Original Research

Low-dose irradiation affects the functional behavior of oral microbiota in the context of mucositis

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Abstract

The role of host–microbe interactions in the pathobiology of oral mucositis is still unclear; therefore, this study aimed to unravel the effect of irradiation on behavioral characteristics of oral microbial species in the context of mucositis. Using various experimental *in vitro* setups, the effects of irradiation on growth and biofilm formation of two *Candida* spp., *Streptococcus salivarius* and *Klebsiella oxytoca* in different culture conditions were evaluated. Irradiation did not affect growth of planktonic cells, but reduced the number of *K. oxytoca* cells in newly formed biofilms cultured in static conditions. Biofilm formation of *K. oxytoca* and *Candida glabrata* was affected by irradiation and depended on the culturing conditions. In the presence of mucins, these effects were lost, indicating the protective nature of mucins. Furthermore, the *Galleria melonella* model was used to study effects on microbial virulence. Irradiated *K. oxytoca* microbes were more virulent in *G. melonella* larvae compared to the nonirradiated ones. Our data indicate that low-dose irradiation can have an impact on functional characteristics of microbial species. Screening for pathogens like *K. oxytoca* in the context of mucosits could be useful to allow early detection and immediate intervention.

Keywords: Irradiation, mucositis, oral microbiota, biofilm, virulence, *Galleria melonella*

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Introduction

Alimentary mucositis is a severe side effect of both chemo- and radiotherapy and causes a range of symptoms throughout the gastrointestinal tract, including ulcers in the oral cavity, nausea, vomiting, diarrhea, and constipation. Accelerated fractionated schedules and concurrent chemotherapy have increased the incidence and severity of mucositis. Apart from being treatment limiting, the impacts on patient quality-of-life and the economic costs of supportive therapies are of significant concern.[1–3] Limited therapeutic effects have been described for a variety of agents including radioprotectors, antibiotics, low-level laser therapy, and growth factors, but the evidence gathered to date does not support the use of any of these agents as a standard treatment. Therefore, there is an urgent need for original strategies to prevent and/or treat oral mucositis. This would not only reduce the suffering of the patients involved but would also dramatically reduce hospital and management costs associated with this condition. Although reports regarding the oral microbiota as a risk factor are scarce, prescription of alkaline mouthwashes is standard practice in the prevention of overgrowth by pathogens such as *Candida albicans* or Gram-negative bacilli.

Recently, we extensively reviewed the scientific literature on the link between oral mucositis and the endogenous microbiota.[4] Overall, there is convincing data to conclude that irradiation changes the oral core microbiome over time[5,6] with Gram-negative species becoming more abundant[7–9] together with *Candida* spp.[8,10–12] and *Lactobacillus* spp.[9,10,12] The number and proportion of *Lactobacillus* spp. were found to be increased in swabs of tongue, buccal mucosa, vestibulum, supragingival plaque and subgingival region, and saliva after radiotherapy subjects, while data on *Streptococcus* spp. were inconclusive.[9,10,12] *C. albicans* fungi seemed to be the most significant oral cavity pathogen in radiotherapy-induced mucositis patients, whereas Gram negatives such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were observed as main pathogens isolated from the blood of radiotherapy-treated patients.[7]

Radiation therapy uses high-energy irradiation to shrink tumors and kill cancer cells. X-rays, gamma rays, and

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charged particles are types of irradiation used for cancer treatment. In this study, we will mainly focus on the effect of gamma rays on microbial behavior. Gamma rays are released during isotopic negative beta decay that involves the conversion of a neutron into a proton, which releases an electron and an antineutrino.[13] Gamma irradiation causes damage to nucleic acids and (phospho)lipids of the microbiota, whereas proteins, carbohydrates, and fats in food are not significantly affected by doses up to 10 kGy.[14] It has further been shown that irradiation significantly changes the expression of heat shock proteins, the 50 S ribosomal protein, the transcriptional regulator (CtsR), and the enzyme formate acetyltransferase at doses >1 kGy.[15,16] Surprisingly, effects of low-dose gamma irradiation on the viability, metabolic activity, and other functional properties of microbiota are hardly investigated. Only a few studies show that doses less than kilogray might indeed induce some effect. Recently, a study by Parikka *et al*.[17] showed that two doses of 25 Gy with a one month's interval were sufficient to reactivate a latent *Mycobacterium marinum* infection in adult zebrafish. In the reactivation group, a steep drop in survival after 16 days concomitant with high bacterial loads was seen and histological analysis of the moribund zebrafish revealed vast areas of free microbiota outside granulomas. Although in the zebra fish model the dampened immune suppression of the host is likely to be the trigger for reactivation, gamma irradiation is likely to have direct effects on *Mycobacterium* as *in vitro* studies with doses above 1 Gy have previously shown that the viability of *M. tuberculosis* is adversely affected.[18]

It is well known that gamma irradiation causes oxidative DNA damage and triggers oxidative stress responses, and compromised DNA repair mechanisms can lead to increased risk of carcinogenesis. It is currently unknown if or to what extent microbiota play a role in regulating radiosensitivity. A recent study by Maier *et al.*[19] showed that the composition of the intestinal microbiome has an impact on the DNA repair response following irradiation. Mice with a conventional intestinal microbiota exhibited lower levels of acute chromosomal DNA lesions and γ-H2AX phosphorylation than mice with a restricted composition after total body irradiation (1 Gy). On the other hand, Crawford and Gordon[20] showed that germ-free mice seem to be markedly resistant to lethal irradiation enteritis. Although limited, these studies indeed suggest a potential role for the microbiota in the host's response to irradiation.

In this study, we wanted to determine whether radiation therapy might result in microbial dysfunction thereby providing a risk factor for developing or exacerbating oral mucositis. Therefore, the aim of this study was to unravel the effects of low-dose gamma irradiation on the functional behavior of a panel of oral species, namely *C. albicans*, *Candida glabrata*, *Klebsiella oxytoca,* and *Streptococcus salivarius*. *C. albicans* is found primarily in the intestines, colon, and the oral cavity and although a commensal, it can become pathogenic if a person's immunity is lowered or if there is a dysbiosis.[21] *C. glabrata* is considered a relatively harmless commensal of mucosal tissues, however, with the increased use of immunosuppressive agents, mucosal and systemic infections caused by *C. glabrata* have increased significantly.[22] *K. oxytoca* is like other *Klebsiella* spp. an opportunistic pathogen and although it tends to colonize along the mucosal membranes, it can be found in all parts of the body. Infections often occur in patients with immunodeficient diseases and those being treated with antibiotics.[23] *S. salivarius* is the principal commensal of the oral cavity in humans, the pioneer in colonizing dental plaque and infrequently pathogenic.[24] To our knowledge, this type of study has not been performed so far, but would provide information on specific microbiota as risk factors for developing or progressive oral mucositis following radiation therapy.

Materials and methods

Cells, chemicals, and irradiation

C. albicans (ATCC 10231), *C. glabrata* (ATCC 2001), *K. oxytoca* (ATCC 49131), and *S. salivarius* (ATCC 7073) were obtained from the New Zealand Reference Culture Collection (ESR, Porirua, New Zealand) and grown on horse blood agar plates (Fort Richard Laboratories, Auckland, New Zealand). For growth studies, cells were cultured in Bacto Todd-Hewitt broth (*Candida* species) (Fort Richard Laboratories) supplemented with 0.5% yeast extract (THY), Bacto Brain Heart Infusion (BHI) broth (*K. oxytoca* and *S. salivarius*), Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Auckland, New Zealand) or DMEM supplemented with 5% (w/v) mucins from porcine stomach type III (Sigma-Aldrich). The live/dead BacLight bacterial viability staining kit was purchased from Life Technologies, and crystal violet, Fluka Calcofluor white and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) from Sigma-Aldrich. For the *in vitro* experiments, 60-cobalt irradiations were performed using an AECL Eldorado Model 'G' (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada) at a dose rate of 0.5 Gy/min. For the experiments with *Galleria mellonella*, microbiota were irradiated with a single 6 MV photon beam generated using a linear accelerator SLi-18 (Elekta, Crawley, UK) at a dose rate of 430 cGy/min.

Determination of viability of test species

In order to assess the effect of different media (THY, BHI, DMEM, DMEM supplemented with mucins) on the viability of all test bacterial species with or without irradiation (10 Gy), different assays were performed. First, growth curves for each test species were prepared in presence of the different media with or without irradiation. The tested species were inoculated in 10 mL of culture broth and grown overnight. A 1:1000 dilution (optical density [OD] at 600 nm ~0.03–0.05) was prepared in a 50-mL V-bottom polypropylene tube in presence of the different media and irradiation was performed prior to incubation at 37°C and 200 r/min. Absorbance at 600 nm was measured spectrophotometrically every 30 min, using cuvettes with a path length of 1 cm, until cultures reached stationary phase (mQuant, Bio-Tek Instruments Inc., Winooski, Vermont, USA).

Second, to test the effect of irradiation upon biofilm formation, 200 μL of a 1:100 dilution of an overnight culture of all test species was transferred into two sterile 24-well microtiter plates (Fisher Scientific, Pittsburgh, PA) and 800 μL of the different media was added to the culture prior to irradiation. Plates were incubated for 24 h at 37°C either statically in an atmosphere of 5% CO_2 in air, or shaking at 200 r/min in an atmosphere of air. After 24 h, planktonic cells were gently removed and the remaining biofilm cells of one plate were stained with the live/dead BacLight bacterial viability kit (Life Technologies) according to manufacturer's guidelines. Briefly, 3 μL of the dye mixture of SYTO9 and propidium iodide (PI) was added to the cells and incubated in the dark at room temperature. After 20 min, fluorescence was measured using a fluorimeter EnSpire 2300 Multilabel Reader (Perkin Elmer, Waltham, MA, USA) at Ex480/Em500 nm for SYTO9 and Ex490/Em635 nm for PI. Metabolic activity and viability of the biofilm cells in the second plate were evaluated using the MTT assay. In short, 1 mg/mL of MTT was added to each well after removal of the medium (containing the planktonic cells) and biofilm cells were incubated at 37°C. After 2 h, all media were discarded and formazan crystals were dissolved in 1 mL dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured spectrophotometrically (mQuant, Bio-Tek Instruments Inc., Winooski, Vermont, USA).

Determination of biofilm formation

Biofilm formation of all test species was measured in 24-well plates and in 50 mL V-bottom tubes. For the setup in plates, 200 μL of a 1:100 dilution of an overnight culture was transferred into 24-well plates to which 800 μL of medium was added. For the setup in tubes, 1 mL of a 1:100 dilution of an overnight culture of the test species was transferred into a sterile 50 mL V-bottom tube and 4 mL of the different test media was added. Subsequently, plates and tubes were subjected to irradiation or not (controls) and incubated statically for 24 h at 37°C and 5% CO_2 in air (plates) or in shaking conditions (plates: 200 r/min; tubes: 80 r/min). After 24 h, planktonic cells were gently removed. Biofilms grown on the bottom (plates) and sides of the wells (tubes: ring formation) were washed three times with tap water and stained with 0.2% crystal violet for 30 min. Excess of staining solution was removed by three washing steps with tap water. After overnight drying of the plates and the tubes, the crystal violet was dissolved by adding a mixture of methanol:acetic acid (1:4) and the OD 540 nm was measured spectrophotometrically (mQuant; Bio-Tek Instruments Inc., Winooski, Vermont, USA).

Calcofluor white staining assay

To test the influence of different media and irradiation on extracellular matrix components, β-linked polysaccharides such as cellulose and chitin, a calcofluor white staining was performed on biofilm cells. A total of 200 μL of the calcofluor white solution was added to the biofilms formed in 24-well plates (see above). Calcofluor staining of *C. albicans* and *C. glabrata* was enhanced by adding one drop of KOH to dissolve keratinized particles and to help emulsify solid, viscous material that may mask fungal elements. After 10 min incubation in the dark at room temperature, the dye was discarded and biofilms were thoroughly washed with filter-sterilized water to remove unbound dye. Fluorescence was measured using a fluorescence microplate reader with an excitation filter of 355 and an emission filter of 430 nm EnSpire 2300 Multilabel Reader (Perkin Elmer, Waltham, MA, USA).

G. mellonella larvae infection and survival assay

G. mellonella larvae (also known as the wax worm) were purchased from a local supplier (De Papegaai, Sint-Niklaas, Belgium), maintained at room temperature in the dark, and used within one week. Overnight bacterial cultures were divided in two equal parts after which one part was irradiated (10 Gy). Cultures were washed three times and resuspended in sterile PBS at the desired concentration. Larvae were each injected with 20 μl of inoculum into the lower left proleg using an insulin syringe. Each group of 9–10 larvae was incubated at 37°C in 9-cm petri dishes without food for up to three days.

For tests with supernatant, supernatant of irradiated and nonirradiated overnight cultures of *K. oxytoca* and *S. salivarius* was collected 4 h postirradiation and cleared from remaining cells by centrifugation (10 min, 2200 g) and passed through a filter (0.2 μm). Larvae were injected with 20 μL of the supernatant or sterile broth as a control. For the experiments with dead bacteria, cells were fixed with ice-cold methanol for 10 min and washed with phosphate buffered saline (PBS) prior to injection.

Statistics

Statistical testing was conducted using Statistical Package for the Social Sciences statistics 22. Normality of the data was checked using the Shapiro–Wilk assay. When normality could be assumed, statistical differences between the growth conditions were determined using Student's *t*-tests or analysis of variance. For nonparametric analyses, Mann–Whitney U or Kruskal–Wallis tests were used. Pairwise *post hoc* analysis was performed with Bonferroni correction. For the *G. mellonella* experiments, the survival between the different groups was compared using the log rank test. A 0.05 statistical significance level was used for all analyses.

Results

Effect of ionizing irradiation on growth of oral planktonic cells

We first investigated the effect of ionizing irradiation (10 Gy) on the growth of different oral species (*C. albicans*, *C. glabrata*, *S. salivarius*, and *K. oxytoca*) under agitation. Growth curves were obtained by culturing planktonic cells in presence of rich culture broth (THY or BHI), glucose-rich DMEM and DMEM supplemented with mucins. As shown in Figure 1, a dose of 10 Gy did not affect subsequent outgrowth of test species. In contrast, comparative

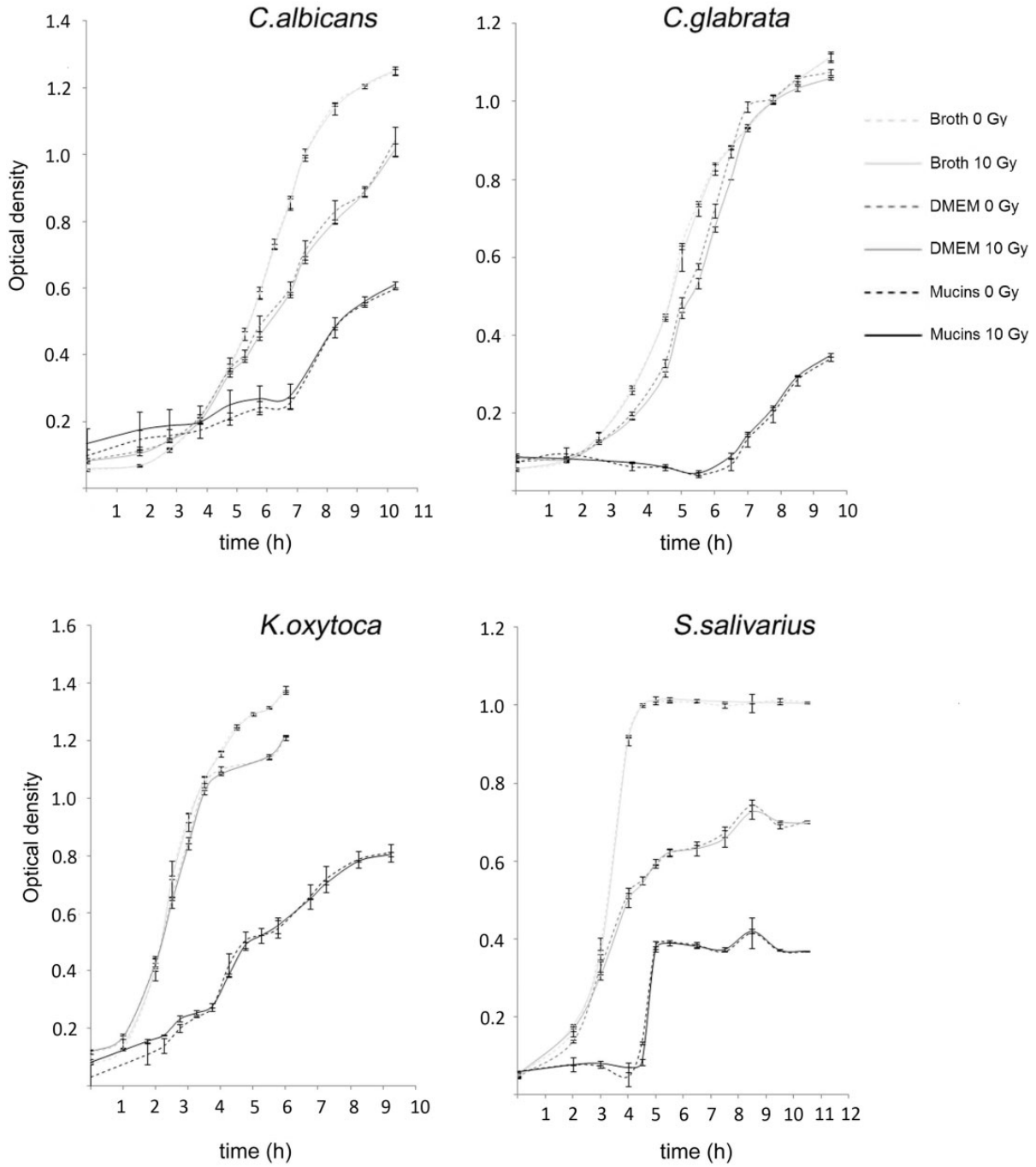

Figure 1 Growth curves of irradiated (10 Gy) and nonirradiated *C. albicans*, *C. glabrata*, *K. oxytoca* and *S. salivarius* planktonic cells cultured in different media (broth, DMEM, or DMEM + mucins). Vertical axis represents the OD of the cells measured at 600 nm using a spectrophotometer. Data of one experiment using two parallel cultures of each species are shown

assessment of the data obtained with the different culture media (BHI or THY, DMEM, DMEM + mucins) showed a differential effect on growth of all test species depending on the culture medium. In general, the presence of mucins had a strong growth inhibitory effect on all test species.

Effect of ionizing irradiation on biofilm formation and viability of oral microbiota

Biofilm formation was assessed after irradiation of the inocula. Biofilms were allowed to form in (1) static conditions, using 24-well plates and (2) dynamic conditions, using 24-well plates or 50 mL V-bottom tubes, in the presence of the different test media during a period of 24 h following irradiation of the inoculum. Crystal violet staining of the biofilms was performed as a measure of biofilm material, including both cells and extracellular matrix components. We observed that irradiation significantly reduced biofilm formation of *K. oxytoca* when cultured in static conditions and in the absence of mucins ($P = 0.002$) and of *C. glabrata* in dynamic conditions in the presence of mucins ($P = 0.017$) (Figure 2). However, thicker biofilms

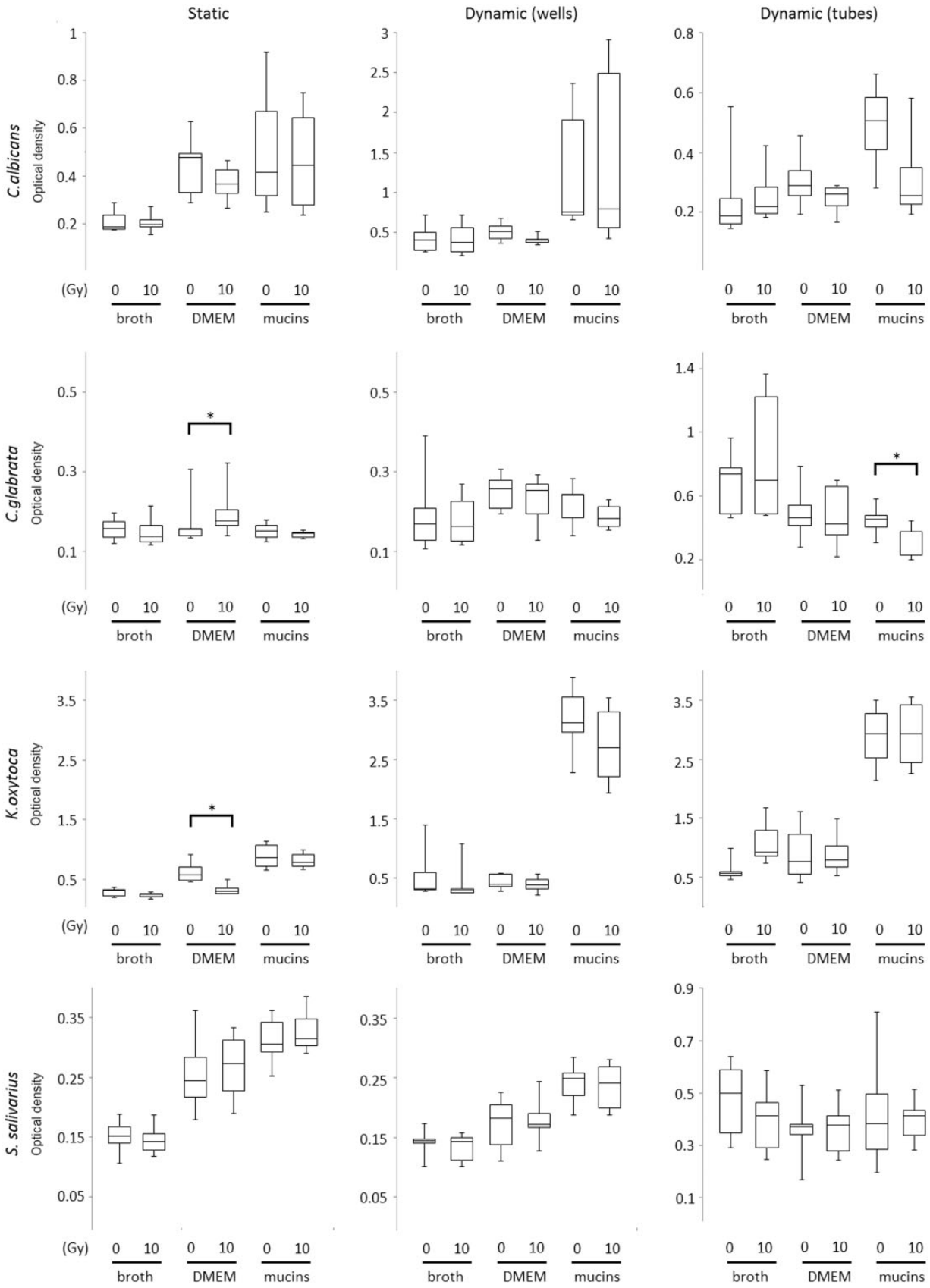

Figure 2 Box and whiskers plot presentation of biofilms of irradiated (10 Gy) and nonirradiated *C. albicans*, *C. glabrata*, *K. oxytoca*, and *S. salivarius* cells formed during 24 h of culture in different media (broth, DMEM, or DMEM + mucins). Biofilms were generated both in static (24-well plates, first column) and dynamic conditions (24-well plates, second column; tubes, third column). Vertical axis represents the OD of dissolved crystal violet molecules measured at 540 nm using a spectrophotometer. Boxes and whiskers show median values and interquartile range of scores. Pooled data of at least four repetitions are shown. $^*P < 0.05$

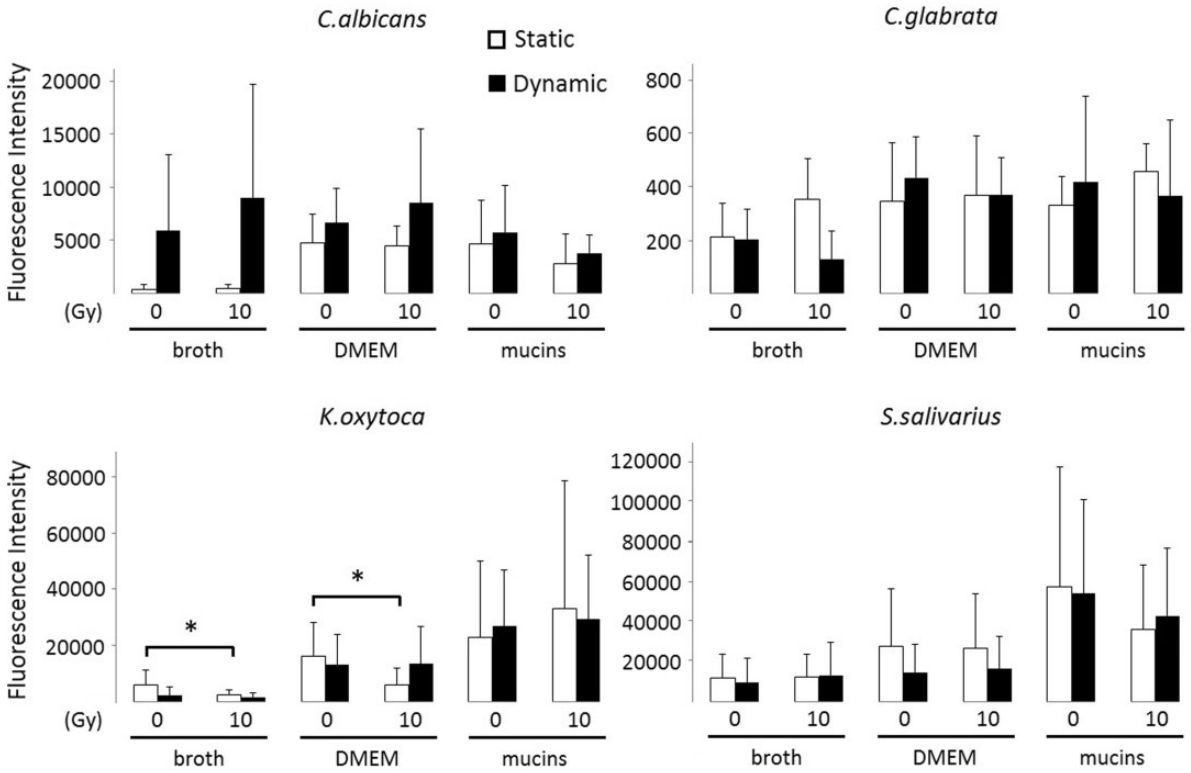

Figure 3 SYTO9-positive cells present in irradiated and nonirradiated biofilms after 24 h in different culture media. Biofilms were generated both in static and dynamic conditions (24-well plates with or without shaking). Vertical axis represents the SYTO9-positivity assessed by measurement of fluorescence intensity at Ex480/Em500 nm. Bars represent the means including the standard deviation. Pooled data of at least three repetitions are shown. $^*P < 0.05$

were observed for *K. oxytoca* cultured in dynamic (broth) conditions (not significant) and for *C. glabrata* in static conditions ($P = 0.029$) after irradiation (Figure 2). In addition, there was an overall positive effect of mucins on biofilm formation for most species, which was significant for *K. oxytoca* (static: $P = 0.022$; dynamic wells: $P < 0.001$; dynamic tubes: $P < 0.001$), *C. albicans* (dynamic wells: $P = 0.012$), and *S. salivarius* (dynamic wells: $P < 0.001$).

Next, we evaluated the number of cells in biofilms of all test species grown for 24 h after irradiation of the inoculum. To this end, we measured the fluorescence intensity of the biofilms after SYTO9 and PI staining. The SYTO9 stain generally labels all microbiota in a population, whereas PI penetrates only microbiota with damaged membranes, causing a reduction in the SYTO9 fluorescence when both dyes are present. We observed that irradiation significantly reduced the number of SYTO9-positive *K. oxytoca* cells, when the biofilms were grown in static conditions and in the absence of mucins (broth: $P = 0.041$; DMEM: $P = 0.008$) (Figure 3). The metabolic activity, measured as MTT reduction, of the *K. oxytoca* biofilms was also reduced in those specific conditions (Figure 4). Figure 3 further illustrates that *Candida* biofilm formation on polystyrene plates is poor, irrespective of the type of medium used, whereas *K. oxytoca* and *S. salivarius* form dense biofilms in all media as was also obvious upon visual inspection. There was a trend for the presence of mucins to enhance biofilm formation by *K. oxytoca* and *S. salivarius*, although this difference was not statistically significant. We were unable to

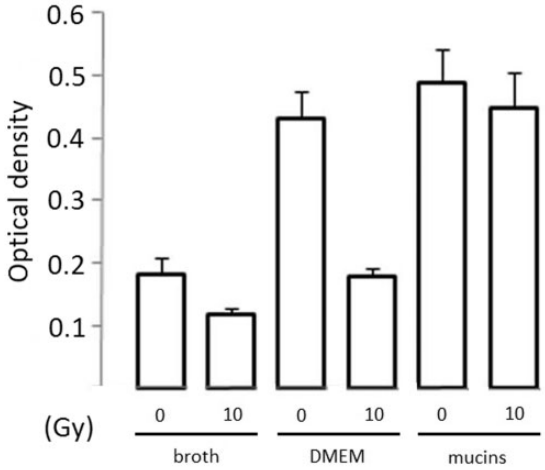

Figure 4 Metabolic activity of irradiated and nonirradiated biofilm cells of *K. oxytoca* after 24 h of culturing in different media (without agitation) as determined by measuring the OD at 490 nm of the dissolved formazan crystals (vertical axis). Bars represent the means including the standard deviation. Data of one representative experiment are shown (three replicates)

detect PI values that significantly differed from the detection limit, which indicates that the majority of the biofilm cells were alive and not affected by irradiation.

We further examined if irradiation had an impact on the production of extracellular matrix components of biofilm cells. The cellulose content of the biofilms grown for 24 h

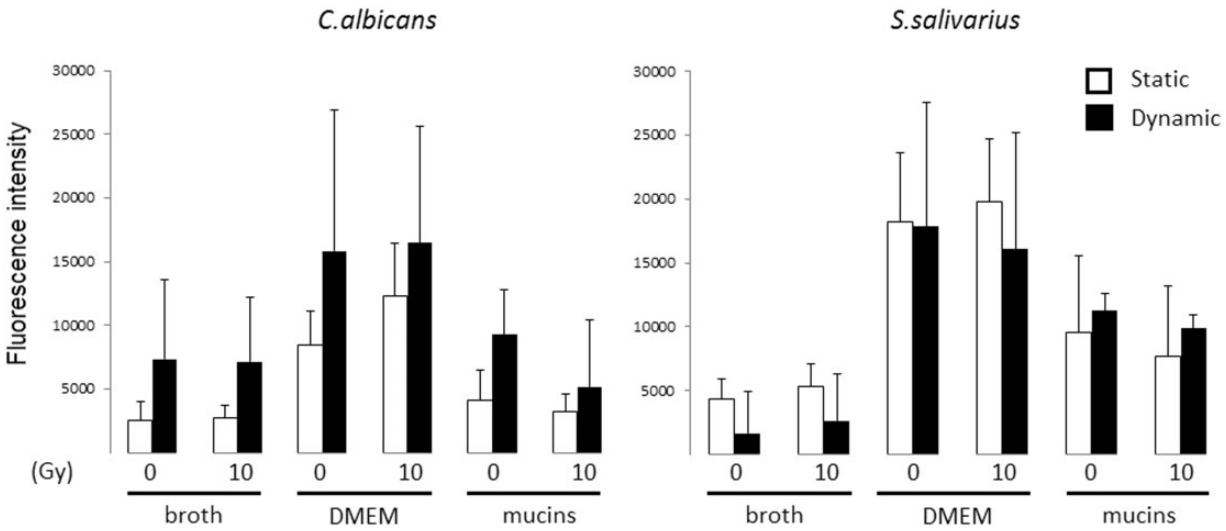

Figure 5 Calcofluor staining of biofilms of irradiated (10 Gy) and nonirradiated *C. albicans* and *S. salivarius* formed within 24 h in static (white bars) or dynamic conditions (black bars) in 24-well plates and in presence of the three test media. Vertical axis represents the calcofluor fluorescence intensity at Ex355/Em430 nm. Bars represent the means including the standard deviation. Pooled data of two repetitions are shown. $^{*}P < 0.05$

after irradiation of the inoculum was evaluated by a calcofluor white staining. In our setup, only *C. albicans* and *S. salivarius* produced an extracellular matrix that contained cellulose (Figure 5). Overall, no effect of irradiation on the cellulose content of the biofilm matrix could be observed.

Effect of ionizing irradiation on virulence of K. oxytoca and S. salivarius

We further questioned if irradiation had an impact on the virulence of the pathogen *K. oxytoca* and the commensal *S. salivarius*. To test this, we used larvae of the wax moth *G. mellonella* as a model host for examining the virulence potential of the species of interest. For survival assays, wax moth larvae were infected and mortality was monitored every 24 h for four days. At a dose of 10^6 cfu/mL, irradiated *K. oxytoca* were significantly more virulent then nonirradiated cells ($P = 0.027$; log rank test, $n = 20$) (Figure 6(a)). At one day postinoculation, the survival probability of the wax moth larvae infected with irradiated cells was lower than with NIR. The time course of survival of the wax moth larvae depended on the pathogen load as injection of a dose of 10^7 or 10^8 cfu/mL resulted in a high mortality rate 24 h postinoculation, while a dose of 10^5 cfu/mL was too low to cause infection (data not shown). In contrast to *K. oxytoca*, the virulence of *S. salivarius* at a dose of 10^7 cfu/mL did not significantly change upon irradiation (Figure 6(b)). In order to find out if the increased virulence of *K. oxytoca* after irradiation was due to the secretion of particular factors, supernatants from nonirradiated and irradiated were collected and injected into the wax moth larvae. However, the supernatant of both groups had no effect on the overall survival of the wax larvae (data not shown). Also injection of dead cells had no influence on larval survival (data not shown), pointing to the need of living, metabolically active bacteria for virulence.

Discussion

Overall, our data indicate that ionizing irradiation can induce behavioral changes in specific oral species (Table 1). Of all species tested, only *K. oxytoca* was consistently affected. Although we failed to detect an effect of irradiation on microbial growth, biofilm formation was clearly induced. We speculate that irradiation triggers a stress response that might be responsible for the induction of microbial adhesion. It is known that upon sensing a stress signal, free-living cells will initiate attachment to a surface and the formation of a biofilm that has a greater ability to withstand environmental challenges.[25] Irradiation might have an effect on the different stages of biofilm formation i.e. initially when adhesion to a surface occurs through organelles including pili, flagella, and external microbial layers, or due to immobilization of microbiota,[26–28] or through the secretion of quorum-sensing molecules such as 3-oxo-C12-homoserine lactone,[29] or in mature biofilms through the increased production of extracellular matrix molecules like specific polysaccharides, proteins, and extracellular DNA (eDNA).[30]

Most importantly, we showed that irradiation significantly increased the virulence of *K. oxytoca in vivo* using the *G. mellonella* model system. This model allows the study of bacterial and fungal infections at 37°C (in contrast to the *Caenorhabditis elegans* and *Drosophilla melanogaster* infection models) and the immune system of the larvae shares functional homology with the human innate immune system, possessing both humoral and cellular immunity.[31,32] This observation is important and relevant in the context of oral mucositis. Indeed, *K. pneumoniae* is a prevalent isolate in blood and oral cavity of cancer patients undergoing (chemo)radiation therapy[7,33] and pediatric patients undergoing high-dose chemotherapy or hematopoietic stem cell therapy.[34,35] Both groups have a very high incidence of developing oral mucositis. Due to their

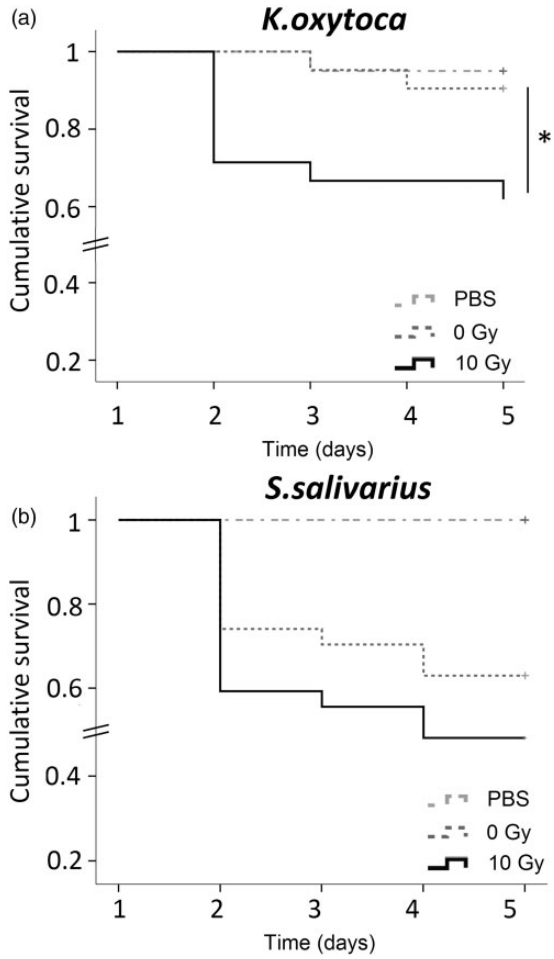

Figure 6 Survival curves of *Galleria melonella* after inoculation with *K. oxytoca* (a) or *S. salivarius* (b), plotted using the Kaplan–Meyer method. For experiments with *K. oxytoca*, two independent tests were performed and pooled results are presented. Test groups were PBS controls ($n = 20$), nonirradiated (0 Gy; $n = 21$) and irradiated (10 Gy; $n = 21$). Vertical axis represents the cumulative survival probability of the wax larvae during four days postinjection. For experiments with *S. salivarius*, three independent tests were performed and pooled results are presented. Test groups were PBS controls ($n = 27$), nonirradiated (0 Gy; $n = 27$) and irradiated (10 Gy; $n = 27$). Vertical axis represents the cumulative survival probability of the wax larvae during four days postinjection. Differences in survival were calculated using the log rank test. $^*P < 0.05$

high incidence rate, *Klebsiella* spp. are considered one of the most significant infectious pathogens, causing urinary tract infections, pneumonia, wound infections, septicemia, neonatal septicemia, and nosocomial infections.[36,37] As *K. pneumoniae* is frequently identified in individuals wearing removable maxillary prosthesis and are assumed to play important roles in denture stomatitis, wearing a removable prosthesis might be an important risk factor for developing mucositis and/or infections during or after radiation therapy.[38] It is therefore likely that the oral ulcerations are gateways for *Klebsiella* spp and other opportunistic pathogens to penetrate the underlying damaged mucosa. We are currently using the *G. mellonella* model to examine effects of radiation on different *Candida* spp. Results may explain the high incidence of local and/or systemic infections through fungal translocation associated with oral mucositis. Remarkably, the supernatant of irradiated bacteria did not mimic the toxic effects of living cells ruling out the interplay of a secretion factor. We speculate that irradiation induces a stress response in the microbial cells that changes the adhesive properties or surface hydrophobicity of the cells walls. Indeed, the microbial cell surface hydrophobicity is known to contribute to the adherence properties, which can be affected by stressors such as subinhibitory concentrations of antibiotics.[39,40] This modified adhesive behavior could be linked to virulence since the number of cells attached to host cells is increased and therefore infection is more likely to occur.

We further observed that irradiated *K. oxytoca* cells formed less biofilm in static conditions where oxygen transfusion is limited. In dynamic, oxygen-rich conditions, an opposite trend could be observed. Interestingly, these changes were not seen when mucins were present in the culture medium. Mucins exhibit protective effects on the alimentary tract through reducing mechanical and chemical stress, preventing bacterial overgrowth and penetration of the mucosa. All oral microbiota are found in complex biofilms on and in the epithelial mucous layer (soft and hard tissues) or on the teeth (dental plaque), or as planktonic cells present in saliva.[41] A number of recent studies[5,6,42] has shown that after radiation therapy, the composition of the core oral microbiome is modulated over time. In this study, however, we show that also the functional behavior of oral microbiota can be affected by irradiation. We speculate that this change in functional behavior might have a dramatic effect on the epithelium, deprived from its protective mucous layer following radiation therapy due to reduced salivary production by the damaged salivary glands. As a consequence, the composition and texture of the mucous layer covering the oral epithelium are dramatically altered and protective proteins such as defensins, immunoglobulins, lysozyme, and trefoil proteins will be less abundant.[43] Moreover, since mucins are important for growth of microbiota[44] and for initiating host–microbiome interactions such as adhesion of microbiota,[45] a mucous-deprived environment will leave the host more vulnerable to opportunistic species. Indeed, during progressive mucositis, desquamation of the epithelium and its overlaying mucin layer results in severe ulcerations often associated with local infections. Moreover, mucin composition and expression have been shown to be altered by chemotherapy and this is thought to be associated with mucositis.[46–48] Therefore, our *in vitro* observations provide biological clues as to why patients with a damaged mucosa have an increased risk of developing infections.

Our data suggest that the overall effect of irradiation on biofilm formation by *K. oxytoca* is dependent on factors such as oxygen availability and the shear stress. In the oral cavity, mucin-poor, static, oxygen-deprived conditions are found in the gingival crevice, which is a reservoir of opportunistic pathogens. *K. oxytoca* has been frequently isolated in biofilms of HIV-positive patients with plaque-associated gingivitis or periodontitis, indicating their ability to colonize oxygen-deprived niches deprived from mucins in these patients[49,50] and after bracket placement.[51] Our data show that in oxygen-deprived conditions, a lower number of *Klebsiella* cells are found in newly formed biofilms, whereas in oxygen-rich conditions, a higher number could be

Table 1 Overview of effect of irradiation on different behavioral characteristics of oral species

		Static	Dynamic
Growth planktonic cells	*C. albicans*		**NA**
	C. glabrata	–	**NA**
	K. oxytoca	–	**NA**
	S. salivarius	–	**NA**
Biofilm formation	*C. albicans*	**NA**	**NA**
	C. glabrata	↑ ($p_{DMEM} = 0.029$)	↓ ($p_{DMEM+mucins} = 0.017$)
	K. oxytoca	↓ ($p_{DMEN} = 0.002$)	↑ (broth: trend)
	S. salivarius	**NA**	**NA**
Number of biofilm cells	*C. albicans*	**NA**	**NA**
	C. alabrata	**NA**	**NA**
	K. oxytoca	↓ ($p_{broth} = 0.041$; $p_{DMEM} = 0.008$)	**NA**
	S. salivarius	**NA**	**NA**
Cellulose content	*C. albicans*	**NA**	**NA**
	C. alabrata	**NA**	**NA**
	K. oxytoca	**NA**	**NA**
	S. salivarius	**NA**	**NA**
Virulence	*K. oxytoca*	↑ ($p = 0.027$)	
	S. salivarius	**NA**	

Note: -: not tested; NA: not affected.

observed. This suggests that radiation therapy might lead to a lower number of potentially harmful cells in the gingival crevices after radiation therapy, whereas the opposite could happen on the teeth or gum line, where oxygen is abundantly available. Therefore, patients with a history of gingivitis/periodontitis might be able to manage their condition (gingivitis/periodontitis) better after radiotherapy when maintaining good oral hygiene (as they are prone to develop denser *K. oxytoca* biofilms on the teeth surface). However, those patients might have an increased risk of developing infections related to *C. glabrata* as our data suggest that irradiation stimulates the biofilm formation of that species in static, oxygen-deprived conditions.

A potential relationship between radiation-induced mucositis and periodontitis has recently been suggested by Khaw *et al.*[52] as both diseases are linked with chronic and systemic inflammation. This is clinically important, since it is estimated that up to 50% of the population is affected by periodontal disease as a result of the presence of multiresistant enterobacteria such as *Klebsiella* spp. in subgingival sites.[53] Therefore, these patients may have a particular risk of developing oral mucositis complicated with systemic infections especially when they are immunocompromised and hospitalized. Various *Candida* species are frequently identified in oral mucositis patients, especially after high-dose chemotherapy and radiotherapy.[11,33,54,55] In our study, no changes in biofilm formation of *C. albicans* were observed after irradiation. Therefore, the mechanism by which *C. albicans* is able to overgrow the affected mucosa during mucositis might not be associated with effects on growth or biofilm formation.

Overall, the risk of developing an infection after irradiation will ultimately also depend on other factors like the virulence of the microbiota, the shift in tempo-regional attachment, and colonization of the microbiota and the complexity of the composition of the biofilm. Recent data indicate that both chemo- and radiotherapy are associated with a decrease in microbial diversity,[5,6,56] which is believed to be correlated with a poor health prognosis. Our data indicate that apart from the composition, radiation therapy can also change the functional behavior of the resident oral microbiome. Therefore, we hypothesize that local and systemic infections associated with mucositis are probably more likely to result from the cumulative effect of changes in composition, function, and behavior of the microbiome than solely from a compromised diversity. Therefore, functional changes in the oral microbiome following chemo- and radiation therapy should be studied more in depth.

Although this work is relevant in the context of identifying microbial risk factors for the development of oral mucositis, it is important to highlight its limitations. Oral biofilms are highly complex multispecies structures and it is the delicate interaction between all its members that will ultimately determine their behavior. Thus, further studies should address (1) if similar observations can be made with other mucositis-associated pathogens besides *K. oxytoca* and in multispecies biofilms containing *K. oxytoca* and (2) what the underlying mechanism of this increased virulence potential of *K. oxytoca* is. Based on our preliminary results, we hope to further clarify if treatments like chemo- and radiation therapy are likely to modify the functional behavior of the oral microbiome in a way that will eventually lead to dysbiosis, bacterial translocation, and the overgrowth of pathogens during the progression phase of mucositis.

Authors' contributions: BVH, TDR, and KDb conducted all the experiments. BVH and TDR performed the data analysis and drafted the manuscript. TDR performed the statistical analysis. TB and BVH conducted the irradiation of the microbial cultures. SW trained BVH in the use of *Galleria mellonella* as a model system. TVdW supervised the work of TDR and SS of BVH, both TVdW and SS revised the manuscript.

Authors' note

Barbara WA Vanhoecke and Tine RG De Ryck contributed equally to this work.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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