

Research Article

Dynamic Shifts in the Oral Microbiome during Radiotherapy

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Abstract

Awareness is growing concerning the potential role of the oral microbiota in radiotherapy-induced side effects like mucositis. In a small-scale patient study, we used denaturing gradient gel electrophoresis to visualize the shifts in the oral microbial community during radiotherapy. Samples of the first weeks of irradiation clustered together and towards the end of the therapy the richness decreased (-14 %) and the oral microbial community became dominated by a small fraction of species. The shifts at the level of the buccal mucosa and the tongue were correlated with different clinical parameters and a significant correlation between shifts in the buccal microbial community and the patients' normal oral functioning (pain, nutrition) was observed. By monitoring the microbial shifts during therapy, the role of the microbiota can be further elucidated and treatment regimes can be adapted, increasing patients' quality of life during therapy.

ABBREVIATIONS

DGGE: Denaturing Gradient Gel Electrophoresis; NOF: Normal Oral Functioning; PCR: Polymerase Chain Reaction; CUP: Cancer of Unknown Primary Origin; CT: Chemotherapy; RT: Radiotherapy; AB: Antibiotics; R: Richness; OSCC: Oral Squamous Cell Carcinoma

INTRODUCTION

Radiotherapy is still one of the most important treatment options for patients with head and neck cancer. Unfortunately, apart from the tumour, ionizing radiation also affects the healthy tissue surrounding the target, resulting in serious side effects and an overall decrease in the patients' quality of life. Irradiation of the oral cavity, for example, can have destructive effects on the salivary glands and often leads to hyposalivation (reduced salivary flow) and xerostomia (dry mouth syndrome) [1,2]. This interruption of the salivary flow and associated xerostomia following radiation therapy have previously been linked with shifts in the oral microbiome, resulting in higher abundances of *Streptococcus mutans*, *Lactobacillus* spp., *Candida* and *Staphylococcus* spp. whereas the number of *S. sanguis*, *Neisseria* spp. and *Fusobacterium* spp. tends to decrease [3-5]. These microbial changes might trigger other side effects. The

overgrowth of potential harmful species such as *Candida* spp. (mainly *C. albicans*) or cariogenic species will explain for example the higher prevalence of candidiasis and caries in patients treated with radiotherapy [6-9].

Up to date, no data exist that correlate the shifts in the oral microbiome with the severity of mucositis. Nevertheless strong evidence exists that also in the context of mucositis the oral microbiota can be very important [10,11]. To enhance our current knowledge on the correlation between microbial shifts and particular radiation-related side effects like mucositis, a small-scale prospective study with 10 head and neck cancer patients was performed. To analyse their oral microbiome, we used 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) profiling, a culture-independent approach, as over 30 % of the oral microbial species have not yet been cultivated [12]. Microbial shifts at the level of the buccal mucosa and the tongue were analysed and the correlation with different clinical parameters was investigated.

PATIENTS, MATERIALS AND METHODS

Patients

Ten patients treated with radiotherapy for head and neck cancer were enrolled in the study. All but one of the patients

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- Gini-coefficient

were male and the age ranged from 49-74 y, with a median of 60 y. All patients were treated with intensity-modulated radiotherapy (IMRT) at the department of Radiotherapy at Ghent University Hospital, Belgium (2011-2012). Five patients received concurrent chemotherapy. All patients had different treatment regimens due to the heterogeneity in their tumours and combined therapy modalities. Although the cumulative tumour doses are comparable for all patients (Table 1), the cumulative dose were the buccal and tongue mucosa differed from patient to patient depending on the location of the primary tumour. This results in more severe grades of mucositis, found in patients treated for cancer of the oropharynx or oral cavity.

Oral sampling

The study was approved by the Medical Ethical Committee (Ghent University hospital, B670201110552) and all patients provided their written informed consent prior to the study. To sample the oral microbial community, the oral cavity of the patients was flushed with drinking water before gently wiping the buccal and tongue mucosa ten times with a cotton swab. The upper part of the swab (cotton part) was stored in a sterile eppendorf tube at -20°C until DNA extraction. Samples were taken in the beginning of radiotherapy (all start samples taken before the fifth fraction) and weekly during the treatment (immediately after irradiation) until the end of the therapy. After the therapy, an extra sample was taken within the first 2 months after the therapy (follow-up appointment).

Clinical records

Weekly, all patients enrolled for the study consulted a radiotherapist. Their oral cavity and oropharynx were inspected and the severity of mucositis was scored according to the grading scale of the world health organization. At the time of sampling, patients were interrogated about their oral pain and their ability to eat solid foods. The time point during therapy when patients first suffered from severe pain and/or when they had difficulties to eat (only liquid food possible or the need for percutaneous endoscopic gastrostomy or total parenteral nutrition) was recorded. At this time point, the normal oral functioning (NOF)

was considered to be significantly affected.

All patients' characteristics are listed in table 1.

DNA extraction

Total DNA extraction was performed as described earlier [13]. Briefly, 500 µL Tris-HCl (10 mM, pH 9) and 1 mg lysozyme (VWR, Leuven, Belgium) was added to the cotton swab and the mixture was incubated at 37°C on a rotary shaker for 10 min (250 rpm). 37.5 µL SDS (20 %) was added to the eppendorfs and gently mixed manually for 5 min. 500 µL chloroform-isoamyl alcohol (24:1 (v/v)) was added to extract the DNA. After homogenization of the suspension, the tubes were centrifuged at 7,000 g for 15 min at 4°C and the water phase was recovered. 0.8 volumes of 100 % isopropanol were added and the DNA was allowed to precipitate during at least 1 h at -20°C. By centrifugation at 7,000 g for 15 min at 4°C the DNA was pelleted. After drying the pellet, it was resuspended in polymerase chain reaction (PCR) water (250 µL), and the DNA was purified with the Wizard® DNA Clean-Up System (Promega, Leiden, The Netherlands) according to the manufacturer's protocol. The purified DNA was stored at -20 °C.

Nested PCR

A nested PCR strategy was applied as described previously [13,14]. For the external PCR reaction, we made use of EUB8F (5'-agagtttgatcmtggctcag-3') and 984yR (5'-gtaaggttcytcgcgt-3') primers during 35 PCR cycles (95°C, 30 s; 50°C, 30 s and 72°C, 1 min). For the internal PCR, the primers PRBA338GC (5'-cgcccgccgcgcggcgggcgggcgacgggggactcctacggag-gcagcag-3') and 518R (5'-attaccgcggctgctgg-3') were used for 25 cycles (95°C, 30 s; 53°C, 30 s and 72°C, 1 min) to obtain 16S rDNA gene fragments of 180 bp suitable for DGGE analysis. The TaKaRa Ex Taq™ kit (Westburg, Leusden, and the Netherlands) was used as master mix. Each reaction consisted of 2.5 µL Ex Taq buffer (10x), 2 µL dNTPs, 0.125 µL Ex Taq enzyme, 0.2 µM of each primer, 17.375 µL of PCR water (Sigma-Aldrich, Diegem, Belgium) and 1 µL of DNA. The PCR product of the external PCR was diluted 1:3000 and 1 µL of this dilution was added to 24 µL of mastermix for the internal PCR. The reaction mixtures were incubated in a Biometra thermocycler (Westburg, Leusden, The Netherlands) at

Table 1: Overview of the patients' clinical characteristics.

Patient	Sex	Age(y)	Tumour site	Treatment	Number of RT fractions	Cummulative dose (Gy)	Mucositis (grade)	Hospitaliza-tion (*)	ΔNOF(*)
1	Male	74	Larynx	RT+CT	30	70.2	0	3	2
2	Female	73	Oral cavity	RT+AB	32	69.12	3	/	0
3	Male	60	Oral cavity	RT+CT	32	69.12	4	4	3
4	Male	60	Oropharynx	RT	32	69.12	3	/	3
5	Male	66	Oropharynx	RT+CT	32	69.12	3	6	3
6	Male	56	Oropharynx	RT+CT	30	69.12	3	/	2
7	Male	65	CUP	RT	33	66	3	4	1
8	Male	60	CUP	RT	32	69.12	2	/	3
9	Male	58	CUP	RT+CT+AB	33	66	3	7	6
10	Male	49	Hypopharynx	RT	32	69.12	1	/	0

Abbreviations: CUP= Cancer of Unknown Primary Origin; RT= Radiotherapy; CT= Chemotherapy; AB= Antibiotics /= no hospitalization; ΔNOF= Significant change in patients' normal oral functioning (NOF); * = time during therapy (weeks after start) at which the patient was hospitalized or when the NOF was affected.

94°C for 5 min, followed by 35 or 25 cycles and a final extension at 72°C for 10 min.

DGGE analysis

For the DGGE analysis [15], the PCR samples were mixed with loading buffer (4:1) before separation of the DNA fragments on a 8 % acrylamide gel with a denaturing gradient from 40 - 60% (100 % denaturant contains 7 M urea and 40 % formamide). Electrophoresis was run for 16 h at 120 V, while the gel was maintained at 60°C using the INGENYphorU System (Ingeny International BV, Middelburg, and The Netherlands). DNA bands were visualized using SYBR Green (0.5x). The DGGE profiles were analysed and clustered with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). For this, the different lanes were defined, background was subtracted and the intensity of the lanes was normalized. The calculation of similarities was based on the Dice correlation coefficient. Clustering analysis was performed using Ward's method to calculate the dendrograms. The same homemade marker was used on every gel, allowing inter-gel (inter-patient) analysis.

Richness, Pareto-Lorenz curves and Gini coefficients

The richness (R) of a sample equals the number of bands that were detected on the DGGE profile. The Pareto-Lorenz curves were used to visualize the evenness of a certain microbial community and were constructed as described by Marzorati *et al.* (2008) [16]. Higher evenness is characterized by a curve close to the 45°C diagonal (the theoretical perfect evenness line). Lower evenness points to the dominance of a small fraction of species in the community. The Gini coefficient equals the normalized area between a given Pareto-Lorenz curve and the perfect evenness line.

For this study, the differences in richness and Gini coefficients between the samples at the end and at the start of the therapy were calculated. When the difference equals 0, no changes were found. Negative values point to a lower richness or evenness at the end compared to the start.

Statistics

Statistical analysis was performed in SPSS statistics 22. Spearman's correlation coefficient was used to search for significant ($p < 0.05$) correlations between the patients' normal oral functioning and the Dice-Ward clusters found after DGGE analysis. After a normality check using the Shapiro-Wilk assay, T-tests were used to evaluate if the differences in richness and Gini coefficients were significantly different from zero ($p < 0.05$).

RESULTS AND DISCUSSION

In this study, band wise cluster analysis was performed after DGGE to gain insights into the microbial shifts that occurred during radiation therapy. In 6 out of 10 patients (patient 1, 2, 3, 4, 5 and 7), 2 main clusters could be observed, in which samples of the first weeks of treatment clustered together (Figure 1). Shao *et al.* (2001) also used DGGE analysis to study the shifts in the oral plaque microbiota during radiotherapy. Comparable to our results, they also reported low similarity of the DGGE profiles within one individual [17].

PCR-DGGE analysis was previously proven useful to investigate the microbial diversity in healthy or diseased states. For example, the microbial colonization of tumour tissue (oral squamous cell carcinoma, OSCC) and healthy tissue was studied using DGGE. In this way, 4 bands could be identified that were unique for tumour tissues and may be associated with different stages of OSCC [18]. Ahmed *et al.* (2012) used DGGE to compare the oral microbial profiles of healthy individuals with those obtained from patients suffering from caries or periodontitis [19]. Although most of the samples of the periodontitis group clustered together, no strict separation was found in that study. As the overall microbial composition was shown to be highly similar over time within one person [20], DGGE analysis is a promising tool to identify shifts due to therapy.

For the buccal samples, microbial community shifts were mainly found after 3 weeks of irradiation (median cumulative dose of 32.94 Gy), whereas for the tongue, shifts could be noticed earlier (median = 2 weeks of IR; median cumulative dose = 23.76 Gy) (Table 2). Previously, pairwise similarity of plaque samples was also reported to decrease after 3 weeks of treatment [17]. No significant differences could be noticed between the lowest similarity values of the buccal mucosa compared to the tongue (data not shown). Due to radiotherapy, the microbial richness (number of bands/sample, median $R_{\text{buccal}} = 21$; median $R_{\text{tongue}} = 20.5$ at start) was negatively impacted both on the buccal and tongue mucosa (median $\Delta R_{\text{buccal}} = \text{median } \Delta R_{\text{tongue}} = -3$, $p_{\text{buccal}} = 0.317$; $p_{\text{tongue}} = 0.532$; Table 2). This decrease in richness confirmed the results of Hu *et al.* [6], who observed a decrease in the number of operational taxonomic units after radiotherapy.

Marzorati *et al.* (2008) described how one could get more data from microbial fingerprints, making this technique more powerful. By use of Pareto-Lorenz curves for example, insights in the evenness can be obtained. A high evenness of a community means that all different species are present in similar amounts and the community is thus not dominated by a smaller fraction of species [16]. As an uneven community is linked with a loss of functionality and an increased risk to get invaded by pathogens, it is important to evaluate this parameter. Pareto-Lorenz curves of the samples at the start and the end of the therapy were constructed and the differences in Gini coefficients were calculated (Table 2). In 6 patients, the oral community remained stable or was dominated by a few species (uneven community), characterised with higher Gini coefficients at the end of the therapy (Figure 2 – left panel; Table 2 – $\Delta \text{Gini} \geq 0$). However, in 4 patients (2, 8, 9 and 10) a higher evenness at the end of the therapy was observed (Figure 2 – right panel; Table 2 – $\Delta \text{Gini} < 0$).

The clinical records of these patients may give an indication why their oral microbial communities behave differently. Prior to the start of the radiotherapy, the tongue of patient 2 showed clear signs of infection for which the patient received antibiotics (clavulin). Probably, the microbial community was able to restore itself due to the antibiotics as evidenced by a higher richness and evenness of the community at the end of the treatment. Also patient 9 received antibiotics (glazidim and vancomycin) during radiation therapy, clarifying its deviant behaviour in our analyses. On the tongue surface of patient 8, a milky spot was observed at the start of the therapy and this was

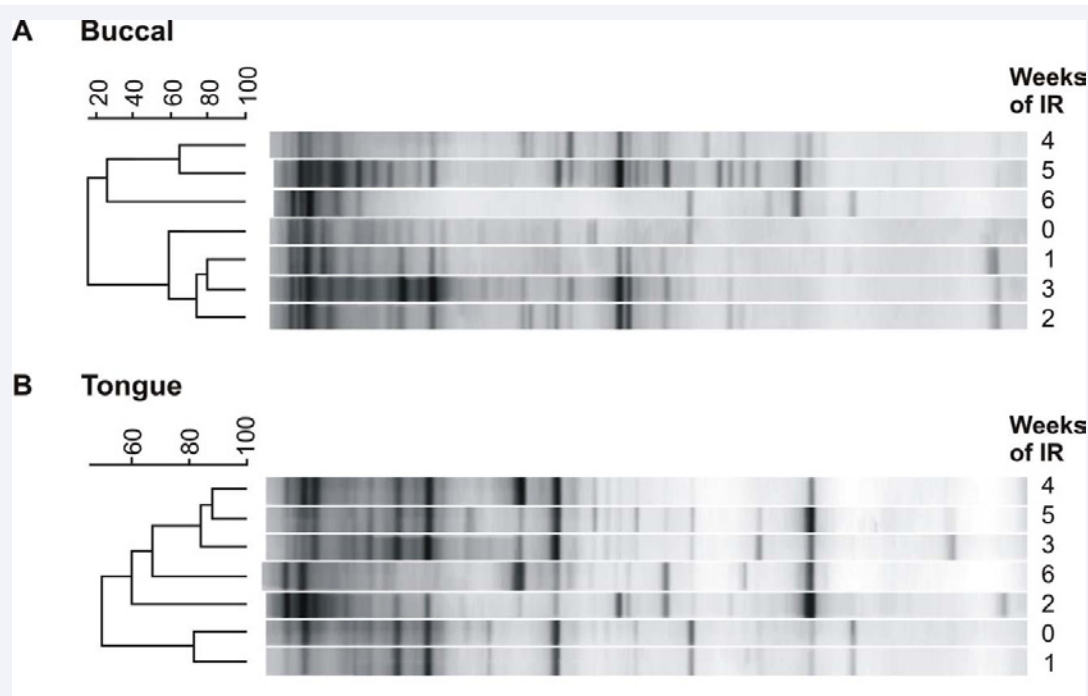


Figure 1 DGGE cluster analysis (Dice-ward) of the buccal and tongue samples of one representative patient during radiotherapy (patient 5). Percentage of similarity is shown on the scale above the dendrogram.

Table 2: Overview of the patients' microbial diversity characteristics.

Patient	First cluster buccal (*)	Cum. Dose first cluster buccal (Gy)	First cluster tongue (*)	Cum. Dose first cluster tongue (Gy)	ΔR (buccal)	ΔR (tongue)	$\Delta Gini$ (buccal)	$\Delta Gini$ (tongue)
1	3	35.64	1	16.2	-8	-9	0.120	0.158
2	1	12.96	2	23.76	N.D	12	N.D	-0.425
3	4	36.72	3	25.92	-4	-3	0.295	0.043
4	3	30.24	3	30.24	-1	-13	-0.002	0.117
5	3	36.72	1	12.96	-9	1	0.225	0.004
6	/	/	/	/	-8	-3	0.035	0.030
7	1	18	4	46	-3	-5	0.053	0.065
8	/	/	/	/	-2	7	-0.117	-0.216
9	/	/	/	/	6	-1	-0.106	-0.006
10	/	/	2	21.6	9	N.D	-0.240	N.D

Abbreviations: IR= Irradiation; ΔR = Richness (end)-richness (start); $\Delta Gini$ = Gini (end)-Gini (start); N.D= Not Determined; *= Number of weeks after the start of radiotherapy that clustered together

probably caused by a fungal infection. Oral rinses with an antifungal solution based on nystatin (5 times/day) were advised, which resulted in the successful elimination of the infection and the aberrant observations in the tongue microbial community. Patient 10 was the only patient who received a tracheostoma prior to the radiation therapy. In the course of the therapy, this patient was able to restart oral feeding. Interestingly, this patient did not suffer from radiotherapy and had no clear signs of oral mucositis in his oral cavity or oropharynx. In all other patients no infection before or during the therapy was noticed wherefore special treatments like antibiotics were obliged. As a preventive measure they were advised to maintain good oral hygiene and

to rinse their oral cavity with the antifungal solution based on nystatin.

A significant correlation was found between the patients' normal oral functioning and the buccal clustering ($p= 0.031$). When patients suffered from severe pain in the oral cavity or when they were unable to eat solids, their microbial population changed significantly. It was already shown before that diet and more specifically the protein and starch content influenced the oral microbial abundance of *Lactobacilli* and *Streptococci* [21]. Also tube feeding will disrupt the indigenous oral microbiota, allowing other bacteria to become more dominant. This results in a higher number of opportunistic pathogens like *Corynebacterium*

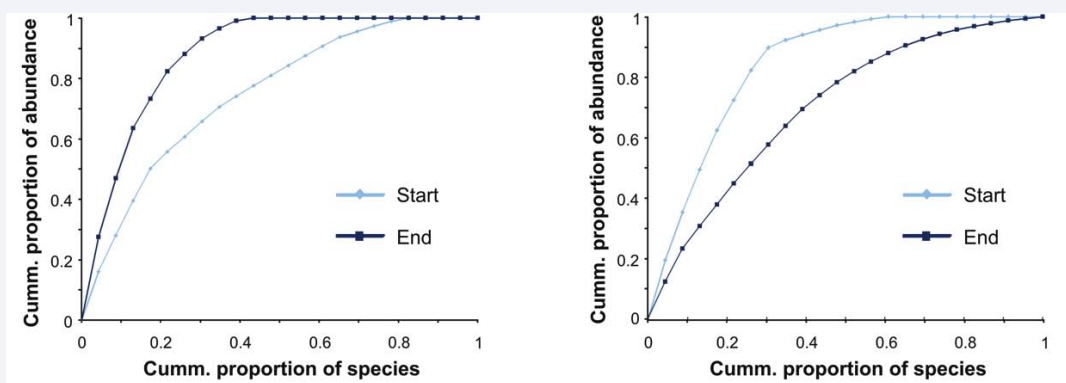


Figure 2 Pareto-Lorenz curves of the buccal microbial community at the beginning and the end of the therapy of two representative patients (left panel= patient 5; right panel= patient 10).

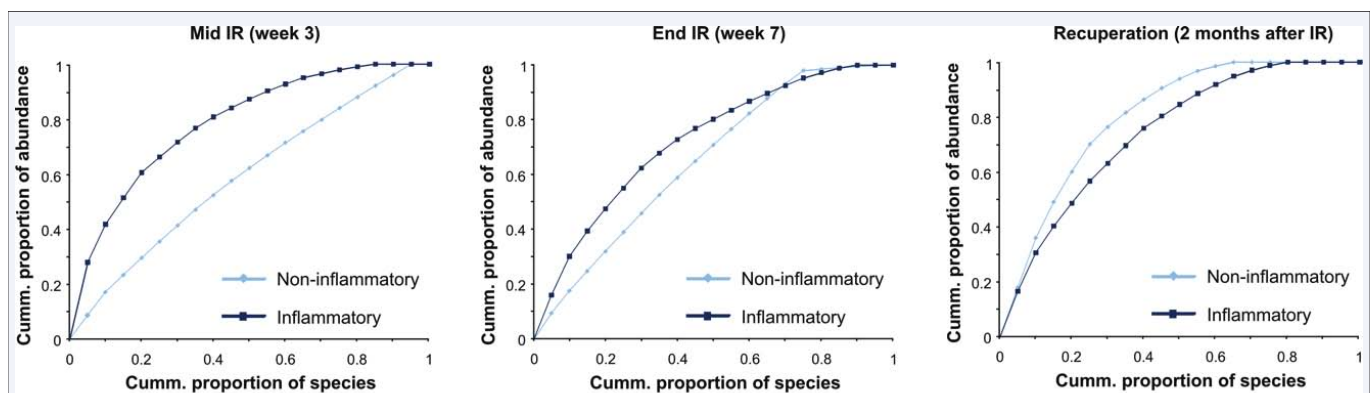


Figure 3 Pareto-Lorenz curve of an inflammatory and non-inflammatory region in the oral cavity of patient 3 during the radiotherapy.

striatum and *Streptococcus agalactiae* in tube-fed patients [22]. For the tongue samples, no significant correlations could be found.

There were no significant correlations between the severity of mucositis and the clustering or the changes in richness and evenness. Nevertheless, the microbial community of a mucosal lesion (inflammatory region) was found to be dominated by a small number of species (low evenness) compared to the community of a non-inflammatory region in the same patient at the same time point during therapy (Figure 3). The higher abundances of Gram-negative microbiota and lactobacilli due to radiotherapy are likely to cause this shift in evenness [4,5,23]. After radiation therapy, the microbial community of the inflammatory region was restored (higher evenness; Figure 3).

CONCLUSION

In this small-scale patient study we investigated the microbial shifts during radiotherapy. In patients that did not receive antibiotics during their treatment, the oral microbial community was shown to evolve to a more uneven community, similar to what was seen in the inflammatory regions. The shifts in the buccal microbiota were found to be significantly correlated with the patients' normal oral functioning. When a certain therapy is affecting patients' ability to eat, doctors should be aware of important microbial shifts and they should monitor the presence

of pathogens to avoid more undesirable side effects.

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