the gut microbiome could be investigated in patients with tumours in a specific site e.g., oral cavity or oropharynx, and treated with a specific treatment e.g., radiotherapy alone or chemoradiotherapy. This will allow studying the gut microbiome impact on both OM and treatment outcomes in a more homogeneous cohort of patients.

6.7 Conclusion

Overall, this study reports novel findings on the microbial composition of patients with HNC and its potential association with OM severity and treatment outcomes. The present study revealed that the three bacterial genera, *Eubacterium*, *Victivallis*, and *Ruminococcus*, are associated with severe OM. Moreover, bacterial genera *Faecalibacterium*, *Prevotella*, and *Phascolarctobacterium,* are associated with better treatment outcomes. This indicates that pre-treatment gut microbiome composition could be exploited to predict the severity of OM and treatment success. Moreover, this provides a foundation for new strategies aiming to modulate the gut microbiota to improve radiotherapy outcomes, including pre-and probiotics, faecal microbiota transplantation, or diet.

Supplementary materials

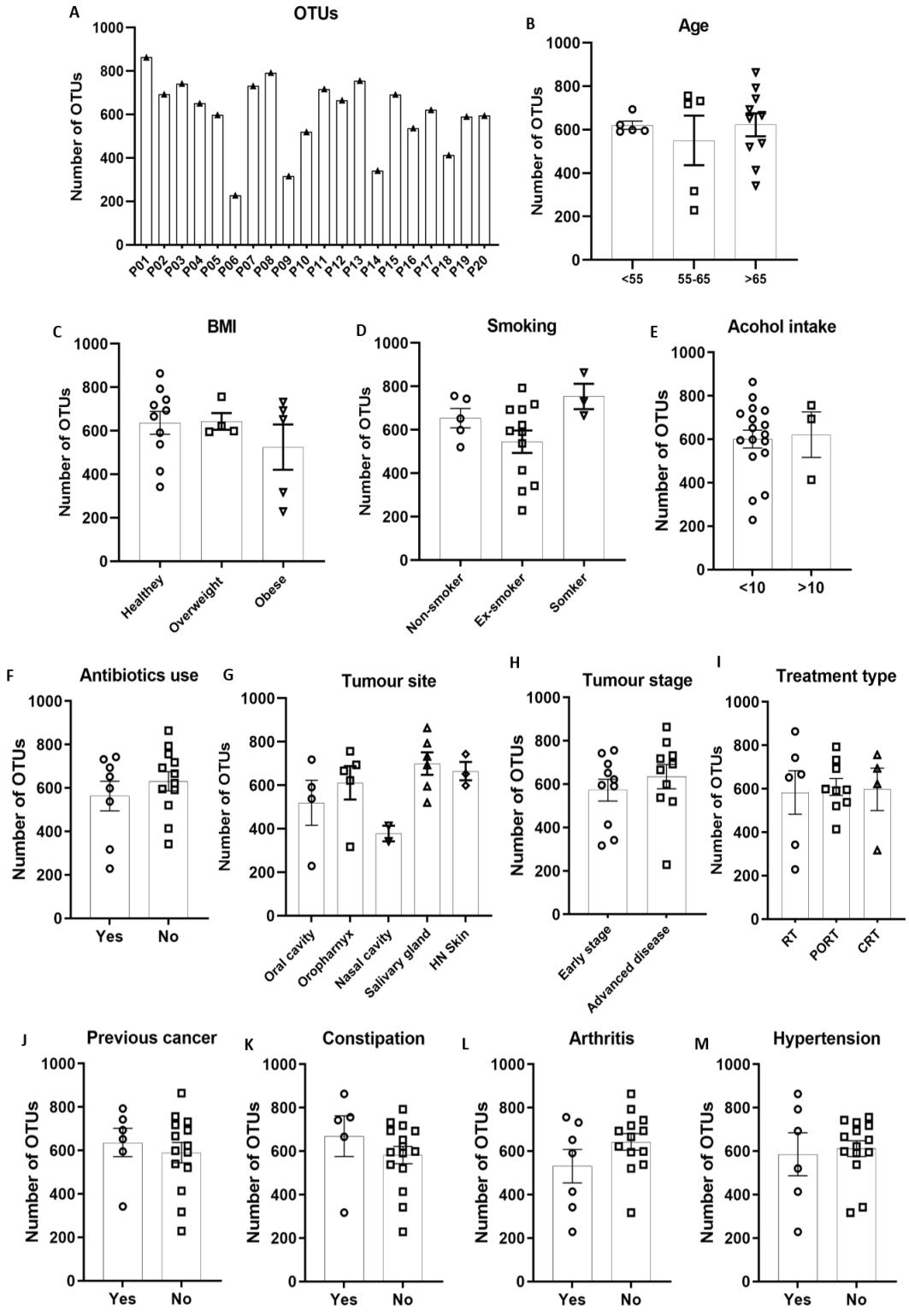


Figure S1: OTUs richness. **A)** The number of positive OTUs of all patients. There was no significant difference in the number of OTUs between patients based on age **(B)**, BMI **(C)**, smoking **(D)**, alcohol intake **(E)**, pre-treatment antibiotic use **(F)**, tumour site **(G)**, tumour stage **(H)**, treatment type **(I),** previous cancer diagnosis **(J)**, constipation **(K)**, Arthritis **(L)** or hypertension **(M)**. Unpaired t-test, one-way ANOVA or Kruskal-Wallis test. CRT, Chemoradiotherapy; PORT, postoperative radiotherapy; RT, radiotherapy.

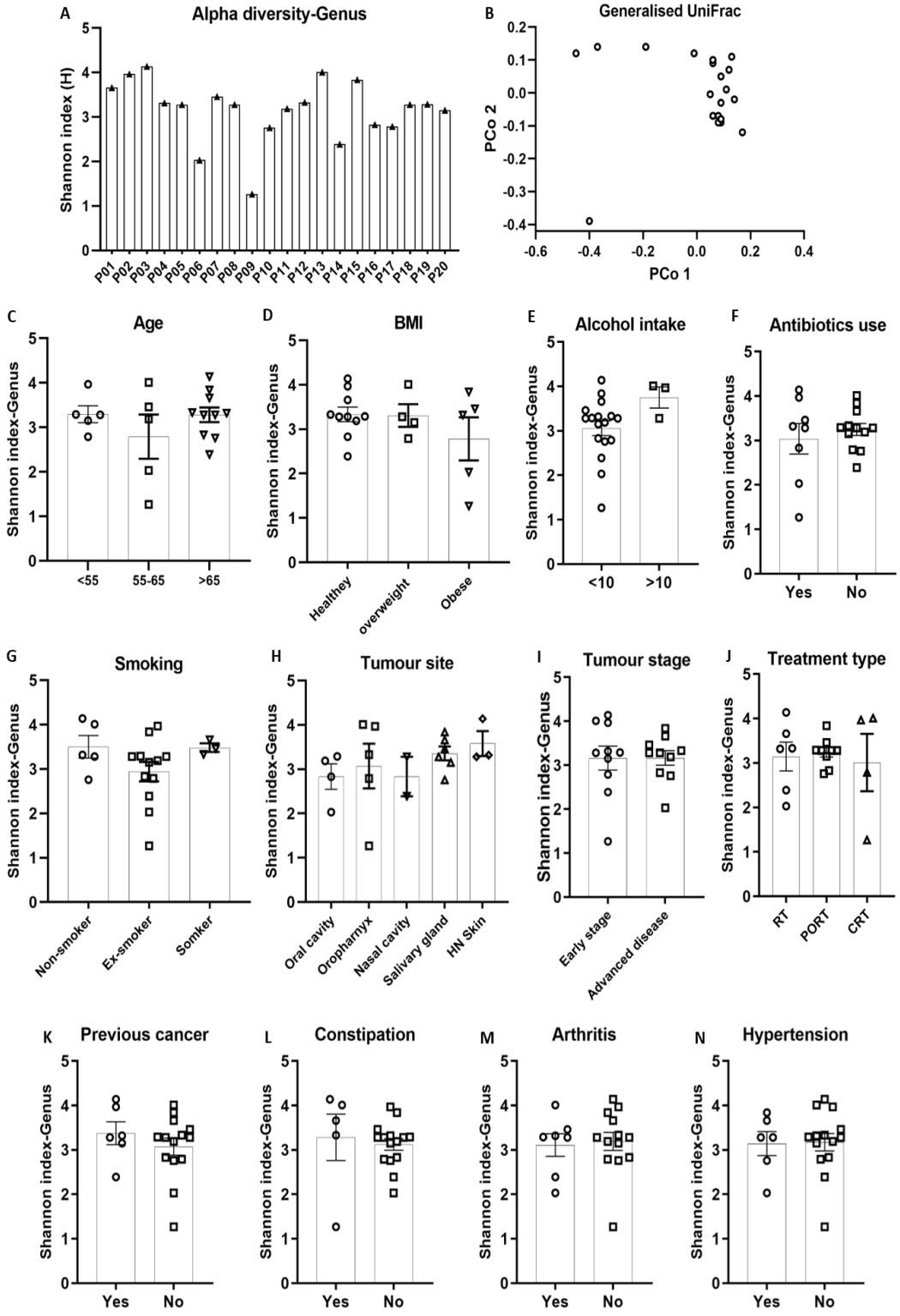


Figure S2: Microbial diversity. **A)** Shannon index values of all patients. **B)** The PCoA (generalised UniFrac distance) of all patients. There was no significant difference in the alpha diversity between patients based on age **(C)**, BMI **(D)**, alcohol intake **(E)**, pre-treatment antibiotic use **(F)**, smoking **(G)**, tumour site **(H)**, tumour stage **(I)**, treatment type (**J),** previous cancer diagnosis **(K)**, constipation **(L)**, Arthritis **(M)** or hypertension **(N)**. Unpaired t-test OR one-way ANOVA. CRT, Chemoradiotherapy; PORT, postoperative radiotherapy; RT, radiotherapy.

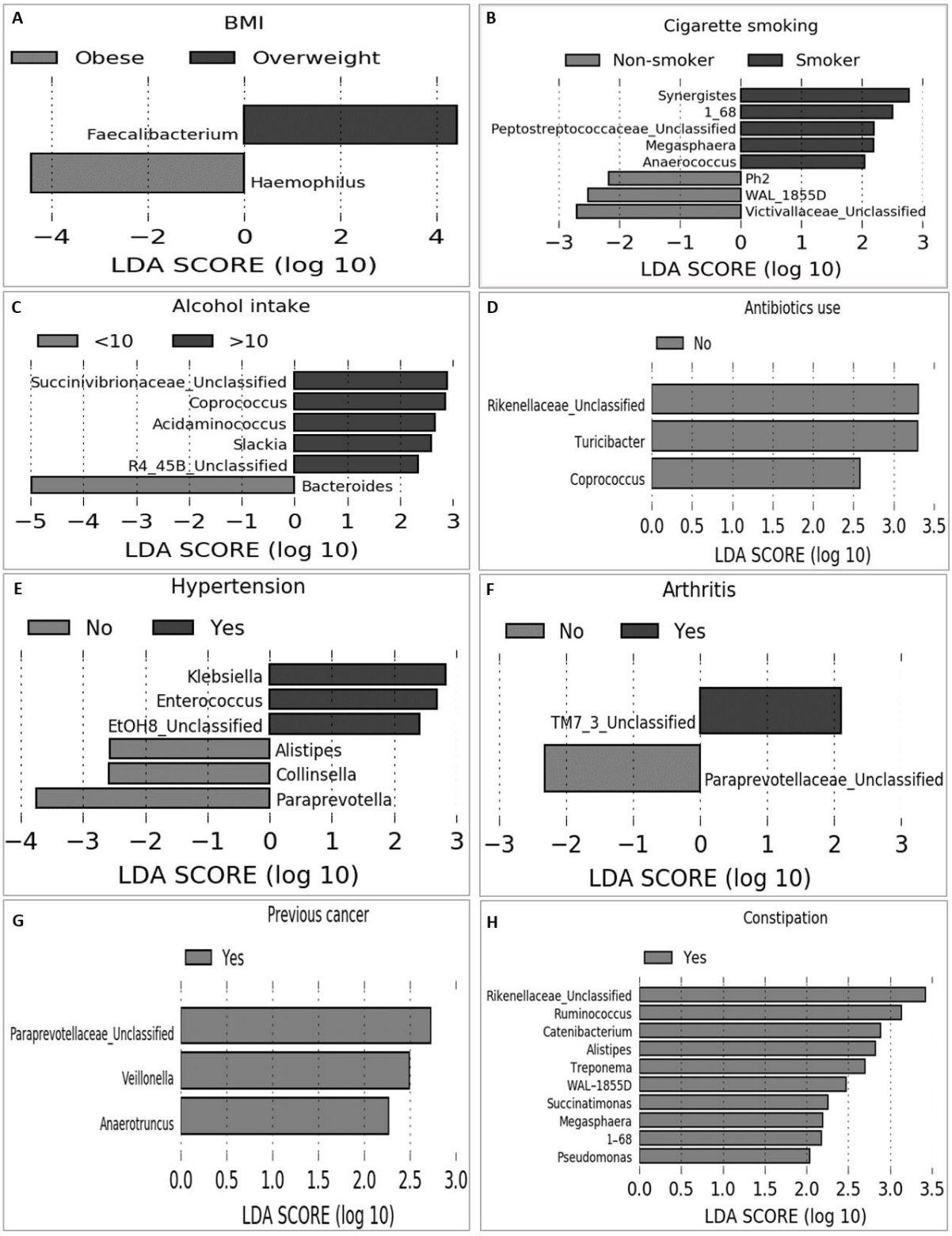


Figure S3: LEFSE analysis. The differential change in microbial genera based on BMI **(A)**, smoking **(B)**, alcohol intake **(C)**, antibiotics use **(D)**, hypertension **(E),** arthritis **(F)**, previous cancer diagnosis **(G)** and constipation **(H)**. LDA; Linear discriminant analysis.

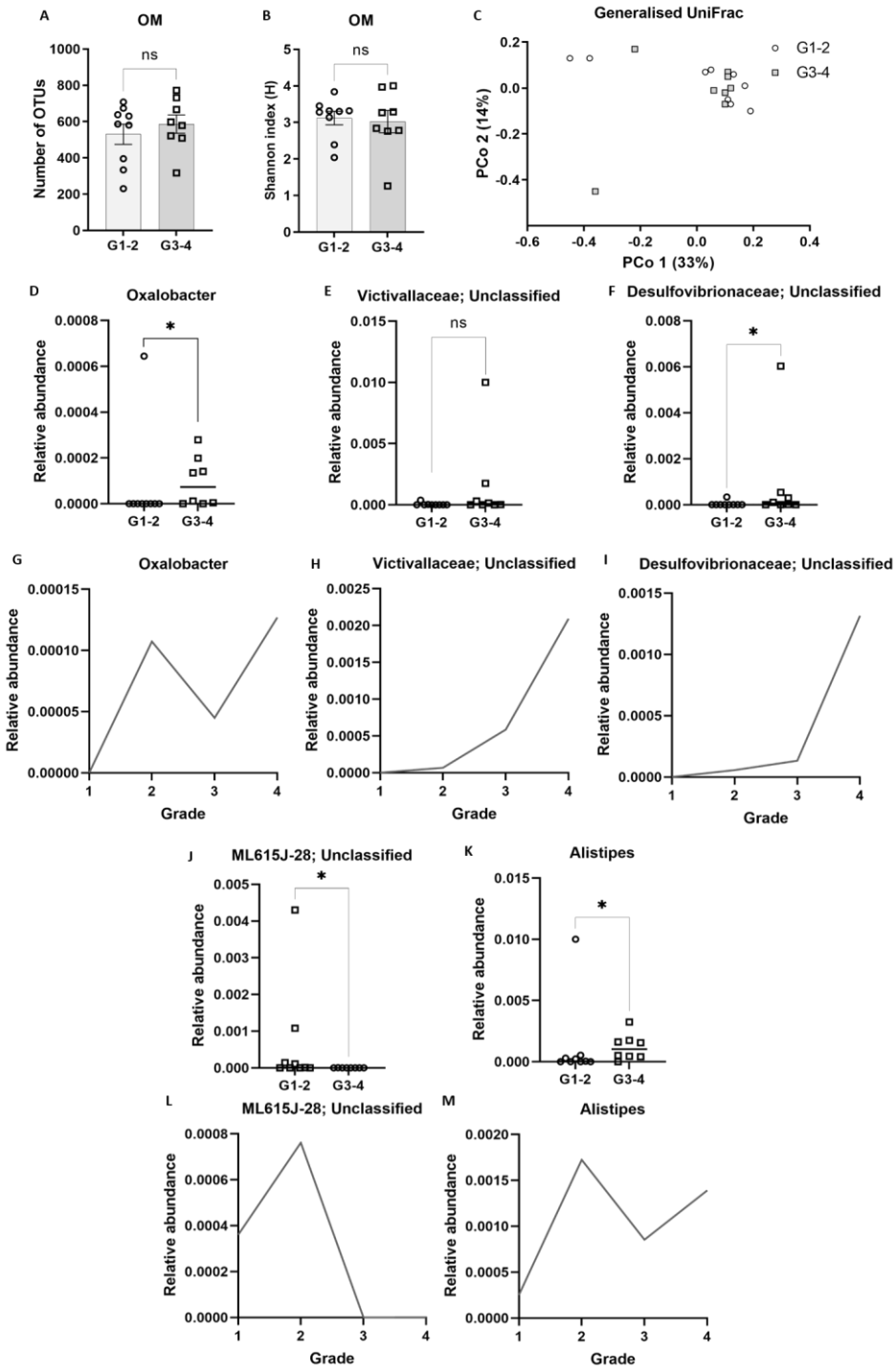


Figure S4: Association between gut microbiome and OM severity. **A)** The number of OTUs in G1-2 and G3-2 OM groups (mean ± SEM). **A)** Shannon index of G1-2 and G3-4 OM groups (mean ± SEM). **C)** The PCoA (generalised UniFrac distance) of G1-2 and G3-4 OM groups. The relative abundance of *Oxalobacter* (**D**), *unclassified Victivallaceae* (**E**), and *unclassified Desulfovibrionaceae* (**F**) of G1-2 group compared to G3-4 group. **G-I)** Change in average relative abundance of *Oxalobacter* (**G**)*, unclassified Victivallaceae* (**H**) *and unclassified Desulfovibrionaceae* (**I**) according to change in OM severity grade. **J-K)** The relative abundance of *unclassified ML615J-28* (**J**) and *Alistipes* (**K**) of G1-2 group compared to G3-4 group. **L-M)** Change in average relative abundance of *unclassified ML615J-28 (***L**) and *Alistipes* (**M**) according to change in OM severity grade. *P ≤ 0.05. Mann Whitney test; line in D, E, F, J, K represents the median.

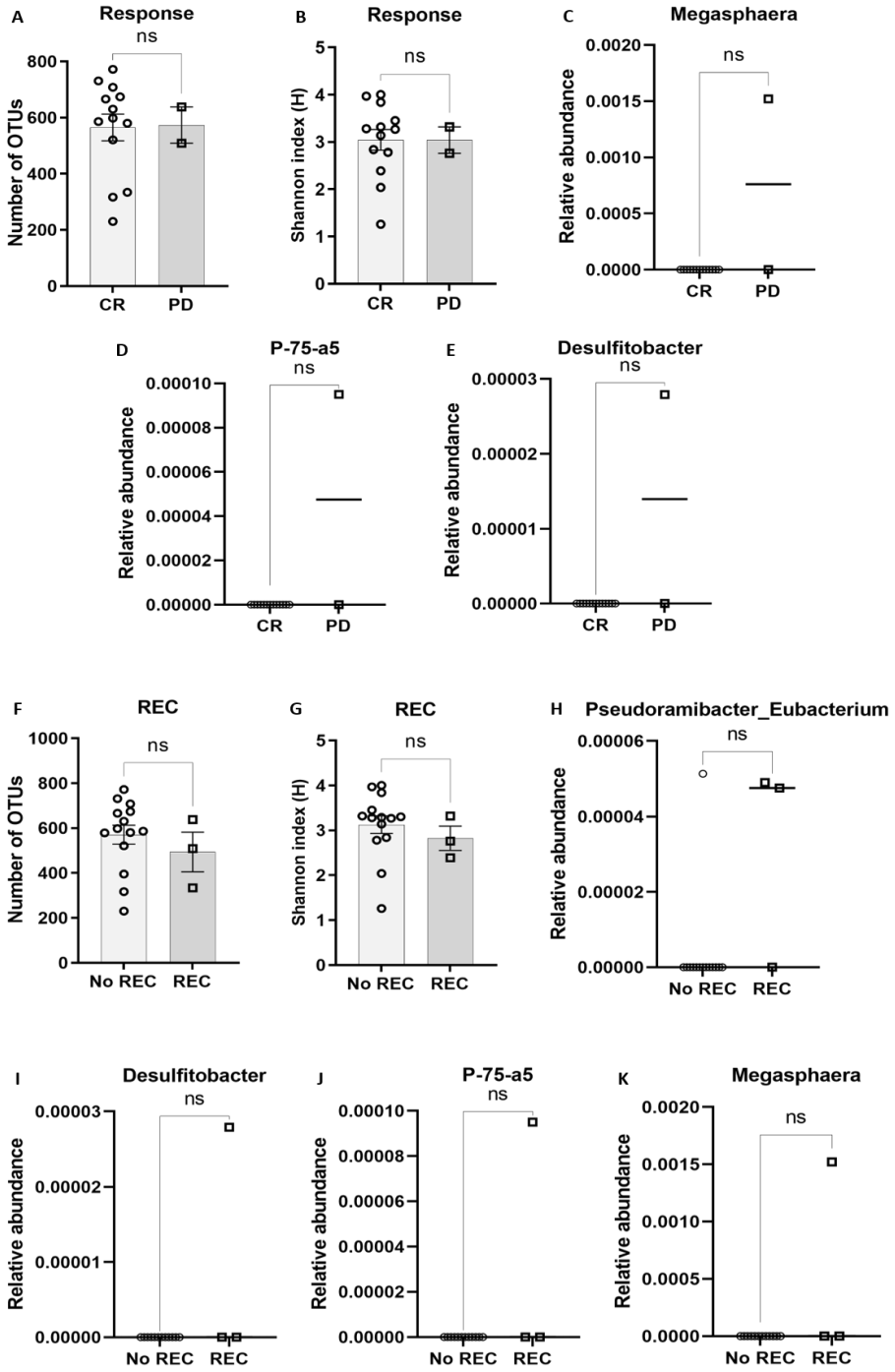


Figure S5: Association between gut microbiome and radiotherapy outcomes. **A)** The number of OTUs in CR and PD groups (mean ± SEM). **B)** Shannon index of CR and PD groups (mean ± SEM). The relative abundance of *Megasphaera* (**C**), *p-75-a5* (**D**), and *Desulfitobacter* (**F**) in CR group compared to PD group. **F)** The number of OTUs in No REC and REC groups (mean ± SEM). **G)** Shannon index of No REC and REC groups (mean ± SEM). The relative abundance of *Pseudoramibacter_Eubacterium* (**H**), *Desulfitobacter* (**I**), *p-75-a5* (**J**) and *Megasphaera* (**K**) in No REC compared to REC groups. Mann Whitney test; line in C, D, E, H, I, J, K represents the median.

Table S1: 10 most abundant genera in G1-2 and G3-4 OM groups

G1-2	**G3-4**
Bacteroides (40%)	Bacteroides (41.9 %)
Parabacteroides (7.8%)	Faecalibacterium (7.9%)
Faecalibacterium (6.9%)	Unclassified Ruminococcaceae (7.2%)
Unclassified Ruminococcaceae (6.8%)	Prevotella (5.5%)
Unclassified Clostridiales (4.7%)	Unclassified Lachnospiraceae (4.2%)
Unclassified Lachnospiraceae (4.1%)	Unclassified Clostridiales (3.9%)
Unclassified RF32 (3.4%)	Parabacteroides (3.6%)
Prevotella (2.8%)	Unclassified Barnesiellaceae (3.2%)
Sutterella (2.6%)	Oscillospira (2.5%)
Ruminococcus-2 (2.5%)	Phascolarctobacterium (2%)

Table S2: 10 most abundant genera in CR and PD groups

CR	PD
Bacteroides (40.6%)	Bacteroides (45.4%)
Faecalibacterium (7.9%)	Unclassified Ruminococcaceae (7.1%)
Unclassified Ruminococcaceae (6.9%)	Blautia (6.7%)
Parabacteroides (6.6%)	Unclassified Clostridiales (6.6%)
Prevotella (4.5%)	Akkermansia (5.2%)
Unclassified Lachnospiraceae (4.1%)	Unclassified Barnesiellaceae (4.4%)
Unclassified Clostridiales (4%)	Unclassified Lachnospiraceae (3.0%)
Sutterella (2.7%)	Parabacteroides (3%)
Oscillospira (2.3%)	Oscillospira (2.5%)
Unclassified Barnesiellaceae (2.12%)	Unclassified Bacteroidales (2.0%)
CR: complete response; PD, progressive disease	

Table S3: 10 most abundant genera in no recurrence and recurrence groups

No REC	REC
Bacteroides (39%)	Bacteroides (50%)
Faecalibacterium (8.9%)	Unclassified Clostridiales (6.4%)
Unclassified Ruminococcaceae (7.2%)	Unclassified Ruminococcaceae (6.0%)
Parabacteroides (5.9%)	Parabacteroides (5.7%)
Prevotella (4.9%)	Blautia (4.5%)
Unclassified Lachnospiraceae (4.4%)	Unclassified Barnesiellaceae (3.9%)
Unclassified Clostridiales (3.9%)	Akkermansia (3.5%)
Sutterella (2.4%)	Unclassified Lachnospiraceae (3%)
Unclassified RF32 (2.3%)	Oscillospira (2.7%)
Oscillospira (2.2%)	Sutterella (1.8%)

Table S4: Differentially increased microbes at different taxa levels in different groups

Phylum	Class	Order	Family	Genus	Species
Differential microbes increased in G3-4 OM group					
Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	Victivallis	Vadensis
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	[Eubacterium]	Biforme
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	[Ruminococcus]	Unclassified
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter	Formigenes
Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	Unclassified	Unclassified
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Unclassified	Unclassified
Differential microbes increased in G1-2 OM group					
Proteobacteria	Alphaproteobacteria	RF32	Unclassified	Unclassified	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes	Massiliensis
Tenericutes	RF3	ML615J-28	Unclassified	Unclassified	Unclassified
Differential microbes increased in PD group					
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megasphaera	Unclassified
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	P-75-a5	Unclassified
Firmicutes	Clostridia	SHA-98	Unclassified	Unclassified	Unclassified
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfitobacter	Unclassified
Differential microbes increased in No REC group					
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium	Unclassified
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	Copri
Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium	Prausnitzii
Differential microbes increased in REC group					
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	Unclassified
Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_ Eubacterium	Unclassified
Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Desulfitobacter	Unclassified
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Eggerthella	Lenta
Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megasphaera	Unclassified
Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	P-75-a5	Unclassified

Table S5: differential features identified by LEFSe analysis in different groups

Genus	LEFSe p-value
Differential microbes increased in G3-4 OM group	
Victivallis	0.018
[Eubacterium]	0.019
[Ruminococcus]	0.027
Oxalobacter	0.031
Unclassified Victivallaceae	0.048
Unclassified Desulfovibrionaceae	0.043
Differential microbes increased in G1-2 OM group	
Unclassified RF32	0.032
Alistipes	0.043
Unclassified ML615J-28	0.043
Differential microbes increased in PD group	
Megasphaera	0.011
P-75-a5	0.011
Unclassified SHA-98	0.043
Desulfitobacter	0.011
Differential microbes increased in No REC group	
Phascolarctobacterium	0.023
Prevotella	0.032
Faecalibacterium	0.032
Differential microbes increased in REC group	
Adlercreutzia	0.013
Pseudoramibacter_Eubacterium	0.029
Desulfitobacter	0.031
Eggerthella	0.010
Megasphaera	0.031
P-75-a5	0.031

Chapter seven

This chapter discusses the results and findings of the thesis and proposes future directions

Chapter 7: General discussion

7.1 Introduction

Radiotherapy is a crucial management method of head and neck cancer (HNC). However, it is associated with several undesirable toxicities including radiotherapy-induced oral mucositis (OM), which is one of the most frequent and troublesome acute toxicities in patients with HNC treated with radiotherapy [72]. Variation in radiotherapy outcomes in terms of tumour response and normal tissue toxicities is another challenge for radiotherapy. The current factors used to predict the risk of oral toxicities and locoregional tumour failure or tumour recurrence has failed to adequately explain most of the variation in radiotherapy outcomes between patients. Accumulating evidence from recent research has revealed an individual's gut microbiota may play a role in several diseases, particularly inflammatory and immune-related conditions [109]. The immune system components play a major role in both the pathogenesis of OM and tumour response to radiotherapy due to OM-associated inflammation and radiotherapy-associated immunogenic cell death, respectively [338]. Hence, gut microbiota modulation of immune responses provides a potential target for OM intervention or radiotherapy response prediction. This thesis, therefore, aimed to investigate the gut microbiome as a new target affecting both the pathogenesis of OM and radiotherapy outcomes.

This thesis provides an in-depth investigation of the role of the gut microbiome on radiotherapy-induced OM and radiotherapy outcomes in clinical and preclinical settings. Chapters 3 and 4 describe model development studies for antibiotic-induced microbiota depletion (AIMD) and radiation-induced OM. In order to investigate the role of gut microbiota on radiation-induced OM, I needed first to validate an antibiotic-induced

microbiota ablation method and establish an OM model in rats. This was achieved by conducting two pilot studies that constitute chapters 3 and 4. Results from these chapters were used to conduct the main preclinical study in chapter 5. This study showed that the gut microbiome is involved in the pathogenesis of radiation-induced OM through the modulation of inflammatory cytokines in lingual tissues. Finally, findings from chapter 6 demonstrated that certain genera in the pre-therapy gut microbiome are associated with severity of OM and tumour recurrence in patients with HNC.

7.2 Model development to study the gut microbiome in murine animals

Research on the gut microbiome in murine animal models has relied on two key methods; 1) germ-free (GF) animals and 2) antibiotic-induced microbiome disruption or depletion methods [403]. Due to the inherent limitations of GF animals such as the developmental defects in the immune system, especially important to maintain for studies such as those presented in this thesis where the immune system is critical to the hypothesis, and logistical difficulties in maintaining GF status, I utilised the AIMD method in this thesis. This method allows studying the impact of the gut microbiota in different physiological and pathological conditions in the absence of the major bacterial taxa. Chapter 3 of the thesis provides a detailed protocol for the use of a cocktail of three antibiotics in drinking water to establish an AIMD model in rats. The findings from this chapter showed that using ampicillin, neomycin and vancomycin successfully ablated the majority of the gut microbiota and significantly reduced the microbial diversity and richness. The AIMD method used in this thesis has many advantages compared to the previously used protocols. First, this method utilised a combination of three antibiotics only compared to a cocktail of four to five antibiotics used in previous studies providing a more cost-effective gut microbiota ablation method. Moreover, the antibiotics used have negligible (neomycin and vancomycin) or low

(ampicillin) oral absorption [244-246]. This is an important feature to ensure that the effects observed of antibiotics are relatively specific to the ablation of the gut microbiota and not as a result of systemic depression of the immune system cells by antibiotics [404]. Furthermore, unlike most of the previous studies, metronidazole was excluded from the antibiotic cocktail. In addition to reducing antibiotic palatability [240], metronidazole has extremely high oral absorption (>90% bioavailability) [405] and can be absorbed into the systemic circulation and hence affecting other organs. Another advantage of this AIMD method is that it did not cause antibiotic-induced diarrhoea observed with other antibiotic cocktails [406], animal welfare, therefore, was not affected by treatment. In addition, the antibiotics were administered in drinking water instead of oral gavage. This does not only reduce gavage-related animal stress but also allows the administration of antibiotics in animals with oral injuries such as OM explored in this thesis.

On the other hand, similar to almost all previous studies, this AIMD did not completely eradicate the gut microbiota. One of the consistent observations across studies is the enrichment of Proteobacteria phylum, particularly the *Enterobacteriaceae* family, after antibiotic treatment [236, 239, 248, 250, 254, 256, 257]. Some have suggested that this taxon is introduced to the gut through food [236, 239]. Another possibility is that this bacterial taxon is resistant to antibiotic treatment. Therefore, further research is needed to determine the exact reason for Proteobacteria enrichment after antibiotic treatment and exploring how to overcome this limitation to make AIMD methods equivalent to GF status as much as possible. Interestingly, despite the increase in this taxon which is gram-negative bacteria, serum lipopolysaccharides (LPS) levels – a danger signal mainly released by gram-negative bacteria – have been reported to decrease after gut microbiota ablation with antibiotics [248, 254]. For instance, Shen et al. demonstrated that treating mice with

broad-spectrum antibiotics for 3 weeks caused > 2 log-fold reduction in faecal bacterial load while the relative abundance of Proteobacteria was increased [254]. This indicates that the LPS levels are more related to the overall bacterial load instead of the relative abundance of Proteobacteria. Hence, the enrichment of Proteobacteria alone might not have a significant biological impact when using AIMD models. Another limitation of the microbiota ablation with antibiotics administered in drinking water is that it will not only deplete the gut microbiota but also can influence the oral microbiota as well. The disruption of oral microbiota, however, will be to a lesser degree than the microbiota in the gut. This will have implications on studying conditions linked to the oral microbiome including radiation-induced OM. Comparing the effects of topical antibiotics to those of antibiotics administered in drinking water may help rule out whether the observations are related to the oral microbiota instead of the gut microbiota. Finally, one important point worth mentioning is that in this AIMD model, I analysed the gut microbiome of the caecal contents instead of the faecal samples. Previous studies have reported some differences in microbial community structure between caecal contents and faeces in both mice and rats [407-409]. Analysing faecal samples might be clinically relevant because it can be easily collected in a non-invasive manner. It also allows the longitudinal assessment of microbiota over time while animals are still alive. However, a faecal sample is associated with high variability in microbial composition because of sample handling and the high risk of cross-contamination. Therefore, the use of caecal contents is preferred in studies where the microbiome is analysed in a single time point.

In general, AIMD methods developed in this thesis provide a simple and cost-effective method to effectively ablate the gut microbiota with a minimal impact on animal welfare.

7.3 Establishment of Radiation-induced OM model in rats

To establish radiation-induced OM in animal models, the radiation should be limited to the oral cavity and cause minimal stress to the animals. In chapter 4, I developed a custom-made lead cylinder that was used as a lead shield. This method allows the irradiation of snouts only, while the rest of the body was protected from radiation exposure. OM has been developed using both single-dose and fractionated-dose radiation. While fractionated-dose radiation is more clinically applicable, it can cause physiological stress to the animals as they will need to undergo multiple radiation sessions (3 to 8 sessions), which each require an anaesthetic. This could lead to changes in systemic inflammation as acute physiological stress has been associated with increased levels of systemic inflammatory cytokines including IL-6 and TNF-α [410]. Furthermore, it has been long identified that anaesthetic agents can impact the immune system function with inhaled drugs, such as isoflurane, induce pro-inflammatory effects while injected drugs, like ketamine, stimulate anti-inflammatory responses [411-413]. Since OM is an inflammatory condition, I used a single-dose of X-ray radiation to minimise stress-induced changes in inflammatory markers. In this thesis, 20 Gy single-dose radiation was the optimal radiation dose to induce OM with macroscopic and microscopic tissue injury observed. Previous studies have used this dose to establish OM models in rats [282, 284, 287], however, Chang et al., for example, used a similar radiation dose but reported high mortality among irradiated rats. This might be due to insufficient radiation shielding. This indicates that radiation shielding is a critical part of OM model development and that the shielding device developed in this thesis was successful to prevent radiation toxicities outside the oral cavity.

Most of the previous studies have used buccal mucosa to assess OM in animal models. This is because buccal mucosa is the most frequent site of OM clinically as it is made of a non-

keratinised epithelium [414] unlike the tongue which is composed of keratinised epithelium. As such, evaluation of the buccal mucosa of animals is more clinically relevant. In this thesis, we assessed OM in the tongue because it allows easier and non-invasive evaluation of temporal development and severity of OM throughout the experimental period. To assess the buccal mucosa, the animals need to be anaesthetised and a more invasive examination is required. Nevertheless, buccal mucosa from this model was collected, when animals were euthanised and analysed for tissue damage and inflammatory cell infiltrations. This was not included in this thesis but presented in an Honours thesis by Dr Gunjan Verma [415]. The assessment of the oral mucosa showed significant mucosal ulceration and infiltration of inflammatory cells on the peak severity time point (day 9) [415] similar to what has been observed in the tongue tissues, presented in this thesis. Furthermore, a similar impact of AIMD was observed in the buccal mucosa characterised by the accelerated healing of mucosal injury [415]. This indicates a similarity in the pathophysiology of OM in both tongue and buccal mucosa. Therefore, we can assume that the assessment of OM in the tongue can reflect changes in the buccal mucosa in this study.

Overall, chapter 3 of the thesis described a novel radiation shielding method and provided a protocol for the development of a radiation-induced OM model in rats using 20 Gy single-dose X-ray radiation.

7.4 Gut microbiome and radiotherapy-induced OM

The gut microbiota is a key modulator of the immune system and the alteration in the gut microbial composition has been extensively linked to the pathogenesis of several inflammatory conditions [109]. In this thesis, I investigated whether the gut microbiome is associated with the pathogenesis or severity of OM in both preclinical and clinical studies. The preclinical study (chapter 5) revealed that irradiation of the rat snout can alter the gut

microbiota composition and induce the expansion of pro-inflammatory microorganisms including *Verrucomicrobiaceae*, *Streptococcaceae*, *Peptococcaceae*, *Erysipelotrichaceae*, and *Staphylococcaceae*. Animal irradiation was also associated with a significant reduction in propionate-producing bacteria (*Rikenellaceae* and *Prevotellaceae*). This dysbiotic microbiome may contribute to the observed increase in inflammatory cytokines in the irradiated oral mucosa.

Conversely, the depletion of gut microbiota prior to radiation resulted in a better OM outcome including a smaller ulcer-like area on the tongue, shorter duration of severe OM, and faster OM healing. Interestingly, gut microbiota ablation mainly influenced the healing of OM rather than the initiation/development phase of OM. Healing is the final phase of mucositis based on the five-phase model described by Sonis [84]. The healing phase is the least understood stage of OM, and the exact signalling pathways involved in this phase are yet to be determined [86]. Overall, this stage is characterised by the resolution of pro-inflammatory responses, activation of anti-inflammatory pathways, differentiation, proliferation, migration of epithelial cells, and restoration of normal tissue integrity [86, 88]. Signalling pathways involving the release of anti-inflammatory cytokines (e.g. IL-10) and growth factors (e.g. TGF-β and VEGF) exert anti-inflammatory responses and stimulate tissue repair and regeneration [88]. In chapter 5, a reduction in the levels of pro-inflammatory cytokines was observed in the tongue tissue of the AIMD group, therefore, we speculate that the protective effects of AIMD are mediated by the suppression of inflammatory responses induced by radiation. However, the exact mechanism by which the gut microbiota modulates OM healing needs further investigation.

To our knowledge, this is the first preclinical study to assess the impact of the gut microbiota on radiation-induced OM. Further preclinical studies are warranted to confirm

how the gut microbiota may influence radiation-induced OM with more focus on the signalling pathways associated with the healing phase. In addition, studies could be designed to compare the effects of antibiotic-induced oral microbiome depletion (AIOMD) using broad-spectrum topical antibiotics to AIMD using systemic antibiotics. This will allow evaluation of whether the improvement in OM outcomes is due to gut or oral microbiota or perhaps both. In addition, further preclinical research is needed to assess the utility of microbiota-based interventions such as probiotics or faecal microbiota transplantation to treat or reduce the severity of radiation-induced OM. Finally, a limitation of the model was that rats were not tumour-bearing, preventing the ability to co-evaluate the effect of antibiotics on both toxicity and radiation efficacy given that the gut microbiota is critical for an optimal anti-tumour response.

In the clinical study (chapter 6), I assessed whether there is an association between pre-therapy gut microbiome and severity of OM in patients with HNC. While there was no difference in the microbial richness and diversity between patients with mild/moderate (G1-2) and severe (G3-4) OM, three microbial genera (*Victivallis*, *Eubacterium,* and *Ruminococcus*) were found to be differentially increased in patients who developed G3-4 OM. Some of these genera have been linked to pro-inflammatory conditions, thus, they may contribute to OM-associated inflammation and increase the risk of severe OM. While this association needs to be further validated in a larger cohort of patients, these preliminary results suggest that the pre-therapy gut microbiome may be useful to help predict the likely severity of OM. Although not designed in the study protocol, I was able to serendipitously evaluate whether antibiotics prior to radiotherapy were associated with the development of OM since 40% of the patients reported taking antibiotics within 2 weeks of starting treatment. However, I found no significant association with the severity

of OM. However, the different types of antibiotics used, their administration (oral, I.V.), duration, and purpose make it difficult to ascertain any conclusions. It would be prudent to expand the cohort with a defined outcome testing the interaction between specific antibiotic class use and OM risk, in addition to other radiotherapy outcomes.

The notion that the gut microbiome can be used to predict or treat radiotherapy toxicities has been gaining increased interest in recent years. For instance, Ferreira et al. reported that the gut microbiome is associated with both acute and late radiotherapy-induced enteropathy in patients undergoing prostate and pelvic radiotherapy and suggested that it can be targeted to predict and treat these toxicities [416]. Collectively, results from our study and other work are promising and will pave the way for research on incorporating the gut microbiome when making decisions regarding radiotherapy planning clinically.

Together, the findings from this preclinical study (chapter 5) suggest that the gut microbiome is involved in the pathogenesis of OM, particularly the healing phase, and with further research may provide a potential therapeutic target to reduce the severity of OM. Furthermore, results for the clinical study (chapter 6) indicate that certain bacteria in the baseline gut microbiome are associated with the severity of OM suggesting that the gut microbiome may be used to predict OM severity in clinical settings, although there was no association between antibiotic use in the lead up to radiotherapy and risk of OM.

7.5 Gut microbiome and radiotherapy response

Activation of immune responses plays a key role in the radiotherapy anti-tumour activity [338]. Since the gut microbiota regulates systemic immune responses, increasing research has been focusing on investigating its role in the modulation of anti-tumour immune response in the context of cancer treatments, including radiotherapy. In chapter 6 of this thesis, I investigated the association between the baseline gut microbiome and

radiotherapy outcome and demonstrated that *Faecalibacterium*, *Prevotella*, and *Phascolarctobacterium* genera were associated with recurrence-free survival while *Adlercreutzia* and *Eggerthella* genera were associated with recurrence development. A limitation was that only 3 patients were classified as experiencing recurrence within 12 months. It is likely that if the time frame was extended to 2 or more years, additional patients would have been added to the recurrence group and improved the power to identify microbial signatures linked to radiotherapy outcomes.

Regardless, to our knowledge, this is the first clinical study that looked at the gut microbiome as a predictive marker for radiotherapy efficacy in the context of HNC. Previous research has shown that the baseline gut microbiome can be used to predict the radiotherapy outcomes in cervical and rectal cancers. In these studies, Sims et al. demonstrated that the baseline gut microbiome diversity is an independent predictor for both recurrence-free and overall survival in patients with cervical cancer treated with chemoradiotherapy [417]. In their study, patients with higher baseline alpha diversity had improved overall and recurrence-free survival. Higher alpha diversity also correlated with the number of certain immune cells, mainly the tumour-infiltrating CD4+ T cells, which play a key role in the anti-tumour response of chemoradiotherapy. This study also reported that *Porphyromonadaceae* was enriched in long-term survival (>2 years) while *Enterobacteriaceae* was increased in short-term survival (< 1 year), however, there was conflicting reporting regarding these results in this paper [417]. Another study by Yi et al. analysed the baseline gut microbiome of patients with locally advanced rectal cancer treated with neoadjuvant chemoradiotherapy and reported that butyrate producing-bacteria *Dorea*, *Roseburia,* and *Anaerostipes*, were enriched in responders, while *Fusobacterium* and *Coriobacteriaceae* were increased in non-responders. They also

generated a response prediction classifier including 10 microbial biomarkers which yielded an area under the curve of 73.5% [418].

In addition to investigating the association between the gut microbiome and radiotherapy responses clinically, there should be further *in vivo* and *in vitro* preclinical research to identify mechanisms by which the gut microbiota modulates the response to radiation. Currently, only two preclinical studies have assessed the mechanism by which the gut microbiota impacts tumour response to radiation [401, 419]. Both Yang et al. and Uribe-Herranz et al. showed that administration of vancomycin, an antibiotic active against gram-positive bacteria, improved tumour control induced by ionising radiation [401, 419]. Interestingly, both studies reported an increase in the abundance of the genus *Akkermansia* after vancomycin treatment. To assess whether gram-positive bacteria compromise radiation response through production of butyrate, an important SCFA, Yang et al. administered GF mice with *Kineothrix alysoides*, a member of the butyrate-producing *Lachnospiraceae* family. This resulted in reduced ionising radiation efficacy and increased systemic and tumour levels of butyric acid. Butyric acid suppresses the expression of type I interferons in dendritic cells and the cross-presentation of tumour associated antigens to tumour-specific cytotoxic T cells and hence reducing the anti-tumour immune response [419]. These results suggest that the SCFA, butyrate, has a detrimental impact on radiotherapy efficacy.

Conversely, clinical studies have reported that butyrate-producing bacteria are associated with a better response to immunotherapy which was observed in this thesis as well in the case of radiotherapy. For instance, *Faecalibacterium prausnitzii* (*F. prausnitzii*) is a butyrate-producing bacteria that have been associated with better treatment response and prolonged progression-free survival in patients treated with immunotherapy [398] and

with recurrence-free survival in patients treated with radiotherapy, as observed in chapter 6 of this thesis. Therefore, researchers are trying to address the correlation between the SCFAs levels, the abundance of *F. prausnitzii,* and immunotherapy efficacy. Coutzac et al., for example, assessed the gut microbiome of a total of 88 patients with metastatic melanoma treated with ipilimumab immunotherapy and confirmed that higher proportions of *F. prausnitzii* were associated with prolonged progression-free and overall survival. Then, they measured the concentrations of serum SCFAs, but surprisingly they showed that *F. prausnitzii* was negatively correlated to serum butyrate. Butyrate concentration was also negatively correlated to progression-free and overall survival. This raises the question of the relationship between butyrate production in the gut and systemic concentrations [420].

In contrast to Coutzac et al., Botticelli et al. analysed the metabolomic profile of 11 patients with non-small cell lung cancer treated with nivolumab immunotherapy and demonstrated higher levels of SCFAs including butyrate and propionate were associated with long responders (> 1-year progression-free survival) [421]. Similarly, Nomura et al. analysed SCFAs from 52 patients with solid tumours treated with immunotherapy (nivolumab or pembrolizumab) and reported that higher faecal SCFAs concentration including butyrate was found in responders compared to non-responders [422]. Altogether, current evidence suggests butyrate-producing bacteria (i.e., *F. prausnitzii*) are linked to the efficacy of both immunotherapy and radiotherapy. However, what is still unknown is the exact pathway by which *F. prausnitzii* improves anti-tumour efficacy and whether SCFAs including butyrate have beneficial or detrimental effects on treatment effectiveness is still inconclusive. Future studies should further assess the effects of SCFA in cancer treatment efficacy. Moreover, other pathways for immunomodulatory effects of bacteria such (*F. prausnitzii*) should be investigated.

7.6 Extended studies from the clinical study

7.6.1 Longitudinal analysis of the gut microbiome for patients with HNC

The gut microbiome composition can be altered in response to different external stressors including cancer treatments. For instance, exposure to pelvic radiotherapy has been found to alter the gut microbiome composition in patients with gynaecological cancer [423]. This is expected as the gastrointestinal tract is within the field of radiation. However, little is known whether radiotherapy for HNC can alter the gut microbiome or not. To date, only one study has evaluated the gut microbiome pre-and post-treatment in patients with HNC. Oliva et al. assessed the gut microbiome for patients with HPV+ oropharyngeal squamous cell carcinoma before and at the end of chemoradiotherapy (70 Gy/35 fractions over 7 weeks with cisplatin) and found that there was no difference in the microbial diversity and taxa composition between different time points [375]. To assess whether this is also the case in our cohort, in addition to the pre-treatment samples presented in chapter 6, I also collected three additional stool samples from the patients in three time points throughout the treatment period (week 3 after commencing radiotherapy, after the last radiotherapy dose, and one month following treatment completion). While these samples could not be included in the current thesis due to time-frames, these samples will be analysed in the same way as the pre-therapy sample (chapter 6) to assess any changes in gut microbiome composition throughout the treatment period. Since four patients were treated with chemoradiotherapy, this may allow the comparison of the effect of radiotherapy alone or chemoradiotherapy on the gut microbiome. Furthermore, this will provide insight into the impact of alterations in the gut microbiome throughout treatment on radiotherapy outcomes. The differences in the ability of an individual's gut microbiome to resist changes imposed by cancer treatment may contribute to variation in response to treatment. Further, gut microbiome resistance to changes during radiotherapy might be more important than

baseline composition for both response and toxicities. Moreover, the variation in the time needed for restoration of the normal gut microbiome could contribute to variation in response to radiation including the duration of OM.

7.7 Targeting the microbiome to improve OM and radiotherapy outcomes

The gut microbiome provides a unique therapeutic target as it can be easily modified using multiple methods such as antibiotics, probiotics, prebiotics, synbiotics, postbiotics, or faecal microbiome transplant [424]. Probiotics are live bacteria with health benefits, prebiotics refers to food ingredients that prompt the growth of beneficial gut bacteria, while synbiotics are the combination of both probiotics and prebiotics. Postbiotics are the soluble biologically-active microbial metabolites and by-products such as SCFAs [425]. Since an intact gut microbiome is required for balanced immune responses and optimal anti-tumour immune response, it is not advised to use antimicrobial-based methods to manipulate the gut microbiome as they are aggressive and non-selective [424]. Therefore, other methods might be better suited to manipulate the gut microbiome in patients with cancer.

Probiotics are the most widely used method to modify the gut microbiome. In the context of OM, a systematic review and meta-analysis study found three studies that evaluated the use of probiotics to reduce the severity of OM in patients with HNC treated with radiotherapy or chemoradiotherapy [340]. Two of these studies used probiotic lozenges containing the same bacteria strain (*Lactobacillus brevis* CD2) but showed conflicting results. Sharma et al. found this probiotic preparation to reduce the incidence of severe OM (grade 3-4) [227], however, Sanctis et al. reported no significant difference in OM severity between intervention and control groups [426]. The third study by Jiang et al. showed that the administration of ingested probiotic capsules containing *Bifidobacterium*

longum, *Lactobacillus lactis*, and *Enterococcus faecium* reduced the severity of OM in patients with locally advanced nasopharyngeal carcinoma [364]. The same research group conducted another study with a different probiotic concoction (*Lactobacillus plantarum MH-301*, *Bifidobacterium animalis subsp. Lactis LPL-RH*, *Lactobacillus rhamnosus LGG-18*, and *Lactobacillus acidophilus*) and found that this improved probiotic combination significantly reduced the severity of chemoradiotherapy-induced OM in patients with nasopharyngeal carcinoma [427]. Overall, these preliminary results suggest that ingested probiotics may provide better results compared to probiotic lozenges, which supports the finding that local antibiotics in lozenges or pastes also don't alter the development of OM. Future studies should focus on bacteria that have been implicated in OM severity. Based on our findings (chapter 5) and previous study findings, propionate-producing bacteria could provide better probiotics to reduce the severity of OM. The propionate itself could be administered as a postbiotic to reduced OM-related inflammation.

On the other hand, how manipulation of the gut microbiome may impact radiotherapy efficacy has not been investigated. Since preliminary results have shown that the use of over-the-counter probiotics is associated with lower alpha diversity, hence, a less favourable response to immunotherapy [428], future microbial-based interventions to augment cancer treatment efficacy, including radiotherapy, should focus on specially targeting those bacteria that have been proven to be associated with treatment outcomes. Based on the evidence from this thesis and previous work, *Faecalibacterium Prausnitzii*, *Prevotella Copri*, *Phascolarctobacterium,* and *Akkermansia muciniphila* provide a potential target to improve radiotherapy efficacy and prognosis.

7.8 Gut microbiome and machine learning in predicting radiotherapy outcomes

Recent years have witnessed an increasing interest in utilising machine learning, a branch of artificial intelligence, to develop predictive models that allow disease diagnosis, and prediction of disease prognosis and treatment outcomes by incorporating different clinical and demographic data related to patients, disease, and therapy [429]. Since certain health conditions have been associated with a distinctive microbial signature, there is growing attention to the use of the gut microbiome profile in the development of such machine learning models for diagnosing and predicting the prognosis and treatment outcomes for several diseases including cancer [430, 431]. However, research in this field is still in its early stages and more work needs to be done to improve the design of microbiome studies, in terms of sample size, sample collection and analysis methodology, and enhancing the performance and reproducibility of machine learning models [431].

One of the future directions for my clinical study (chapter 6) is to utilise machine learning tools to generate a predictive model for OM severity and radiotherapy outcomes (response and recurrence). This model could include patient demographics, tumour, and treatment-related factors in addition to the gut microbiome data (diversity indices and abundance). This will help to identify patients at higher risk of developing OM and those with an unfavourable response to radiotherapy. This will allow personalising treatment based on the need of each patient. For instance, for those at high risk of OM, prophylactic measures such as feeding tube insertion or photobiomodulation could be implemented before commencing treatment. Furthermore, for those at risk of recurrence, specific therapy planning before treatment or more frequent screening after treatment could be applied.

In addition to these measures, the gut microbiome of high-risk patients could be manipulated to improve OM and radiotherapy outcomes.

7.9 Conclusion

This thesis explored the role of the gut microbiome in radiotherapy-induced OM and its association with radiotherapy outcomes. The findings of the present studies showed that gut microbiota can influence the healing of radiation-induced OM through the modulation of pro-inflammatory cytokines. Furthermore, the difference in baseline microbial composition is associated with OM severity and treatment outcomes. The gut microbiome can be targeted to improve radiotherapy outcomes in patients with HNC. Probiotics that include propionate-producing bacteria could provide a potential way to reduced tissue injury and inflammation associated with OM. Moreover, targeting bacterial taxa associated with a preferred response to radiotherapy could be a way to improve radiotherapy efficacy

Chapter eight

Chapter 8: References

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Appendices

Appendices

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REVIEW

Gut microbiota: implications for radiotherapy response and radiotherapy-induced mucositis

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ABSTRACT

Introduction: Radiotherapy is a mainstay of solid tumor management but can be associated with unacceptable levels of off-target tissue toxicity which impact treatment outcomes and patients' quality of life. Tumour response to radiotherapy and the frequency and severity of radiotherapy-induced toxicities, especially mucositis, varies among patients. Gut microbiota has been found to modulate both the efficacy and toxicity of some types of cancer chemotherapies and immunotherapies but has yet to be investigated thoroughly in the setting of radiotherapy.

Area covered: In this review, we discuss the potential role of gut microbiota on modulating radiotherapy-induced oral and gastrointestinal mucositis and the anti-tumor response to radiotherapy through modulation of immune responses.

Expert opinion: The gut microbiota plays a major role in the modulation of systemic immune responses, which influence both radiotherapy response and gastrointestinal toxicities such as mucositis. Hence, investigating the gut microbiota link to the variation in radiotherapy responses and toxicities among patients is warranted. Future targeting of these responses with a patient-tailored restoration of optimal microbial composition could lead to a new era of mucositis prevention and enhanced tumor responses.

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1. Introduction

Radiotherapy is a core modality used for the treatment of brain, head and neck, breast, lung, abdominal and gynecological cancers [1]. Around 50% of all patients with cancer are treated with radiotherapy during the course of their disease [2] and 60% of these are treated for curative purposes [3]. It is a cost-effective method that accounts for only 5% of the total cancer care expenses [4]. It is also a minimally invasive modality that allows flexible adjustment of the dose regimen required for each disease stage and for each individual patient [5]. Despite technological advances in radiotherapy, there are a number of limitations that affect radiotherapy treatment success. Currently, tumor radioresistance and recurrence are major clinical challenges of radiotherapy [6]. Tumors vary in their response to radiation and recurrence rate from one patient to another. Some of these variations can be explained by clinical factors such as tumor size, stage of disease, or failure to determine the exact tumor spread leading to reduced local control. However, these variations do not adequately explain the differences in treatment response considering that among tumors with the same size, stage, and grade and treated with same radiation dose, some will have a recurrence and some will not [7]. Furthermore, there is accumulating evidence that biological factors such as intrinsic radioresistance [8], hypoxia [9], inflammatory cell infiltration [10], and recruitment of bone marrow-derived cells [11] into the tumor microenvironment can also affect radiotherapy outcomes.

Radiotherapy is also associated with several toxicities that impact patients' quality of life. These toxicities can be acute; developing during or immediately following radiotherapy and may affect the likelihood of treatment course completion, including mucositis, dermatitis, cystitis, and bone marrow suppression. In contrast, chronic toxicities appear months or years after the completion of the treatment and include fibrosis, vascular damage, or atrophy of the affected tissue or organ [3]. Late chronic toxicities may develop as consequential effects of severe non-healing acute toxicities, for instance, prostate cancer patients treated with radiotherapy who experience acute gastrointestinal (GI) toxicities are significantly more likely to develop late GI toxicities such as long-term diarrhea [12]. However, there is a variation in the incidence and severity of radiotherapy-induced toxicities between patients [13]. Among patients with the identical tumor size, site and stage, who receive the same treatment schedule, some patients will develop severe toxicities while some will not [14]. Among the identified risk factors of developing toxicities are therapy-related factors (radiation dose, volume, fraction and site, and concomitant therapies) and patient-related factors (age, gender, smoking, and comorbidities) [13]. In addition, studies have shown that genetic variations also may contribute to the risk of severe toxicities following radiotherapy [15]. However, these factors still poorly estimate the risk of toxicities and there is a need for more accurate and sensitive predictive markers.

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Article highlights

- Radiotherapy is a key treatment modality for solid tumors, but the variation in patients' response to radiotherapy and severity of radiotherapy-induced mucositis is still largely unexplained.
- Gut microbiota plays a major role in the development and modulation of intestinal and systemic immune responses.
- Previous studies have found that gut microbiota contributes to the pathogenesis of radiotherapy-induced gastrointestinal mucositis.
- Research has revealed that gut microbiota composition can be used as a predictive marker for the development of radiotherapy-induced diarrhea and fatigue.
- Individuals' gut microbiota composition potentially influences their response to radiotherapy through the modulation of immune responses.
- Future research to investigate gut microbiota impact on the incidence and severity of radiotherapy-induced oral mucositis is warranted with a view to modulate composition to improve cancer therapy outcomes.

This box summarizes key points contained in the article.

In recent years, we have witnessed a growing interest in the impact of the gut microbiota on modulating cancer treatment efficacy and toxicity. Gut microbiota refers to a collection of microorganisms such as bacteria, viruses, archaea, and eukarya that reside in the human GI tract [16]. It is estimated that more than 100 trillion microbes, mainly bacteria, with 500–1000 different species, are found in the GI tract [17,18]. These microorganisms can be found in the mouth, stomach, small and large intestine but the diversity and load substantially increase distal to the ileocecal junction [19]. The microbiota composition varies along the different sites of the GI tract and the dominant phyla of gut microbiota are *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria*, with around 90% of them belonging to *Firmicutes* and *Bacteroidetes* [19]. However, there is a significant interindividual variation in the gut microbiota composition and even a single individual's microbes composition changes over time [20]. Host genetics [21] and several environmental factors such as age, diet, disease, medications, and lifestyle can influence the composition of gut microbiota [22]. The gut microbiota exists in a mutualistic symbiotic relationship with the host, in which the host provides nutrients and environment necessary for the microorganisms' survival. In turn, microbiota contributes to several physiological processes of the host such as digestion, carbohydrate fermentation, immune response modulation and prevention of pathogenic colonization [18].

The alteration of the gut microbiota composition, also known as dysbiosis, has been linked to the pathogenesis of intestinal diseases, such as inflammatory bowel disease and celiac disease, and extra-intestinal diseases including obesity, allergies and type 2 diabetes [23,24]. The microbiota contributes to the development of these disorders primary through the interaction between the microbiota or their metabolic products and the host immune system. For instance, inflammatory bowel disease is associated with alterations in the abundance and diversity of gut microbiota. Changes in the microbial composition can lead to disruption in metabolites production, intestinal barrier and intestinal immune and inflammatory responses, therefore, contributing to inflammatory bowel disease pathogenesis [25]. Moreover, dysbiosis can enable overgrowth of pathogenic microorganisms such as *Clostridium difficile*. The loss of colonization resistance and imbalanced immune responses during dysbiosis contributes to the pathogenesis of *Clostridium difficile* infection [26]. Recently, it has been revealed that gut microbiota influences tumor response [27–30] and severity of cancer treatment-induced GI toxicities [31–34]. The gut microbiota has been shown to affect both the efficacy and toxicity of various chemotherapies and immunotherapies via several mechanisms, primarily through the modulation of immune responses [35]. However, little is known about the role of the microbiota in response to radiotherapy [36]. In this review, we will explain how immune responses affect cellular responses to radiation. In addition, we will discuss the potential impact of gut microbiota on radiotherapy anti-tumor activity and its role in radiotherapy-induced GI mucositis through immunomodulation.

2. Effect of immune signaling on cellular response to radiotherapy

Radiotherapy causes tumor cell death by depositing high energy radiation in the cell which induces DNA damage. Ionizing radiation causes DNA damage directly or indirectly through the production of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [37]. Exposure to ionizing radiation can induce tolerogenic or immunogenic cell death depending on radiation dose and cell type. Exposure to low dose radiation (< 1 Gy) causes tolerogenic apoptosis in which dying cells are engulfed by macrophages and is associated with the release of anti-inflammatory cytokines including Transforming growth factor beta 1 (TGF-β), interleukin-10 (IL-10) and prostaglandin E2. Conversely, the exposure to a higher radiation dose, like doses received during cancer treatment, can lead to immunogenic cell death which is associated with the release of pro-inflammatory cytokines (e.g. IL-1, IL-6, tumor necrosis factor-alpha (TNF-α)) [38,39]. The activation of inflammatory responses prolongs the radiation response by generating more ROS, cytokines and growth factors [40]. This has been implicated in both radiotherapy-induced tumor cell death and normal tissue toxicities.

2.1. Radiation-induced immunogenic cell death

Radiation can induce tumor cell death via apoptosis, senescence, mitotic catastrophe and necrosis [41]. Generally, apoptosis is considered to be non-immunogenic, however accumulating evidence has demonstrated that, in some settings, apoptosis can be immunogenic [42]. In animal models, it has been found that ionizing irradiation and specific chemotherapy agents such as anthracyclines and oxaliplatin can induce an anti-tumor immune response and results in immunogenic cancer cell death [43–46]. Immunogenic cell death (ICD), or immunogenic apoptosis, involves stimulation of the immune response against dying tumor cells and is mediated by 'damage-associated molecular patterns' (DAMPs), which are molecules released or expressed by dying tumor cells [47]. Expression or release of these molecules leads to the activation of antigen-presenting cells (APCs), particularly dendritic cells (DCs). Activated APCs then engulf tumor cell antigens, process them and present them to cytotoxic T lymphocytes and subsequently induce a tumor-specific immune response [48] (Figure 1).

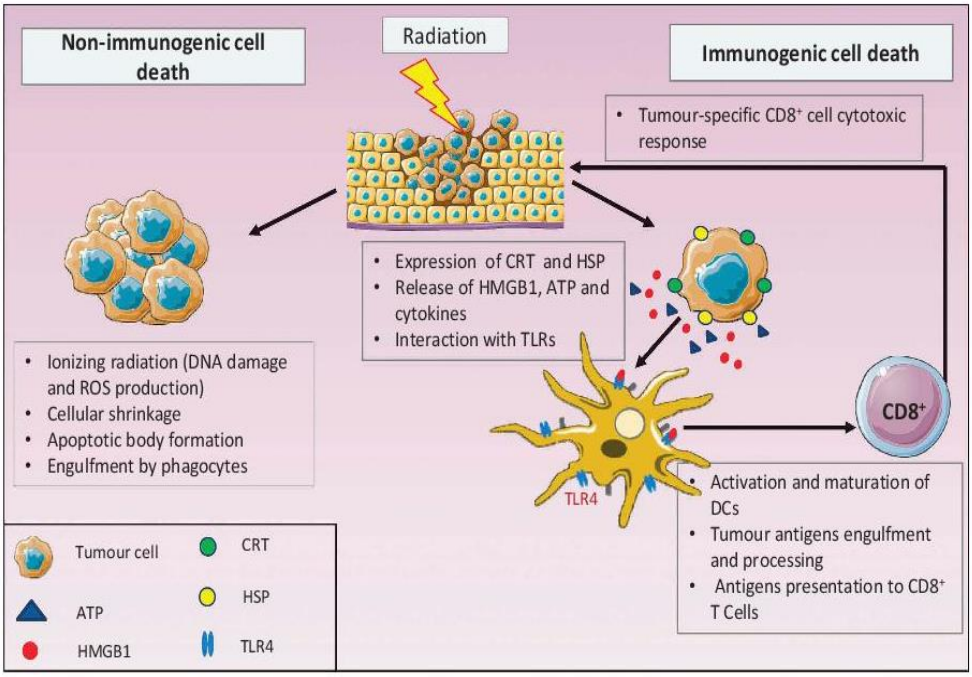

Figure 1. Immunogenic and non-immunogenic tumor cell death caused by radiation. For non-immunogenic cell death, radiation causes direct cell death through DNA damage and ROS production leading to cellular shrinkage and apoptotic body formation. Cellular debris is engulfed by phagocytes without eliciting an immune response. In immunogenic cell death, radiation causes cellular injury leading to the release of DAMPs that interact with TLRs resulting in DC activation. Activated DCs stimulate CD8+ cells, which in turn induce a tumor-specific anti-tumor response. (HAMG1: high-mobility group box-1; CRT: calreticulin; HSP: heat-shock proteins; TLR4: Toll-like receptor 4).

The best-characterized DAMPs include ATP, genomic DNA, calreticulin (CRT), high-mobility group box-1 (HMGB1), heat-shock proteins (HSPs), and pro-inflammatory cytokines such as IL-1α and IL-6 [47]. These molecules interact with vesicular or membrane-bound pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) resulting in activation of the immune response [49]. Gameiro et al. investigated radiation-induced immunogenic modulations *in vitro* and *in vivo* settings. The irradiation of three different tumor cell lines (lung, breast, and prostate human carcinoma) induces the release of ATP and HMGB1 from dying and surviving cancer cells. In addition, when injecting nude mice with prostate carcinoma cells, radiation increases the expression of antigen-processing machinery and CTR in tumor cells which enhances their sensitivity to cytotoxic T-cell killing [50]. Moreover, radiation can induce the production, cell surface expression and extracellular secretion of HSPs leading to the stimulation of anti-tumor immune response through the activation of T and natural killer cells (NKs) as reviewed by Multhoff et al. [51]. Furthermore, Yoshimoto et al. demonstrated that local radiotherapy induces a systemic tumor-specific immune response and that anti-tumor immunity is essential for radiotherapy efficacy. In tumor-bearing mice, they found that depletion of $CD8^+$ cells significantly reduces radiotherapy-induced delay in tumor growth and mice survival time, hence reducing radiotherapy efficacy [43].

TLRs are a class of PRRs that play a key role in ICD and mediate the interaction between DAMPs and immune cells [52]. They are transmembrane proteins expressed by different types of cells including DCs, macrophages and epithelial cells. TLR signaling is mediated through myeloid differentiation primary response protein-88 (MyD88)-dependent pathway or MyD88-independent pathway. The MyD88-dependent signaling pathway is used for all TLRs except TLR3 and is required for the production of pro-inflammatory cytokines. The Myd88-independent signaling pathway is mediated by TIR-domain-containing adaptor protein inducing interferon-β (TRIF) and is required for TLR3 and TLR4 signaling and leads to the production of type I interferons (IFN) [53]. TLR4 is a cell surface homodimer protein that recognizes microorganism-derived 'microbial-associated molecular patterns' (MAMPs) such as lipopolysaccharides (LPS) or DAMPs such as HMGB1, resulting in the release of pro-inflammatory cytokines [54,55]. One study has found that that TLR4 expression by DCs is essential for the efficient presentation of dying tumor cells antigens to immune cells. The release of HMGB1 by cancer cells and activation of the TLR4–MyD88 dependent pathway enhances anti-tumor immune response and radiotherapy and chemotherapy efficacy [55]. However, this study demonstrated that mice with deleted TRIF have a similar response to chemotherapy as wild-type mice [55]. Moreover, in another study, it has been shown that radiotherapy increases the production of type I IFNs which enhances the cross-priming ability of DCs and improve tumor response to radiotherapy. However, the increase in IFN-β production was independent of TRIF-dependent TLR signaling [56]. Together this may suggest that TLR4–MyD88 dependent but not TLR4–MyD88 independent pathway has an impact on tumor response to cancer treatments.

Radiation-induced ICD indicates that the immune system plays a role in radiotherapy activity and tumor control, thus, the efficacy of radiotherapy may be improved through the

modulation of immune responses and communication between tumor cells and the immune system. Given this, research on factors that may influence immune responses to radiotherapy, such as interaction with microbial factors, is warranted and may lead to improving individual's response to radiotherapy.

Although immune responses can improve radiotherapy responses through ICD, some studies have found that inflammatory cell infiltration and secreted inflammatory mediators may contribute to tumor progression by promoting tumor growth and angiogenesis [57]. Different types of immune cells are recruited to the tumor microenvironment including NKs, tumor-infiltrating lymphocytes ($CD4^+$ and $CD8^+$), B cells, and a range of myeloid-derived cells (tumor-associated macrophages (TAM), DCs, and neutrophils) [58]. Infiltrating lymphocytes and NKs are important for anti-tumor immune responses. However, myeloid-derived cells can enhance or reduce radiation anti-tumor immune responses. DCs have a critical role in the cross-priming of tumor antigens to cytotoxic $CD8^+$ T cells leading to tumor-specific immune response [59]. A recent review has demonstrated that although some evidence indicates that radiation induces pro-tumorigenic phenotypes of TAM that enhance angiogenesis, tumor growth and invasion, others have shown that radiation induces the programming of TAM toward pro-inflammatory phenotypes that enhance the anti-tumor response [60]. Therefore, to maximize radiation response, we need to overcome the pro-tumorigenic effect of TAM and enhance the communication between tumor and APCs.

2.2. *Immune signaling and radiation-induced normal tissue toxicities*

Immune signaling pathways, primarily inflammatory responses, are involved in acute and late radiotherapy-induced normal tissue toxicities including gastrointestinal mucositis, pneumonitis, and fibrosis [38,61]. The development of these toxicities is caused by direct exposure to ionizing radiation, ROS production, and release of inflammatory cytokines. Inflammatory mediators cause amplification of ROS production through stimulation of ROS and RNS-producing enzymes such as cyclooxygenase-2 (COX-2), NADPH oxidase, Nitric oxide synthase (NOS) [62].

Overproduction of free radicals causes oxidative damage to both irradiated and non-irradiated bystander cells and contribute to acute and chronic complications [63]. Thus, targeting inflammatory responses and redox system pathways is a potential approach to ameliorate normal tissue toxicity following radiotherapy.

3. Gut microbiota modulates intestinal and systemic immune responses

Gut microbiota plays a critical role in the development and modulation of both mucosal and systemic immune responses. Germ-free (GF) mice are associated with many defects in the intestinal immunity at the tissue, cellular and molecular levels [64]. GF mice have significantly small Peyer's patches and reduced numbers of IgA-producing plasma cells and $CD4^+$ T cells in the lamina propria [65]. Moreover, they have a systemic T-cell deficiency, reduced production of IFN-γ, and $CD4^+$ T cells are biased toward T helper 2 (Th2) cells [66]. Furthermore, the secondary lymphoid organs such as peripheral lymph nodes and spleen of GF mice are less cellular and have poorly developed T and B cells zones [67]. Gut microbiota modulates intestinal immunity through the interaction with PRRs, mainly TLRs. In the intestine, TLRs that are expressed by enterocytes and DCs, recognize several MAMP molecules on the bacterial cell surface such as LPS and peptidoglycan [68]. The microorganisms maintain intestinal homeostasis by regulating the balance between anti-inflammatory and pro-inflammatory signals. For instance, it has been demonstrated that commensal *Bacteroides fragilis* facilitates the development of Regulatory T (Treg) cells, which produce anti-inflammatory IL-10 [69]. Conversely, the segmented filamentous bacterium (SFB) induces the differentiation and generation of Th17 cells, which produce pro-inflammatory cytokines in the small intestine [70].

Systemically, microbiota metabolites such as short-chain fatty acids (including butyrate and propionate) have been found to promote Treg extrathymic generation, suggesting that by-products can mediate the gut microbiota communication with the immune system and modulate the pro- and anti-inflammatory response equilibrium [71]. Furthermore, gut microbial dysbiosis is associated with several immune-mediated disorders such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis, as well as obesity, autism, and type 2 diabetes [24,72]. In GF mice, arthritis was attenuated due to the reduction in autoantibodies, splenic Th17, and autoantibodies-secreting cells. However, the introduction of SFB into GF mice led to the development of arthritis and restored Th17 cells and autoantibody production [73].

4. Gut microbiota and radiotherapy efficacy

The gut microbiota has recently been suggested to play a major role in cancer pathogenesis and response to cancer treatment [36,74]. Gut microbiota interacts with cancer treatments in a bidirectional manner. Anticancer treatments disrupt intestinal microbiota composition and promote dysbiosis. Kim et al. characterized the mouse gut microbiota and revealed that radiation causes significant alteration in both the abundance and diversity of microbiota with an increase in *Alistipes* and a decrease in *Mucispirillum* genus [75]. In addition, a clinical study showed that exposure to pelvic radiotherapy results in the remodeling of overall gut microbiota composition, with 10% decrease in phylum *Firmicutes* and 3% increase in phylum *Fusobacterium* [33]. Generally, the most significant alterations in the gut microbiota associated with cytotoxic chemotherapy or radiotherapy are the increase in *Bacteroides* and *Enterobacteriaceae* and the decrease in *Bifidobacterium, Faecalibacterium prausnitzii* and *Clostridium cluster XIVa* [76].

Evidence also indicates that gut microbiota affects anticancer treatment activity and side effects. The gut microbiota modulates chemotherapy efficacy through 'TIMER' mechanisms which include 'Translocation, Immunomodulation, Metabolism, Enzymatic degradation, and Reduced diversity' [35]. Immunomodulation is an important mechanism by

which gut microbiota influence the response to chemotherapies (cyclophosphamide and oxaliplatin) and immunotherapies (CpG-oligodeoxynucleotides, CTLA-4 inhibitors, and anti-PD-L1) [35]. Viaud et al. demonstrated that Gram-positive gut bacteria induces Th17 and Th1 cell immune responses which are critical for anti-tumor activity of cyclophosphamide in tumor-bearing mice [27]. Another study, in mouse models, demonstrated that the disruption of gut microbiota impacts the response of subcutaneous tumors to oxaliplatin, due to reduced production of ROS by myeloid cells, and to CpG-oligonucleotide due to the poor response of myeloid-derived cells and reduction in cytokine production [28]. Moreover, gut bacteria are critical for CTLA-4 blockade (ipilimumab) anti-tumor response. Vétizou et al. showed that in antibiotic-treated and GF mice, Ipilimumab anti-tumour activity was compromised compared to specific pathogen-free mice. However, treating mice with *Bacteroidales spp* induces immunostimulatory effects, mediated by IL-12–dependent Th1 immune response, resulting in better tumor control [29]. Furthermore, commensal *Bifidobacterium* induces a tumor-specific immune response and enhances the response to anti-PD-L1 immunotherapy in melanoma mouse models. This effect is mediated by enhancing DC function which increases the accumulation of $CD8^+$ T cells in the tumor microenvironment [30]. Clinically, the use of antibiotics against Gram-positive bacteria impairs patients' response to cisplatin and cyclophosphamide chemotherapy. Among patients with chronic lymphocytic leukemia and relapsed lymphoma, the use of gram-positive active antibiotics was associated with early tumor progression and lower overall survival [77].

Currently, no studies have investigated the impact of gut microbiota on radiotherapy efficacy. However, some studies have found that gut microbiota impacts normal tissue radiosensitivity [78–80]. Crawford and Gordon (2005) investigated the role of gut microbiota in intestinal radiosensitivity and found that the small intestine of GF mice is resistant to radiation injury. The deficiency of fasting-induced adipose factor, which is normally suppressed by microbiota, enhances the villus endothelium and lymphocytes radiation-induced cell death [78]. Moreover, a study conducted by Cui et al. demonstrated that gut microbiota disruption impacts radiosensitivity in conventional mice. It was found that disruption of the circadian rhythm of mice is associated with a reduction in the abundance of gut microbe species and that the change in the gut microbiota composition increases mouse sensitivity to gamma-ray irradiation. When mice were exposed to 5 Gy total body irradiation (TBI), those with altered circadian rhythm have a lower survival rate compared to those which housed in a 12 h dark/12 h light cycle. This suggests that circadian rhythm may have a gut microbiota-dependent effect on the radiation response [79]. Evidence from clinical studies also showed that circadian rhythm may impact radiotherapy local control and toxicities. Patients treated in the morning have better local control and less severe toxicities [81]. Moreover, a preclinical study showed that gut microbiota is important for the efficiency of TBI preconditioning. In this study, mice were injected with melanoma cells and exposed to 5 Gy of TBI followed by adoptive transfer of pmel-1 $CD8^+$ T cells, which are splenocytes activated *in vitro* in the presence hgp100 tumor antigen that is highly expressed in human melanomas. Lymphodepletion using TBI before adoptive transfer of tumor-reactive $CD8^+$ T cells enhances tumor control. However, depletion of gut microbiota with antibiotics, LPS neutralization or TLR4 deletion, reduces the effectiveness of TBI, subsequently decreasing the efficiency of adoptively transferred $CD8^+$ T cells to attack tumor cells. TBI induces the translocation of the gut microbiota, and elevates LPS which interacts with TLR4 leading to innate immune activation; hence, enhancing $CD8^+$ T cell activation and improving tumor regression [80].

Although this work does not prove direct causality in terms of microbiota and radiotherapy efficacy, it does indicate a biological signal needing further exploration. Therefore, based on the current evidence and given that immune responses are involved in the radiation-induced cell death, it can be hypothesized that the gut microbiota plays a role in radiotherapy immunostimulatory effect hence, impacting the tumor response to radiotherapy. This immunostimulatory effect of gut microbiota is potentially mediated by Th17 and $CD8^+$ T immune responses (Figure 2).

5. Gut microbiota and radiotherapy-related GI toxicities

Preclinical and clinical studies have revealed that the gut microbiota contributes to the pathogenesis of chemotherapy, radiotherapy, and immunotherapy-related GI toxicities. The exposure to cytotoxic agents or radiation therapy causes intestinal crypt cell apoptosis, disruption of the mucosal barrier and changes the microbiota composition. This results in bacterial translocation and subsequently immune system activation and gut inflammation. The gut microbiota has been found to play a role in chemotherapy and immunotherapy-related GI toxicities such as irinotecan-induced diarrhea and ipilimumab-induced colitis [35]. Furthermore, gut microbiota plays a role in radiation-induced toxicities including alimentary tract mucositis.

5.1. Radiotherapy-induced alimentary tract mucositis

Mucositis is defined as inflammation or ulcerative lesions that affect the mucosa of the GI tract or oral cavity [82]. Radiotherapy can cause both GI mucositis (GIM) and oral mucositis (OM) depending on the structures receiving radiation. The exposure to ionizing radiation leads to the initiation of GIM and OM which develop through a five-stage model [83]. Mucositis pathobiology has been described elsewhere extensively [83,84] but involves in brief; the initiation of tissue injury by radiation followed by inflammation upregulation and amplification which involve the production of pro-inflammatory cytokines such as IL-1β and IL-6 and TNF-α. This leads to ulceration and enhanced inflammation due to interactions with microbial products crossing the breached epithelium. Healing is the final stage which involves extracellular matrix signaling and the proliferation of epithelial cells restoring mucosal integrity. Three signaling pathways have been

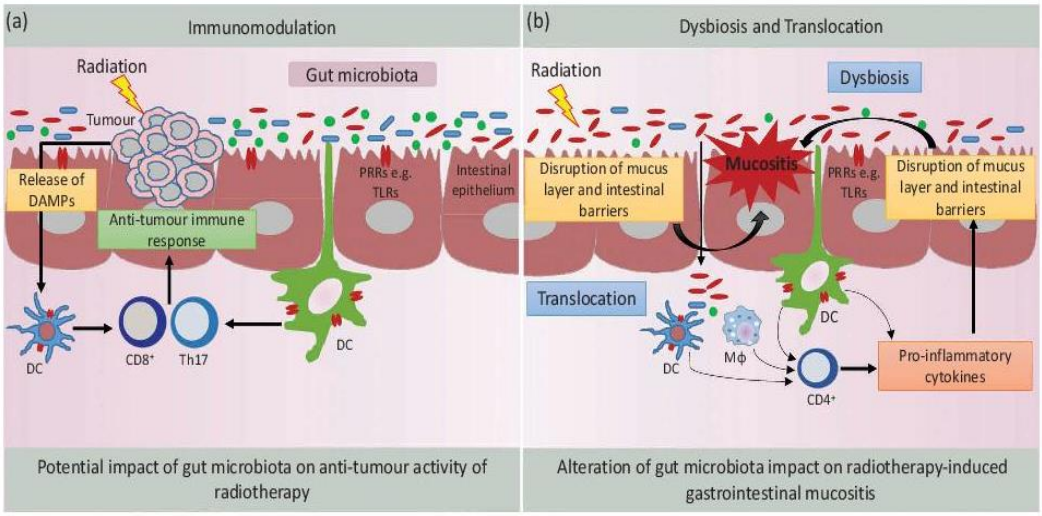

Figure 2. Gut microbiota impact on radiotherapy response and radiotherapy-induced gastrointestinal mucositis through modulation of immune responses. (a) gut microbiota potentially impacts the response to radiation by interacting with immune cells (e.g. DCs) in the intestine and enhancing the innate immune responses; hence improving the anti-tumor immune response which is mediated by Th17 and CD8+ cells. (b) radiation causes gut microbiota translocation and dysbiosis which disrupt intestinal immune homeostasis and release of pro-inflammatory cytokines. This leads to further damage of intestinal barriers; therefore, enhancing radiation-induced gastrointestinal mucositis. (DC: dendritic cells; M?: macrophages; PRRs: pattern recognition receptors, TLRs: Toll-like receptors; DAMPs: damage-associated molecular patterns; Th17: T helper 17 cells).

implicated in radiation-induced mucositis; 1) nuclear factor-κB (NF-κB), 2) NLR-related protein 3 nucleotide-binding domain leucine-rich repeat containing receptor-related protein 3 (NLRP3) inflammasome, and 3) mitochondrial dysfunction. Ionizing radiation causes the production of ROS which activates the NF-κB pathway leading to the release of pro-inflammatory cytokines (IL-1β and IL-6 and TNF-α) and hence inducing an inflammatory response. This inflammatory response and ROS contribute to mitochondrial dysfunction which leads to amplified ROS production from impaired mitochondria resulting in the activation of NLRP3 inflammasome pathways. Activated NLRP3 leads to the production of more IL-1β through the activation of caspase-1 [85].

The frequency and severity of mucositis differ from one patient to another. Currently, conventional patient-related factors such as genetics, age, gender, lifestyle or therapy-related factors, like treatment type, dose, and schedule, are used for the prediction of those at of higher risk of developing GIM and OM. These factors are unreliable and still underestimate the toxicity risk, hence new alternative risk predictive markers are needed [86]. The gut microbiota has been found to play a role in the pathogenesis of radiation-induced GIM [76]. This impact is potentially mediated through influencing and modulating the oxidative stress and inflammatory process, intestinal permeability, mucus layer composition, epithelial repair and harmful stimuli resistance, and expression and release of immune effector molecules in the intestine [87]. The gut microbiota may contribute to radiation-induced GIM through two mechanisms; translocation and dysbiosis. Radiation disrupts the intestinal barriers and mucus layer and causes bacterial translocation resulting in inflammatory response activation. Moreover, dysbiosis due to radiation or due to other factors can influence both local and systemic immune responses (Figure 2).

5.2. Radiotherapy-induced gastrointestinal mucositis

GIM is a debilitating side effect of radiotherapy that significantly impacts patients' quality of life [88], and may lead to treatment delays or dose reductions, compromising treatment outcomes. GIM is associated with several symptoms including abdominal pain, rectal bleeding, diarrhea, fatigue, and infections [76]. One of the most common symptoms of radiotherapy induced-GIM is diarrhea. It affects more than 80% of cancer patients receiving pelvic radiotherapy [89]. Interestingly, some cancer patients develop severe diarrhea following radiotherapy and some do not [32]. One of the investigated factors of radiotherapy-related diarrhea is gut microbiota dysbiosis (Table 1). In a pilot clinical study, which involved 10 patients receiving pelvic radiotherapy and 5 healthy controls, the development of diarrhea (in 6 patients) was associated with a significant modification in gut microbiota composition compared to patients who did not develop diarrhea. *Firmicutes* phylum diversity was increased among patients with diarrhea, however, *Actinobacteria* phylum was not detected in the samples of the patients who didn't develop diarrhea. In addition, the analysis of the pre-radiotherapy stool samples of those who developed diarrhea indicated that their microbiota composition was different from the control group and patients who did not develop diarrhea. This suggests that pre- radiotherapy microbiota composition could be a predictive marker for radiation-induced diarrhea [32]. In another small clinical study, Nam et al. investigated the impact of gut microbiota composition in 9 patients receiving pelvic radiotherapy for gynecological cancers. This study revealed that there are significant differences in the gut microbiota of patients and healthy individuals before radiotherapy. This study also showed that, following radiotherapy, the 8 patients that developed diarrhea had a significant change in their gut

Table 1. Clinical studies to investigate impact of microbiota in the pathogenesis of radiotherapy toxicities.

Study	Study subjects	Treatment type	Toxicity	Key findings
Gut microbiota				
Manichanh et al. [32]	10 patients with abdominal cancer 5 healthy controls	Pelvic radiotherapy (1.8–2.0 Gy/ day, 5 times/ week, 5 weeks)	Diarrhea	Controls and patients without diarrhea had a stable microbial diversity over 7 weeks period Patients who developed diarrhea had a significant modification in their microbial diversity *Actinobacteria* phylum not detected in patients without diarrhea Higher diversity in the *Firmicutes* phylum in patients with diarrhea
Nam et al. [33]	9 female patients with gynecological cancer Data of six healthy controls	Pelvic radiotherapy (50.4 Gy/day, 5 times/ week, 5 weeks)	Diarrhea	Significant differences in gut microbiota between cancer patients and controls Patients who developed diarrhea had a marked change in gut microbiota after radiotherapy
Wang et al. [34]	11 patients with colorectal, anal, cervical cancer 4 healthy controls	Pelvic radiotherapy (1.8–2.0 Gy/day, 5 times/ week, 5 weeks)	Fatigue and diarrhea	Significant differences in relative abundance of some genera between patients who developed or those who did not Patients who developed diarrhea had lower alpha diversity and higher *Firmicutes to Bacteroides* ratio
Oral microbiota				
Zhu et al. [108]	41 patients with Nasopharyngeal carcinoma 49 healthy controls	Radiotherapy (2.0 Gy/day, 5 times/ week, 6–7weeks) (alone/with concomitant therapies)	Oral and oropharyngeal mucositis	Healthy controls have more diverse and more similar bacterial composition Increase in relative abundance of Gram-negative bacteria (mostly belong to phylum *Proteobacteria*) as mucositis develop to peak severity Patient with severe mucositis had a significantly higher abundance of *Actinobacillus spp* and lower bacterial diversity

microbiota composition [33]. Furthermore, Wang et al.'s study of 11 patients receiving pelvic radiotherapy and 5 healthy controls found that gut microbiota dysbiosis both pre and post radiotherapy was associated with an increased risk of fatigue and diarrhea. Moreover, significant differences in the relative abundance of selected genera such as *Veillonella, aecalibacterium*, and *Clostridia clusters XI and XVIII* was observed between patients who developed diarrhea and those who did not. Patients with low microbiota diversity and high *Firmicutes/Bacteroides* ratio are more likely to develop diarrhea [34]. Although these are small studies, they clearly indicate that gut microbiota composition is involved in the pathogenesis of radiation-induced GIM.

Furthermore, evidence from clinical studies has shown promising results for the use of different probiotic preparations to prevent or alleviate radiotherapy-induced GIM, mainly diarrhea [89–91]. Moreover, MASCC/ISOO guidelines support the use of probiotic preparations of Lactobacillus species for the prevention of radiotherapy-induced diarrhea [92]. However, the findings of a recent meta-analysis indicated that the current evidence shows no benefits of the use of probiotics for the prevention of radiotherapy-induced diarrhea and suggests that future research should focus in pairing the GI toxicities with certain microbial phenotypes to allow targeted microbiota manipulation [93].

5.3. Toll-like receptors and radiotherapy-induced gastrointestinal mucositis

The gut microbiota interacts with TLRs expressed on epithelial and immune cells to maintain intestinal homeostasis. The depletion of gut microbiota using broad-spectrum antibiotics in mice has been associated with increased susceptibility to methotrexate-induced gastrointestinal injury, which is suppressed by the administration of TLR2 ligands [31]. Conversely, the knockout of TLR4 in mice has been shown to reduce irinotecan-associated pain and gut toxicity [94]. Additionally, in mice, the administration of LPS (a component of Gram-negative bacteria membrane) before radiation, protects intestinal crypts via induction of COX-2 and the production of prostaglandins [95]. LPS stimulates TLR4-expressing cells, leading to the release of TNF-α which interacts with the TNF receptor on the surface of subepithelial fibroblasts, leading to the production of prostaglandins that reduced radiation-induced apoptosis of epithelial stem cells [96]. Another potential mechanism of TLR protection from radiation is the activation of NF-κB pathway [87]. Egan et al. showed that the activation of NF-κB signaling was essential for the protection of the gut against radiation-induced apoptosis. In addition, this study showed that NF-κB activation mediates LPS radioprotection [97]. This suggests that TLRs may impact the intestinal response to radiation-induced epithelial damage through the NF-κB pathway.

5.4. Radiotherapy-induced oral mucositis

Oral mucositis (OM) is a common adverse effect of cancer treatments and it impacts 20% to 100% of cancer patients depending on the type of treatment they receive [98]. Among head and neck cancer (HNC) patients receiving radiation doses between 50–54 Gy, more than 90% develop OM, and about 60% of these develop severe OM (grades 3 and 4) [99]. OM negatively affects patients' quality of life, therapy, and economic outcomes. Most patients with OM cannot eat by mouth due to severe pain, and often require parenteral nutrition, subsequently, patients also experience severe weight loss [99,100]. Furthermore, OM is dose-limiting and it may cause therapy interruption which negatively affects tumor outcome

and patient survival. Some patients with OM may require extended hospitalization; hence, increasing economic cost. In one study, 11% of the HNC patients treated with radiotherapy had unplanned treatment interruption and 16% of them required hospitalization due to OM [100].

To date, there is no broadly effective treatment or preventive measure for OM. Management of OM is mainly supportive care to reduce pain, provide patients with nutritional support, and treat secondary infections [101]. Several risk factors have been found to impact the incidence and severity of OM including patient-related factors (genetics, age, gender, body mass index (BMI), oral hygiene, and tobacco use) and therapy-related factors (radiation site, dose, and fractionation, and combined therapies) [102]. Recent studies have investigated the genetic polymorphisms associated with the risk of OM development. Single nucleotide polymorphisms (SNPs) in radiosensitivity gene (*XRCC1*) [103], DNA repair gene (*Ku70*) [104], TNF receptors gene (*TNFRSF1A*) [105] and transcription factor gene (*ZNF24*) [106] were associated with increased risk of OM among HNC patients. Although many factors have been identified, there is still a lack of clear predictive markers for OM that can be modified to reduce incidence and severity.

For radiation-induced OM, no study has investigated the impact of gut microbiota composition in OM incidence and severity. However, few studies have examined the association between oral microbiota and OM development. It has been found that radiation changes the diversity and functional behaviors of the oral microbiota in patients with radiotherapy-induced OM [107]. In addition, Zhu et al. found that alteration of the oral microbiota is associated with the progression and severity of OM. Their study included 41 nasopharyngeal carcinoma patients receiving 3D-conformal radiation therapy. The oral microbiota analysis revealed that the Gram-negative bacteria relative abundance increased as mucositis reached peak severity, and those with severe OM had lower bacterial alpha diversity and higher abundance of *Actinobacillus spp* [108] (Table 1). However, the use of antibiotics, in the form of paste [109] or lozenges [110], to selectively eliminate oral microbiota have failed to reduce or prevent radiation-induced OM among HNC patients who were treated with radiotherapy. Moreover, the use of topical (lozenges/paste) antimicrobial agents is not recommended for the prevention of OM [111]. However, Sharma et al. demonstrated that the use of lozenges of *Lactobacillus brevis* CD2 in HNC patients receiving chemo-radiotherapy is associated with reduced OM incidence and higher treatment completion rate [112]. This may suggest that probiotics may have a better impact on alleviating OM compared to antibiotics, and further studies are warranted.

Oral microbiota may play a role in the pathogenesis of radiotherapy-induced OM; however, it is not an ideal predictive marker for the OM risk. There are several challenges for the use of oral microbiota composition as a predictive marker for radiation-induced OM. First, different sites of the mouth harbor different microbiota species which results in sampling variations and biases and make it difficult to determine which species is actually associated with OM. Additionally, radiation changes saliva composition and volume which impacts microbiota diversity. Salivary glands are radiosensitive and the exposure to radiation therapy can cause gland damage, resulting in hypofunction and xerostomia [113]. Radiotherapy in patients with HNC is associated with hyposalivation, reduction in saliva buffering pH and an increase in *Lactobacilli* and *Candida* species [114]. The changes in saliva quality and quantity result in less flushing, buffering, and immune function. Moreover, radiotherapy-induced xerostomia reduces the oral environment proteins and immunological properties and increases the acidity leading to the increase in the acidogenic microbes such as *Lactobacillus spp, Actinomyces Streptococcus mutans*, and a decrease of other types of microorganisms such as *Fusobacterium, Neisseria* and *Streptococcus sanguis* [115]. Therefore, oral microbiota is not a good marker for predicting the risk of radiation-induced OM.

6. Conclusion

Gut Microbiota has been found to contribute to cancer chemotherapy and immunotherapy response by modulation anti-tumor immune responses. However, how it impacts radiotherapy response is yet to be explored. Radiotherapy can induce an anti-tumor immune response, hence, there is a potential that gut microbiota impacts the variation on radiotherapy response through immunomodulation. Moreover, gut microbiota has been shown to contribute to the pathogenesis of radiotherapy-induced GIM by influencing intestinal barriers and the modulating inflammatory responses in the intestine. In addition, it potentially impacts the severity of radiotherapy-induced OM by influencing inflammation and ulceration stages of OM, therefore, future research to investigate this effect is warranted.

7. Expert opinion

Radiotherapy is an essential cancer treatment modality, but the variation in tumor response and recurrence among patients is still largely unexplained. The identification of potential factors that contribute to this variation and their eventual modulation may help to improve tumor response to radiotherapy and reduce radiotherapy toxicities. Gut microbiota composition is a potential factor that may be a determinant of tumor response to radiation. First, previous work has revealed that radiation can induce immunogenic cell death through stimulation of tumor-specific immune responses that enhance tumor control. Second, studies have demonstrated that gut microbiota is critical for the anti-tumor activity of some chemotherapies and immunotherapies which also induce immunogenic cell death. Finally, animal studies have found that gut microbiota is an important determinant of intestinal radiosensitivity. Therefore, future preclinical and clinical studies should be conducted to investigate how an individual's gut microbiota may impact tumor response to radiotherapy.

Furthermore, OM is one of the most frequent side effects of radiotherapy and chemotherapy. To date, there are no clear predictive markers for OM risk. Previous research has focused on oral microbiota and its impact on OM. However, since radiation causes substantial damage to the salivary glands and induces xerostomia, the changes in the oral microbiota may be due to hyposalivation and reduced immune functions

of saliva leading to changes in the oral local environment. Thus, oral microbiota is not an ideal predictive marker of OM. The gut microbiota may offer a better marker because the variety and abundance of bacteria are greatly increased in the distal intestine. Furthermore, the impact on the systemic immune response is more related to the gut microbiota compared to oral changes. Hence, local inflammation in the mouth may be modulated more profoundly by gut microbiota impacts on the immune system compared to oral microbiota. Future studies are needed to study the impact of gut microbiota on OM risk and severity.

Due to advances in cancer treatment modalities and technology, survival rates across many cancers is markedly increasing. However, many of these survivors are living with chronic or late toxicities which impact their quality of life. Toxicities may develop due to unnecessary treatment or due to individual risk factors. Therefore, personalized treatment planning and identification of markers to predict individual patients likely to respond to treatment or at risk of developing severe toxicities will help to provide personalized treatment options to improve treatment outcomes. Thorough investigations into the role of the gut microbiota and tumor response to radiotherapy and its impact on the development and severity of radiotherapy-induced tissue injury may help to incorporate the gut microbiota into personalized radiotherapy risk prediction algorithms. Unlike genomic or proteomic biomarkers, gut microbiota can be modified to maximize the response to treatment and minimize adverse effects. This can be done by using personalized probiotics, prebiotics, or fecal microbial transplantation. Although some individual studies have shown promising results of the use of probiotics to prevent or reduce radiotherapy-induced gastrointestinal damage, meta-analyses found these results are not clinically significant. Therefore, future research should focus on the identification of a specific association between radiation-induced toxicities and certain microbial phenotypes to enable the individualized modification of gut microbiota composition. Moreover, research on how to improve the formulation, administration, and absorption of probiotics or prebiotics-based therapies is warranted.

8. Five year view

The inter-individual variations seen in cancer treatment responses and the severity of treatment-related toxicities are major challenges to cancer treatment success. Past research has attempted to identify potential factors that may explain this variation. More recently, we have witnessed a growing interest in studying the relationship between gut microbiota and cancer treatment response and toxicities. Hence, in the coming years, research will focus on targeting the gut microbiota to enhance cancer treatment, including radiotherapy anti-tumor activity. The gut microbiota can be manipulated by various techniques, and with an understanding of what makes an optimal composition, this is an exciting avenue for novel therapies. Moreover, gut microbiota can be used as a prognostic marker to predict the risk of cancer treatment-related toxicities and can be targeted to prevent or reduce cancer treatment-induced toxicities, particularly gastrointestinal toxicities such as mucositis.

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Declaration of interest

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