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Keywords

bacteria, microbiome, chemotherapy, host-microbe interactions, *in vitro* model, oral mucosa

Abbreviations

5-FU = 5-fluorouracil ; DGGE = Denaturing Gradient Gel Electrophoresis ; DMSO = dimethyl sulfoxide **;** nuclear factor-kappa B (NF-κB); MTT = 3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ; SRB = sulforhodamine B ; DMEM = Dulbecco's modified Eagle's Medium ; PBS = phosphate buffered solution ; NMDS = Non-metric distance scaling ; CFU = colony-forming units

Abstract

Oral mucositis is still one of the most painful side effects of chemotherapeutic treatment with a major impact on quality of life for cancer patients. A mounting body of evidence suggests a key role for the oral microbiome in mucositis development. However, the underlying mechanisms remain elusive. In this work, we have investigated the interactions between the host, the microbiome and chemotherapeutic treatments in more detail. To this end, the effect of 5-fluorouracil (5-FU), commonly inducing mucositis, was assessed on a co-culture model that consists of an epithelial cell layer and a biofilm derived from oral microbiota from different types of samples (saliva, buccal swabs and tongue swabs) and donors (healthy individuals and patients suffering from mucositis). After 24 h of co-incubation, all oral microbial samples were found to reduce wound healing capacity with 26 ± 15 % as compared with untreated condition. Compared with saliva and tongue samples, buccal samples were characterized by lower bacterial cell counts and hence higher wound healing capacity. For samples from healthy individuals, an inverse correlation was observed between bacterial cell counts and wound healing capacity, whereas for patients suffering from mucositis no correlation was observed. Moreover, patient-derived samples had a less diverse microbial community and higher abundances of pathogenic genera. No major impact of 5-FU on wound healing capacity or the composition of the microbiome was seen at physiologically relevant concentrations in the mouth. In conclusion, bacterial cell count is inversely correlated with wound healing capacity, which emphases the importance of oral hygiene during oral wound healing in healthy individuals. However, future research on extra measures besides oral hygiene is needed to assure a good wound healing during mucositis, as for patients the bacterial composition seems also crucial. The direct effect of 5-FU on both the microbiome and wound healing is minimal, pointing to the

48 importance of the host and its immune system in chemotherapy-induced microbial
49 shifts.

Introduction

Oral mucositis is a painful and debilitating complication of cancer treatment with a major impact on the quality of life of the patient. Its frequency is high but varies depending on the type of treatment with around 20-40 % incidence in conventional chemotherapeutic treatment of solid tumours, to almost 100 % for high-dose chemotherapy prior to hematopoietic stem cell transplantation or radiotherapy for head and neck cancer (Lalla et al., 2008; Sonis, 2007; Villa and Sonis, 2015). Although it is one of the most studied toxicities of cancer treatment, only few therapeutic agents are available for oral mucositis (Villa and Sonis, 2016). One of the chemotherapeutic agents with high risk of developing mucositis is 5-Fluorouracil (5-FU) (Villa and Sonis, 2015), an antimetabolite that inhibits thymidylate synthase (TS) and is incorporated in DNA and RNA (Grem, 2000; Longley et al., 2003). The incidence of developing grade 3-4 oral mucositis (i.e. confluent ulcers and unable to eat solids) in case of 5-FU treatment is more than 15 % (Sonis et al., 2004). During continuous infusion (22h), plasma levels of 5-FU range from 3 to 10 μM and saliva levels from 0.08 to 0.8 μM (Joulia et al., 1999; Takimoto et al., 1999). Previous research has indicated that some oral species are sensitive to 5-FU starting from 0.4 μM (Vanlancker et al., 2016).

The pathogenesis of mucositis is described by the 5-stage model of Sonis (2007). Shortly, reactive oxygen species (ROS) are generated in the initiation phase, followed by the activation of transcription factors, such as nuclear factor-kappa B (NF-κB). These induce the production of pro-inflammatory cytokines and activate other signalling pathways. Feedback-loops induce more inflammation and apoptosis which lead to the ulceration phase, in which bacteria colonise the ulcers and can penetrate to the submucosa. In most cases, spontaneous healing takes place within two to three weeks after completion of the treatment. Although this last phase is of great importance in

terms of recovery and further continuation of the cancer treatment, it is also the least understood (Sonis, 2007).

More and more evidence is emerging on the role of the oral microbiome in the pathogenesis of oral mucositis (Stringer and Logan, 2015; Vanhoecke et al., 2015; Vasconcelos et al., 2016). Microbiota can play a negative role in mucositis and induce infection of the ulcers which encourages the use of antimicrobial agents. However, no clinical guidelines have been formulated regarding the use of antimicrobial agents due to insufficient and conflicting scientific data (Saunders et al., 2013; Vanhoecke et al., 2015). Microbiota may also be involved in phases other than the ulceration phase, and this role can be both positive and negative (van Vliet et al., 2010). Microbiota are for example able to influence the activation of Toll like receptors (TLR), NF-κB and mitogen-activated protein kinase (MAPK), which are all proteins involved in important signalling pathways regulating mucositis. This way, microbiota might contribute to a higher tissue inflammation level and therefore increase apoptosis rate (Stringer and Logan, 2015).

Clinical studies have shown shifts in the oral microbial profile of patients, both after chemo- and radiotherapy. However, the great variability in patient population, sample collection and technical methods to analyse the microbiota makes it difficult to generalise conclusions (Vanhoecke et al., 2015). It seems that for blood cultures and oral swabs taken during chemotherapy, the most frequently isolated Gram-negative species are *Enterobacteriaceae* spp., *Pseudomonas* spp., and *E. coli*, whereas *Staphylococcus* spp. and *Streptococcus* spp. are the most frequently isolated Gram-positive species (Napenas et al., 2007; Vanhoecke et al., 2015). Not only microbial composition, but also functional factors such as the mucus layer and microbial adhesion can be affected by the cancer treatment (Stringer et al., 2009; Vanhoecke et al., 2015). Moreover, oral microbiota may regulate wound recovery, with positive or negative effects depending on the species and

100 the bacterial density (De Ryck et al., 2015; Edwards and Harding, 2004; Laheij et al.,
101 2013). These factors will depend on both the donor and on the specific site in the oral
102 cavity, as they each have their own microbial community (Segata et al., 2012). For
103 example, the saliva microbiome resembles the tongue microbiome but is distinct from
104 the buccal mucosal microbiome (Segata et al., 2012).
105 In this study, we further investigated the role of oral microbiota on wound healing
106 capacity and the effect of chemotherapy on both the microbiota and wound healing in an
107 *in vitro* co-culture model that was previously optimized (De Ryck et al., 2014). First, the
108 toxicity of 5-FU towards oral epithelial cells was determined using the MTT/SRB
109 cytotoxicity tests. Next, the impact of oral microbiota and 5-FU, and the combination
110 thereof, on epithelial wound healing were studied in the co-culture model for 24 h, with
111 a special focus on the potential impact of the type of oral sample and donor variability.

Material and methods

Cell culture

The TR146 cell line, obtained from the Laboratory of Experimental Cancer Research (Ghent University Hospital), is an oral squamous cell carcinoma cell line isolated from a local lymph node metastasis (Rupniak et al., 1985). Cells were cultured at 37 °C, 10 % CO_2 and 90 % relative humidity in Dulbecco's modified Eagle's Medium (DMEM) (Gibco) with 10 % heat inactivated fetal bovine serum (Greiner Bio-one), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 2.5 µg/ml amphotericin B (Gibco).

Oral samples

Oral samples were obtained from healthy children or patients suffering from oral mucositis (Ethical approval from Ghent University hospital, Belgian Registration number B670201112526), all aged 6-14 years. All patients were treated for haematological malignancies. Three types of samples were collected: saliva, buccal swab and tongue swab. All samples were collected at least 2 h after eating or brushing teeth and before sampling the oral cavity of the individuals was flushed with drinking water. For the buccal and tongue samples, a sterile cotton swab was gently wiped ten times along the inner cheek or on the dorsal side of the tongue and subsequently dissolved in 1 ml of phosphate buffered solution (PBS).

Chemicals

A filter-sterilized stock solution of 100 mM 5-Fluorouracil (5-FU) (Sigma Aldrich) was prepared in dimethylsulfoxide (DMSO) and further diluted to 75, 50, 20, 10, 5, 1, 0.1, 0.01 mM in DMSO. Stock solutions were further diluted (1:1000) in culture medium for the experiments.

MTT/SRB test

To test the cytotoxicity of 5-FU, an MTT/SRB test was performed. The MTT assay (Mosmann, 1983) was used to measure the mitochondrial activity and the SRB assay (Vichai and Kirtikara, 2006) to measure cellular protein content. TR146 cells were seeded in 96 well plates at a density of 40 000 cells/well (100 µl DMEM with serum/well). After 24 h, medium was discarded and 100 µl serum-free, antibiotic-free DMEM was added together with different 5-FU concentrations (0.01-100 µM). DMSO (1:1000) was used as a control. All plates were incubated at 37 °C and 5 % CO_2. After 24 h, 48 h and 5 days an MTT and SRB test was performed. Six biological replicates were included for each 5-FU concentration and for each time point. For the MTT-assay, 20 µl MTT (3-(4,5-demethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml in PBS^{D-}) was added and incubated for 2 h at 37 °C. All medium was removed and formazan crystals were resuspended in 100 µl DMSO. The absorbance was measured at 570 nm. For the SRB (sulforhodamine B) assay, cells were fixated by adding 25 µl 50 % trichloroacetic acid (TCA) and incubated for 1 h at 4 °C. After removal of the TCA, the plate was rinsed with water and dried. Next, 75 µl SRB solution (0.4 % in 1 % glacial acetic acid) was added and the plate was incubated for 30 minutes at 4 °C. The plate was then rinsed with 1 % glacial acetic acid and dried. The stained cells were resuspended in 200 µl 10 mM Tris buffer and the absorbance was measured at 490 nm.

Co-culture model

To investigate the interactions of oral microbiota and oral epithelial cells without direct contact, we used an oral *in vitro* model described by De Ryck et al. (2014) (Figure 1). Briefly, the model consists of a 24-well Transwell® plate with removable inserts with a polycarbonate membrane of pore size 0.4 µm (Corning Incorporated). In the apical part, 20 µl of the bacterial suspension was brought on top of a solidified agar/mucin solution (75 µl, 5 % porcin mucin Type II, 0.8 % agar). PBS was used as a control. In the

basolateral side, epithelial cells were seeded at a density of 250 000 cells/well and at confluency a wound healing assay was performed (see below). During co-culture, the inserts with the microbiota were transferred to the wells containing the epithelial cells and incubated at 37 °C, 10 % CO_2 in serum-free, antibiotic-free DMEM (Gibco) with 5-FU (10 µM) or DMSO as a control (1:1000). After 24 h of co-culture, inserts were removed and 100 µl PBS was added to collect the bacteria for further analysis. For each of the seven donors (4 healthy individuals and 3 patients suffering from mucositis), a buccal sample, a saliva sample and a tongue sample as well as a blank (without microbiota) was tested in this co-culture model, each with and without 10 µM 5-FU (Figure 1). Each condition was tested in triplicate or quadruplicate.

Wound healing assay

During co-incubation, a wound healing assay was performed based on the protocol by De Ryck et al. (2014) (Figure 1). TR146 cells were stained with Vybrant DiI cell labelling solution (Life Technologies) before seeding in 24 well Transwell® plates at 250 000 cells/well. At the start of the experiment, two scratches were made in the confluent monolayer using a sterile 100 µl pipette tip. Cell medium was discarded to remove cellular debris and 1 ml of new serum-free, antibiotic-free DMEM was added to the cells. At four selected fields per well and at each time point, images of the wound were acquired using a fully automated widefield fluorescent microscope (Nikon Ti, Nikon Instruments), equipped with a 4x/0.15 Plan Achromat objective and EM-CCD camera (Andor Ixon+, Andor Instruments). The surface area of the wound was calculated for each time point using a home-written script for FIJI freeware (http://fiji.sc) that is available upon reasonable request (www.uantwerpen.be/Cell-group/scripts). In brief, the DiI counterstained time-lapse images are first pre-processed by background subtraction and local contrast enhancement, after which the non-damaged part of the

cell monolayer is detected by a combination of variance, maximum and Gaussian blur filtering, and segmented using a user-defined or automatic threshold. The inverse of this mask is selected as wounded area. The relative wound size was calculated by normalizing to the wound area at 0 h.

At the end of the wound healing experiment, metabolic activity and viability of the epithelial cells was evaluated with an MTT-assay. To each well, 1 ml of serum-free, antibiotic-free DMEM and 200 µl MTT (5 mg/ml in PBS^{D-}) was added and incubated for 2 h at 37 °C. After removal of the medium, the formazan crystals were dissolved in 1 ml DMSO. Absorbance at was measured at 540 nm (200 µl) (Infinite F50 Tecan). Percentage of viability, compared to the control, was calculated.

Colony-forming units (CFU)

To measure the number of viable cells present in the insert, the oral samples (saliva, oral swab, tongue swab) were plated using Brain Heart Infusion (BHI)-agar plates. A dilution series was made and 10 µl of bacterial suspension was plated in triplicate.

Flow cytometry

The number of intact and damaged bacterial cells in the insert after 24 h was measured by flow cytometry as described by Van Nevel et al. (2013). The samples were diluted in a filter sterile (0.22 µm) phosphate buffered solution to obtain cell numbers within the detection range (10^4-10^6 cells/ml). Next, the samples were stained with SYBR Green I (10000x diluted from stock, Invitrogen) and propidium iodide (final concentration 4 µM, Invitrogen) and incubated for 13 min at 37 °C before measurement. The flow cytometer (BD Accuri C6 flow cytometer, BD) was equipped with a 488 nm solid-state laser and Milli-Q was used as sheath fluid. Signals were detected in fluorescent channels FL1 (green) and FL3 (red), respectively equipped with a 518-548 nm and 670 nm bandpass filter. Cells were counted by measuring the number of particles in a set volume after

gating on green vs. red fluorescence plots in the BD CSampler software. Quality control of absolute cell counting was done with standardised beads. Background was monitored by measuring a filtered sample, equally diluted as the test samples.

Microbial community analysis

All protocols concerning microbial community analysis are further described in SI (S3). Shortly, DNA extraction was performed based on Vilchez-Vargas et al. (2013) and the quality of the DNA samples was analysed by gel electrophoresis. On all samples, Denaturing Gel Electrophoresis was performed using the PRBA338F-GC and 518R primers targeting the V3 region (Muyzer et al., 1993; Ovreas et al., 1997). Illumina sequencing was performed on one replicate of each condition for the saliva samples of all individuals by LGC Genomics (Berlin, Germany) on the MiSeq platform. The Illumina sequencing data were deposited to the European Nucleotide Archive (SRA) with study number PRJEB20819.

Statistical analysis

All statistical analyses were performed in R (version 3.3.2). Mixed-model regression of MTT and SRB data was performed for each time point with the concentration as categorical predictor. A random intercept effect was incorporated for each replicate measurement. In order to make correct statistical inference, all models were evaluated for normal distributed residuals with homogenous variance, by Shapiro Wilk tests ($p>0.05$) and visually by Q-Q plots. Model parameters were estimated by maximum likelihood. When a significant concentration effect was present (ANOVA, $p < 0.01$), the categories were compared pair-wise by posthoc analysis using Tukey's Honestly Significant Difference (HSD) method. All tested concentrations were compared with the control condition (0 μM) and differences were considered significant at $p < 0.05$.

For all other basic statistics, differences between three or more groups were defined via ANOVA and Tukey as post-hoc test, if the data were normally distributed (tested with Shapiro-Wilk test) and homoscedastic (tested with Levene test); if not, Kruskall Wallis test with Tukey post-hoc testing was used as a non-parametric alternative. For the comparison between two groups, a t-test was used for normally distributed data and the Wilcoxon Rank Sum test as a non-parametric alternative. Differences were considered significant at $p < 0.05$.

The packages phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen, 2016) were used for microbial community analysis. Heatmaps were generated with the pheatmap package and order-based Hill's numbers (Hill, 1973) were calculated. Non-metric distance scaling (NMDS) plots of the bacterial community data were created based on the Bray-Curtis distance measures. Significant differences were identified by means of Permutational ANOVA (PERMANOVA) using the *adonis* function (vegan).

Results

5-FU toxicity to oral epithelial TR146 cells

To assess the direct toxicity of 5-FU towards TR146 oral epithelial cells, an MTT/SRB test was performed after 24 h, 48 h and 5 days of treatment (Figure 2). The SRB test showed a significant decrease ($p<0.05$) in protein content starting from 10 µM for all time points. These decreases ranged from a drop with 10 % for 10 µM after 24 h to 63.6 % for 100 µM after 5 days. The MTT test showed a small but significant ($p<0.05$) increase in mitochondrial activity for some time points at low concentrations of 5-FU (0.01-1 µM). At higher concentrations (starting from 10 µM), small decreases were observed after 24 h and 48 h. Viability dropped to less than 50 % after 5 days of treatment with 5-FU at levels higher than 20 µM ($p<0.05$). Together these data show that 5-FU was toxic for TR146 cells starting from 20 µM after 24 h and starting from 10 µM after 48 h or 5 days.

Bacterial cell counts are determined by sample and donor type

For seven donors (4 healthy individuals and 3 patients suffering from mucositis), three types of samples (saliva, buccal swabs and tongue swabs) were investigated in a co-culture model (Figure 1). In this model, the microbial sample was incubated for 24 h on an agar/mucin layer in indirect contact with oral epithelial cells. Each sample was tested in absence of presence of 10 µM 5-FU in the basolateral compartment. Both the initial (directly after taking the sample, t = 0 h) and final (after 24 h of co-culture, t = 24 h) bacterial cell counts were evaluated (Figure 3A). Depending on the type of sample and the type of donor, the initial bacterial concentration ranged between 1 and 5 log colony-forming units (CFU). With regards to the different oral sample types, a clear distinction was observed between buccal swabs on the one hand and saliva and tongue swabs on the other. The initial (t = 0 h) bacterial concentration in buccal swabs (1.9±1.3 log CFU)

was significantly lower compared to saliva (3.7±1.8 log CFU, p=0.002) and tongue swabs (3.6±1.0 log CFU, p=0.023). Despite this variation in initial number, all samples were able to grow up to a concentration of 7-8 log CFU after 24 h of co-culture in the *in vitro* model. The difference in concentration, depending of the sample type, was still present after 24 h, with slightly lower bacterial cell counts for the buccal swab amended wells (7.4±0.4 log cells) compared to saliva (7.9±0.4 log cells, p<0.001) and tongue swab amended wells (7.8±0.4 log cells, p<0.001). Also the type of donor affected the bacterial cell counts. While patient samples displayed a 2-3 log lower initial bacterial concentration compared to healthy individuals (p<0.001), no significant differences were noted after 24 h in the co-culture model (p=0.14). Surprisingly, treatment with 5-FU did not alter bacterial cell counts at 24 h (p=0.92). Thus, bacterial cell counts are determined by both sample type and donor type, but are not affected by 5-FU.

Buccal-derived samples have lower microbial diversity, compared to saliva and tongue amended samples

DGGE (Denaturing Gradient Gel Electrophoresis) analysis (Figure 3B and SI 2) showed that the microbial community of the buccal swab amended samples was lower in richness and evenness, compared to saliva (for both p<0.001) and tongue amended samples (for both p<0.001) at 24 h. For each donor, Bray-Curtis analysis of DGGE profiles also showed significant differences between the different sample types (Table S1).

Patient-derived samples are less diverse and enriched in pathogenic genera as compared to healthy donor samples

With regards to donor type, DGGE showed a lower richness (p=0.0024) and evenness (p=0.015) for patient-derived samples at 24 h, compared to wells with samples from healthy individuals (Figure 3B). The high cell density in saliva samples allowed for

299 performing Illumina sequencing (guaranteeing high-quality data acquisition). As could
300 be expected, the results showed clear differences between donor types (Figure 4 and
301 Figure S7). In correspondence with DGGE results, diversity parameters were lower for
302 patient-derived samples, compared to samples from healthy individuals (Hill number
303 order 0, p=0.0047; order 1, p=0.0024; order 2, p=0.0037) (Figure 4C). Bray-Curtis
304 analysis at OTU level revealed that 16.0 % of the variation in the composition of the
305 saliva samples could be attributed to the type of donor (p=0.0016). Visualization by
306 NMDS plots confirmed the major impact of donor type as all patient-derived samples
307 cluster to one side of the plot (Figure 4B). At 24 h, patient-derived saliva samples were
308 more dominated by Lactobacillales (containing *Streptococcus*, *Abiotrophia* and
309 *Enterococcus*) (95.3 ± 6.9 %) compared to samples derived from healthy individuals
310 (45.3 ± 23.0 %) ($p<0.001$). In contrast, *Veillonella* is more abundant in samples derived
311 from healthy individuals at 24 h (50.8 ± 24.3 %) in comparison with patient-derived
312 samples (3.8 ±6.4) ($p<0.0023$). The initial (t = 0 h) samples from patients contained also
313 more pathogenic genera, for example 25.5 % of *Porphyromonas* for patient 1, 2.8 % of
314 *Enterococcus* and 3.3 % of *Staphylococcus* for patient 2 and 40.8 % of *Porphyromonas*
315 and 12.7 % of *Mycoplasma* for patient 3. These genera were not (*Enterococcus* and
316 *Mycoplasma*) or at much lower abundances (*Staphylococcus* 0-0.04 %, *Porphyromonas*
317 0.2-2.3 %) detected in the samples derived from healthy individuals. Interestingly, the
318 initial microbial composition of the saliva samples of healthy individuals 1 and 2 on the
319 one hand, and 3 and 4 on the other hand were very similar. This can be explained by the
320 fact that these were samples from siblings, living in the same environment and having
321 similar eating habits. In brief, patient samples had lower microbial diversity and higher
322 abundance of pathogenic genera.

323 **5-FU had no major impact on bacterial composition**

DGGE showed that 5-FU did not affect richness (p=0.87) nor evenness (p=0.14) of the bacteria. Bray-Curtis analysis showed that only for patient 2, a significant effect of 5-FU on the microbial profile could be detected based on the DGGE profile (p=0.0014) (Table S1). For all sample types of this patient, two dominant bands clearly disappeared following 5-FU treatment (Figure S6). Similar to DGGE, Illumina sequencing showed that 5-FU treatment did not significantly affect the bacterial diversity (Figure 4C). However, following 5-FU treatment, a general trend in increased *Streptococcus* abundance (from 40.6 ± 26.7 % to 68.1 ± 25.5 % ; p=0.099) and of decreased *Veillonella* abundance (from 44.7 ± 34.8 % to 26.8 ± 23.4 % ; p=0.32) was observed (Figure 4A). In contrast to the other individuals, wells derived from patient 3 were dominated by *Abiotrophia* after 24 h of co-culture both with and without 5-FU. More specifically, *Prevotella* abundance increased following 5-FU treatment for samples derived from healthy individual 3 and 4 (0.4 % to 3.8 % and 0.4 % to 2.2 % respectively). For patient 2, *Enterococcus* and *Streptococcus* were the most abundant genera in the untreated wells (70.4 % and 29.2 % respectively), whereas in presence of 5-FU *Streptococcus* dominated with 98.9 %. This result confirmed the changed DGGE profiles of patient 2 following 5-FU treatment (Figure S6). Altogether, these results indicate small yet non-significant changes in the composition of the biofilm following 5-FU treatment (based on Bray-Curtis dissimilarities on OTU level, p=0.66).

Bacterial composition changes after 24 h of co-culture

Finally, a significant change in bacterial composition was observed with Illumina sequencing attributed between sampling time points (t = 0 h vs. t = 24 h) (p=0.01), which explained 13.3 % of the variation in all samples (based on Bray-Curtis dissimilarities on OTU level). This difference was also visible in the NMDS plot (Figure 4B). Moreover, all Hill numbers showed a decrease in diversity at 24 h compared to the

initial samples (Hill number order 0, p=0.0051; order 1, p=0.0023; order 2, p=0.0031) (Figure 4C). *Streptococcus* and *Veillonella* were the dominating genera in the saliva samples after 24 h in the *in vitro* model (together 95.6 ± 4.2 %), apart from the control sample derived from patient 2, which was dominated by *Enterococcus* and the samples derived from patient 3, which were dominated by *Abiotrophia* (Figure 4A). Next to *Streptococcus* and *Veillonella*, the initial saliva samples were also populated by *Prevotella*, *Neisseria*, *Granulicatella*, *Haemophilus*, *Actinomyces*, *Porphyromonas*, *Fusobacterium*, and *Megasphaera*, of which levels depended on the donor. A lot of this diversity was lost during the 24 h incubation in the co-culture model. For some donors, most genera were still present albeit at relatively low abundances.

Epithelial wound healing is reduced by oral microbiota, irrespective of the presence of 5-FU

To investigate the closure of artificially induced wounds in an epithelial monolayer over time, we followed wound healing in a separate set-up with and without microbiota derived from a buccal swab from a healthy individual in presence or absence of 5-FU (Figure 1). Compared to the (unchallenged) control wells, epithelial cell wound healing slowed down in presence of microbiota starting from 16 h, eventually resulting in a 16 % lower wound healing after 25 h (Figure 5A). The presence of 5-FU had no effect on the wound healing capacity and this was independent of microbial presence.

This experiment showed that co-culture with microbiota reduces wound healing of oral epithelial cells. This effect might, however, be caused by different bacterial cell counts and composition, which have been shown to depend on the type of donor and the sample type. Indeed, although a general reduction (25.9 ± 15.1 %) of wound healing capacity was observed by the addition of oral microbiota, differences could be noticed between the sample and donor types (Figure 5B).

First, addition of microbiota derived from saliva and tongue swabs had a more detrimental effect on wound healing in comparison with buccal-derived microbiota ($p=0.0048$ for saliva; $p=0.070$ for tongue). Regarding the type of donor, no difference in wound healing capacity was noticed ($p=0.95$). However, plotting the wound opening at 24 h as a function of the bacterial cell counts revealed two different trends between healthy and patient samples (Figure 5C). Microbial samples from healthy individuals displayed a linear relationship with each additional log CFU of bacterial cells resulting in a 15.2 % increase in wound opening ($p=0.00082$). Independent of microbiome composition, this is indicative (adjusted $R^2 = 0.17$) of a higher wound healing capacity at lower bacterial loads. However, no such trend could be observed for patient samples ($p=0.13$). Again, no modulating effect of 5-FU on wound healing was observed after 24 h in the presence ($p=0.62$) and absence ($p= 0.21$) of microbiota. An MTT assay performed after 24 h of co-culture showed no effect of sample type ($p=0.41$) or type of donor ($p=0.14$) on the cell viability of TR146 cells. A small but significant increase in epithelial cell viability was observed following 5-FU treatment in presence of microbiota (89.5 ± 11.0 % to 95.3 ± 11.4 %, $p=0.015$), whereas no effect was observed in absence of microbiota ($p=0.94$). Together these data indicate that wound healing potential is determined by both bacterial cell count and bacterial composition.

Discussion

Oral mucositis is a debilitating side effect of chemotherapeutic treatment in which microbiota are more and more shown to play an important role. In this study, we investigated the interactions between the oral microbiome, oral epithelial cells and a chemotherapeutic (5-FU) using an *in vitro* co-culture model. As wound healing is crucial in recovering from mucositis, this was one of the functional endpoints in the model apart from microbial numbers and composition.

Our data showed that oral microbiota reduced wound healing capacity for all seven donors with 25.9 ± 15.1 %. Previous research using the same *in vitro* model, showed that oral microbiota had similar negative effects on wound healing (De Ryck et al., 2014). However, this reduction appeared to be species- and concentration-dependent (De Ryck et al., 2015; Edwards and Harding, 2004). It has been shown for chronic wounds that low amounts of microbiota can improve wound healing, whereas in infectious conditions with high bacterial loads wound healing capacity is significantly reduced (Edwards and Harding, 2004). Our data confirmed that for healthy individuals, lower bacterial cell counts correlated with higher wound healing capacity. This encourages the use of good oral hygiene during mucositis, shown previously to be of high importance in oral mucositis, as colonization of the ulcers by microbiota may prolong the healing phase (Keefe et al., 2007; Villa and Sonis, 2016). However, for patients that are in the acute mucositis phase, more measures might be needed, as we have shown that for such patients wound healing capacity was independent of the bacterial cell counts. This indicates that also bacterial composition might be important in acute mucositis patients. De Ryck et al. (2015) indeed showed that wound healing capacity seems to be species-dependent with *Klebsiella oxytoca* having a deleterious effect on wound healing, whereas *Streptococcus mitis* and *S. oralis* stimulated wound healing.

Further, we observed differences in the composition and diversity of oral microbiota derived from patients suffering from mucositis compared to healthy individuals. The abundance of *Lactobacillalles* was higher in patient samples in comparison with healthy individuals and the diversity of samples derived from patients was lower. Our results are in accordance with a prospective study with 454-sequencing of mucosal samples also showing a lower diversity in patient samples compared to reference individuals (Ye et al., 2013). Moreover, the Illumina data from our study revealed the presence of larger numbers of genera containing pathogenic species, like *Porphyromonas*, *Enterococcus* and *Staphylococcus*, in the patient-derived samples, which could lead to a higher infection risk. *Porphyromonas gingivalis* was shown previously to be predictive for the development of oral ulcerations in hematopoietic stem cell transplantation patients (Laheij et al., 2012).

Different sites in the oral cavity are colonised with distinct microbial communities (Segata et al., 2012). In our study, buccal swabs had lower bacterial cell counts, compared to saliva and tongue swabs, leading to a higher wound healing capacity, which is in line with the previous results. DGGE also indicated lower richness and evenness in the buccal samples. This lower diversity of buccal microbiome compared to saliva and tongue samples has already been explained by extensive data derived from The Human Microbiome Project by the dominance of *Streptococcus* in buccal samples (Segata et al., 2012).

We also investigated the effect of 5-FU on different endpoints in the co-culture model. We chose to work with a dose of 10 µM, as this was the highest non-toxic concentration for TR146 cells after 24h. Similar toxicity profiles have been recorded for other cell lines such as for Caco-2 cells (Fang et al., 2014). *In vivo* concentrations range from 3 to 10 µM in plasma and 0.08-0.8 µM in saliva following continuous treatment (Joulia et al., 1999;

Takimoto et al., 1999), but significantly increase in case of dihydropyrimidine dehydrogenase (DPD) deficiency (Saif et al., 2009). Previous research showed a variable sensitivity among oral species towards 5-FU (Vanlancker et al., 2016). However, in our system which comprises a plethora of oral species cultured in a biofilm, we did not see an impact of 5-FU on both bacterial cell counts or wound healing. Further, 5-FU had only a minor impact on bacterial composition with an increasing trend in *Streptococcus* and a decreasing trend in *Veillonella*. *Streptococcus oralis, S. mitis* and S. *salivarius* have been shown in a previous study to be resistant to 5-FU at 10 µM, (Vanlancker et al., 2016) which might explain their ability to increase in abundance. No data are available on the sensitivity of 5-FU to *Veillonella*, however our data suggest that *Veillonella* is sensitive towards 5-FU. The results for patient 2 indicated high sensitivity of *Enterococcus* towards 5-FU, confirming previous research (Stringer et al., 2009). Moreover, patient 2 was the only donor for which a significant effect of 5-FU on the microbial composition was shown. This indicates a donor-specific effect of 5-FU which encourages the use of a personalized approach.

At 24 h, the biofilm formed in the model was mainly dominated by *Streptococcus* and *Veillonella* for the saliva samples. Although this indicates a loss of diversity of the original saliva sample when cultured in the *in vitro* model, this loss might be due to biofilm formation. *In vivo* growth of an oral biofilm on enamel-dentin slabs in the mouth of healthy volunteers also showed a dominance of *Streptococcus* (62 %) and *Veillonella* (27%) after 48 h (Klug et al., 2016). Although we used saliva samples, the high abundance of *Streptococcus* is more similar to buccal samples (Segata et al., 2012). We hypothesize that the use of an agar/mucin layer as a substrate promotes biofilm formation of a buccal community, despite the use of a saliva sample as a microbial source. This immature biofilm is formed by *Streptococci*, known to be initial colonizers

of the oral biofilm (Kolenbrander et al., 2010). With respect to *Veillonella*, dependency on the lactic acid produced by *Streptococci* has been shown (Kolenbrander, 2000) and therefore these species are likely to co-occur.

In conclusion, oral microbiota reduce wound healing capacity of epithelial cells with higher bacterial cell counts linked to lower wound healing capacity in healthy individuals. However, for patients suffering from mucositis this is not the case, most probably due to their disturbed oral community with higher abundances of pathogenic genera. More research on the link between oral microbial composition and wound healing capacity is needed to fully understand their role in the wound healing process in patients suffering from mucositis.

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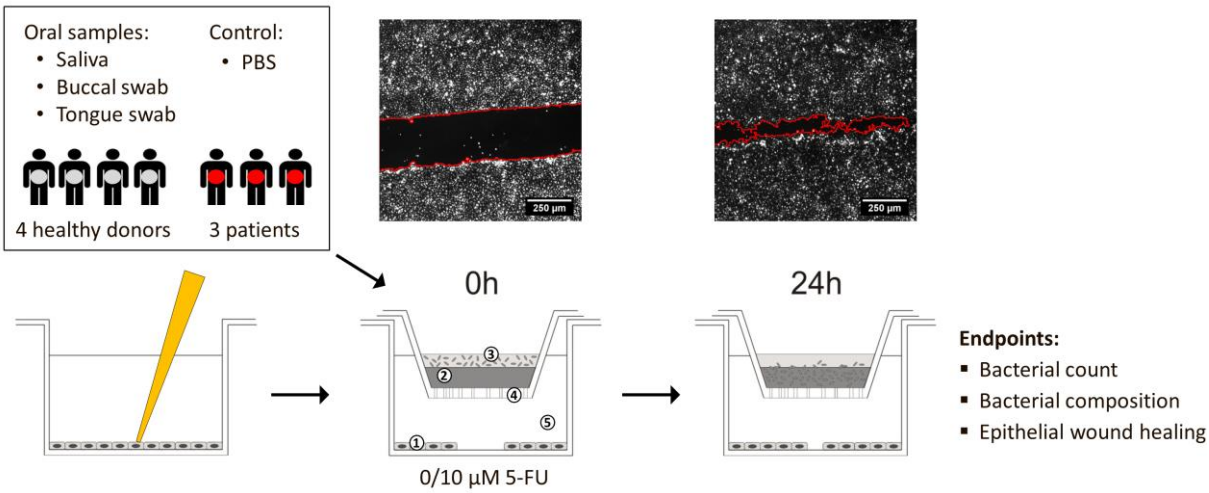


Figure 1: Experimental set-up of the co-culture model with 1) oral epithelial TR146 cells stained with DiI, 2) agar/mucin layer, 3) microbial biofilm or PBS as a control, 4) polycarbonate membrane with 0.4 µm pores, 5) DMEM with 0 µM or 10 µM 5-FU. Fluorescent images show examples of wounds (red line) at 0h and 24h. (based on De Ryck et al. (2014))

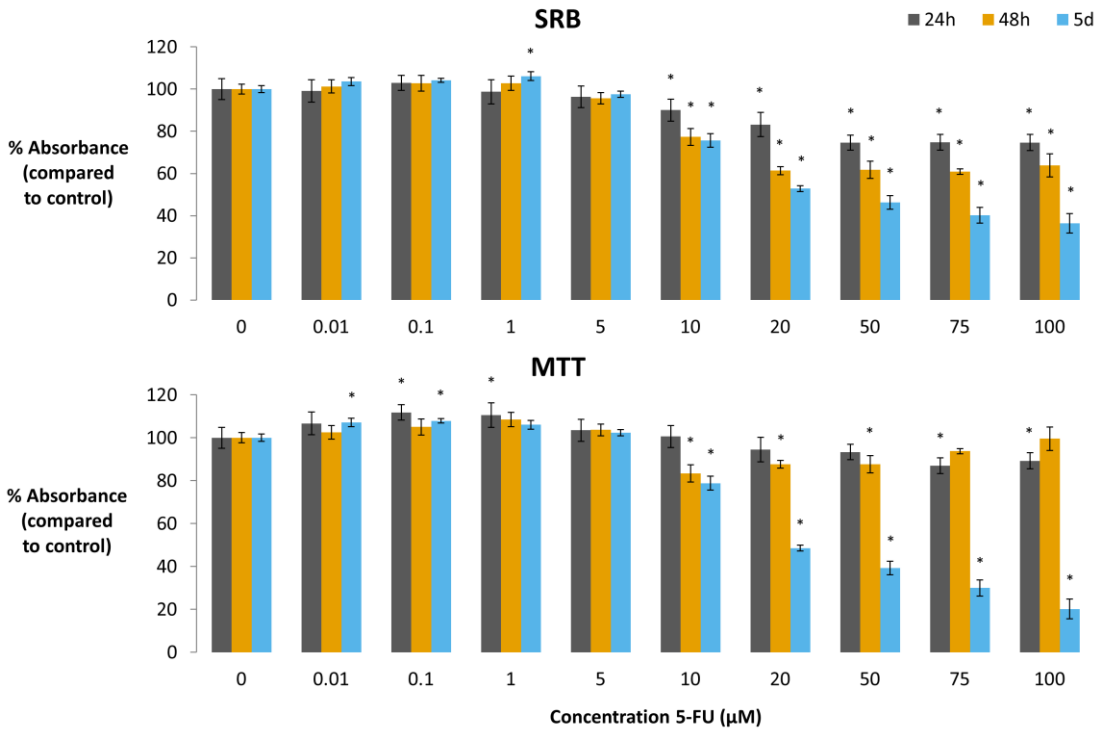


Figure 2: MTT and SRB toxicity test of 5-FU (0.01-100 µM) on oral epithelial TR146 cells (AV±SD, n=6). Significant deviations from the control condition (0 µM) are indicated by the asterisks ($p<0.05$).

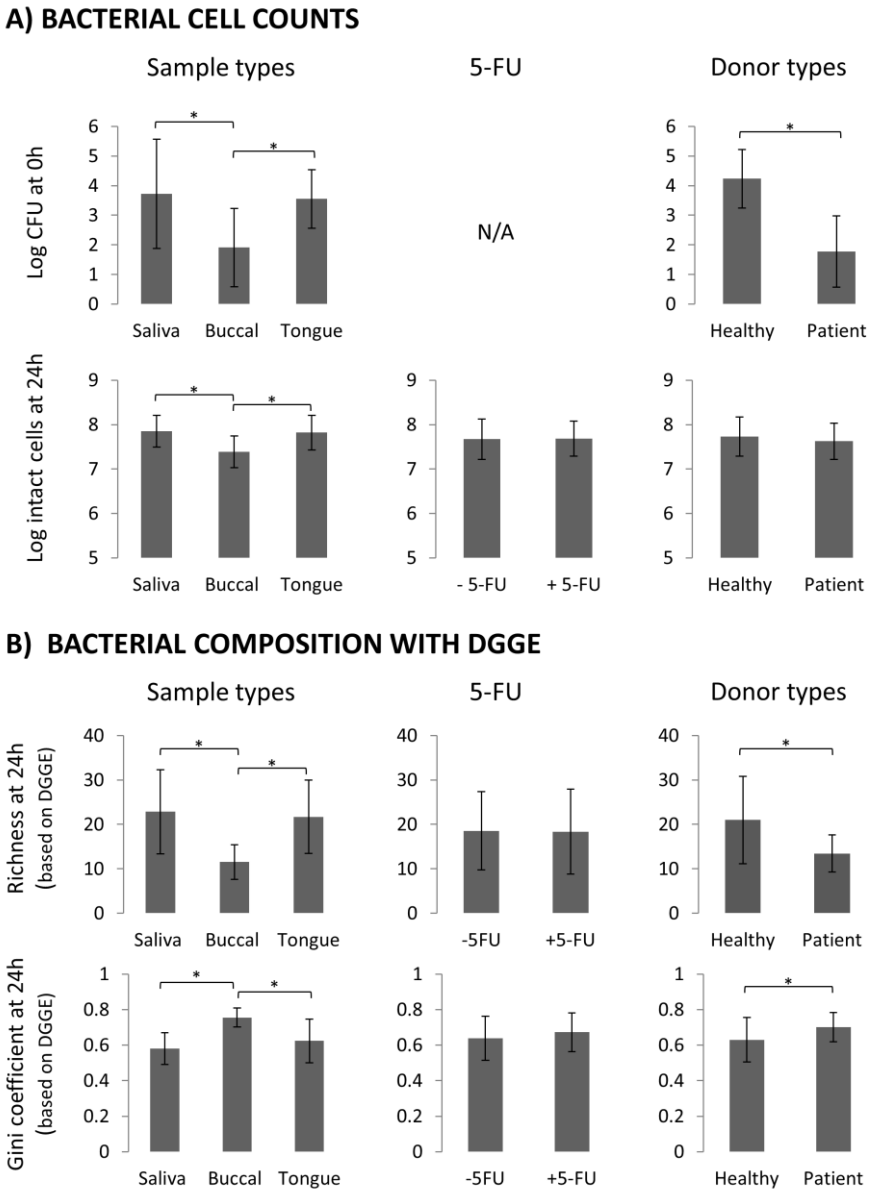
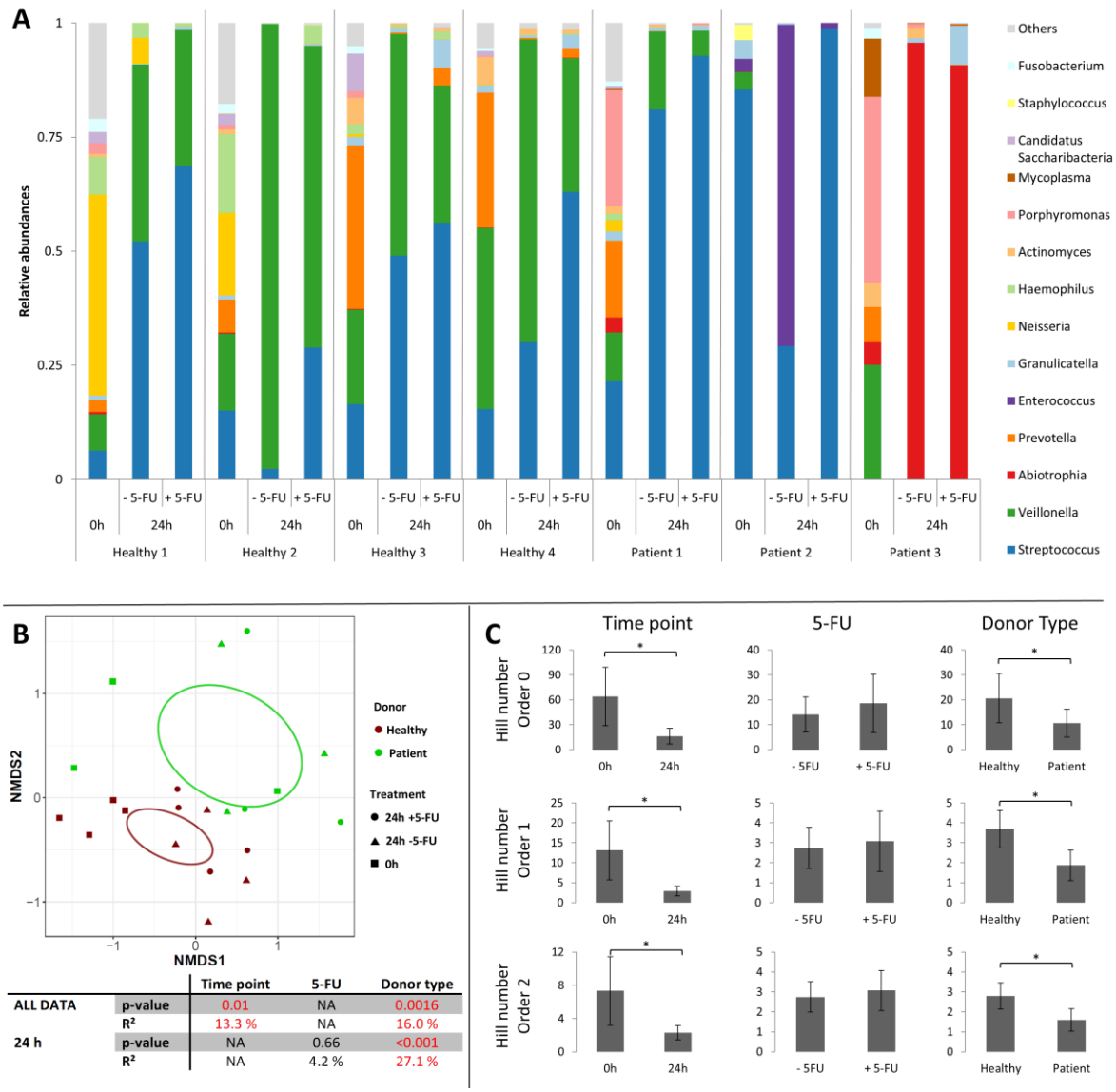


Figure 3: Bacterial cell counts and composition of microbiota derived from different sample and donor types cultured in the oral co-culture model in presence or absence of 5-FU. A) Bacterial cell counts at t=0 h and t=24 h (AV±SD); B) Richness and Gini coefficient as measure for bacterial diversity by DGGE (AV±SD). Significant differences between groups are indicated by the asterisks ($p<0.05$).



		Time point	5-FU	Donor type
ALL DATA	p-value	0.01	NA	0.0016
	R^2	13.3 %	NA	16.0 %
24 h	p-value	NA	0.66	<0.001
	R^2	NA	4.2 %	27.1 %

Figure 4: Illumina sequencing of the 16S rRNA gene of the microbiota in the saliva samples. A) Barplot representing the 14 most abundant genera; B) NMDS plot with 95 % confidence ellipsoid, p-values and R^2 for different confounding factors based on Bray-Curtis dissimilarities ; C) Hill numbers order 0, 1 and 2 representing richness, evenness and diversity respectively (AV±SD). Significant differences between groups are indicated by the asterisks ($p<0.05$).

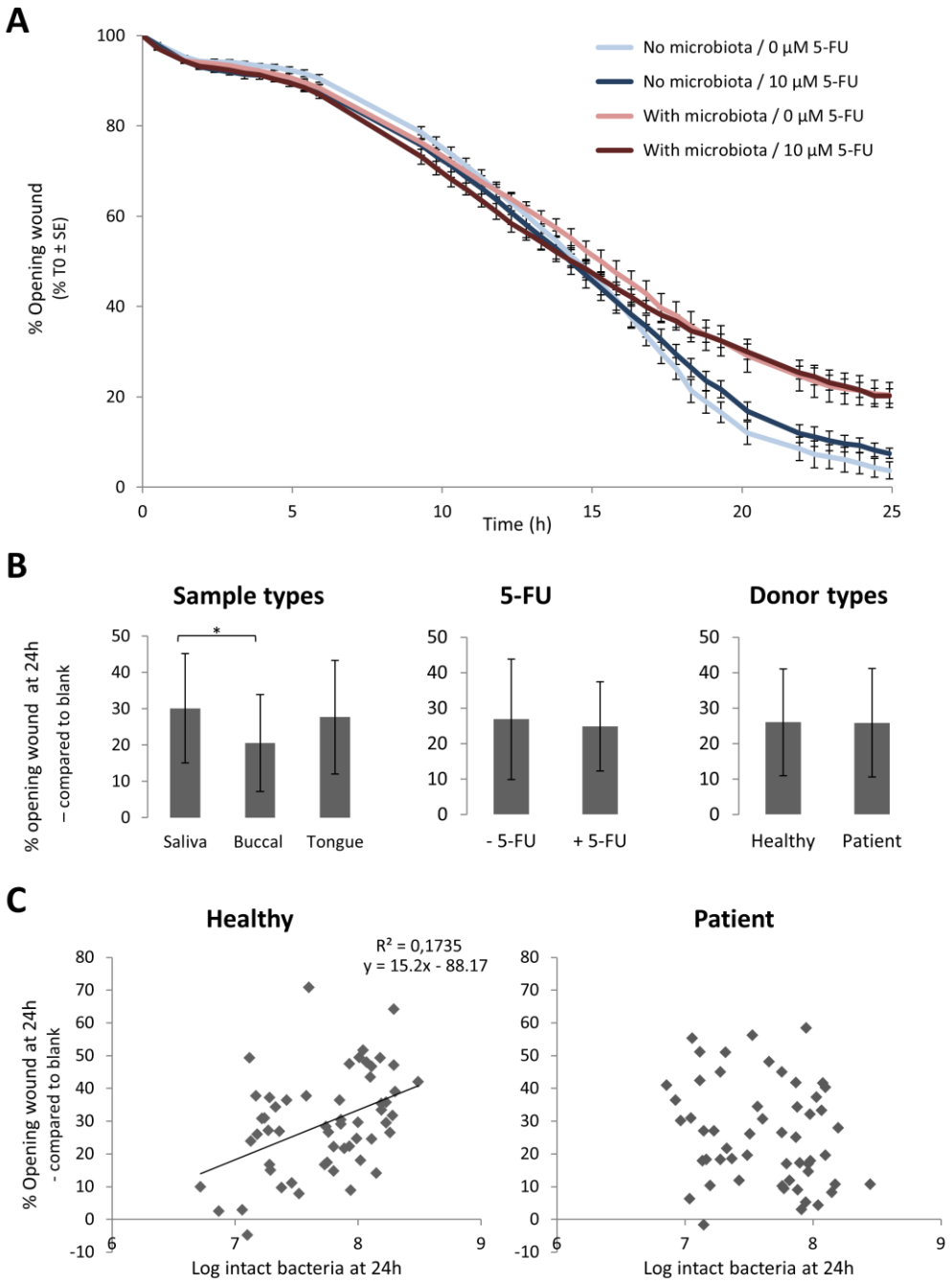


Figure 5: A) Oral microbiota derived from a buccal swab reduces wound healing capacity of oral epithelial cells in an *in vitro* mucosa model, irrespective of the treatment with 5-FU (10 µM) (AV±SE); B) Sample type affect wound healing capacity, whereas donor type and presence of 5-FU do not (AV±SD); C) For healthy individuals, a positive correlation between the opening of the wound and bacterial cell count at 24 h was observed, but for patients no such trend was noticed.

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