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Fungal mitochondrial oxygen consumption induces the growth of strict anaerobic bacteria

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Abstract

Fungi are commonly encountered as part of a healthy oral ecosystem. Candida albicans is the most often observed and investigated fungal species in the oral cavity. The role of fungi in the oral ecosystem has remained enigmatic for decades. Recently, it was shown that C. albicans, in vitro, influences the bacterial composition of young oral biofilms, indicating it possibly plays a role in increasing diversity in the oral ecosystem. C. albicans favored growth of strictly anaerobic species under aerobic culture conditions. In the present study, the role of mitochondrial respiration, as mechanism by which C. albicans modifies its environment, was investigated. Using oxygen sensors, a rapid depletion of dissolved oxygen (dO₂) was observed. This decrease was not C. albicans specific as several non-albicans Candida species showed similar oxygen consumption. Heat inactivation as well as addition of the specific mitochondrial respiration inhibitor Antimycin A inhibited depletion of dO₂. Using 16S rDNA sequencing, it is shown that mitochondrial activity, more than physical presence of C. albicans is responsible for inducing growth of strictly anaerobic oral bacteria in aerobic growth conditions. The described mechanism of dO₂ depletion may be a general mechanism by which fungi modulate their direct environment.
Introduction

The healthy oral cavity houses a diverse and numerous collection of microorganisms (Simon-Soro et al., 2013). The exact number of different microorganisms that reside in the healthy oral cavity remains unknown but it is estimated that there are over 500 different bacterial species present (Zaura et al., 2009). In addition to bacteria, more than 100 species of fungi have been identified in healthy oral cavities (Ghannoum et al., 2010). The large number of different microorganisms serves as protection against extrinsic pathogens that could affect our health. However, some of the commensal microorganisms living in the oral cavity are themselves opportunistic pathogens. These microorganisms can become pathogenic as a result of ecological changes induced by long-term changes in the microenvironment or by impairment of the immune functionality in the oral cavity.

*Candida albicans* is an opportunistic pathogen commonly found in the oral cavity. *C. albicans* is a polymorphic fungus which resides as a commensal in the oral cavity of 50-60% of healthy individuals (Ghannoum et al., 2010). This, otherwise harmless, fungus can become pathogenic in immune-compromised individuals, for example in HIV infected patients (Metwalli et al., 2013). To prevent rapid removal from the oral cavity due to swallowing, most bacterial and fungal species reside in biofilms on the hard (*e.g.* teeth, dentures) or soft surfaces (*e.g.* mucosa of the gingiva, cheek and tongue) (Gibbons and Houte, 1975). Formation of a biofilm provides a protective environment for microorganisms; they are more tolerant towards antibiotics and the host immune defense (Costerton et al., 1999). In biofilms, the microorganisms are in close proximity to each other and have intimate interactions on different levels. The interactions between bacteria and fungi take place on a physical (*i.e.* adhesion), chemical (*i.e.* signaling and secondary metabolites) and metabolic level (reviewed in
(Krom et al., 2014)). Due to their relatively large biomass/cell ratio, fungi have been proposed to have a significant impact on bacterial behavior in biofilms while at relatively low level of colonization acting as a keystone commensal (Janus et al., 2016).

Recently, it was shown that *C. albicans* facilitated the growth of strictly anaerobic bacteria under oxygen rich conditions (van Leeuwen et al., 2016). Also in *in vitro* oral biofilms, *C. albicans* allowed strict anaerobes to grow, probably by creating anaerobic/microaerophilic microniches (Janus et al., 2017). It is currently not known how *C. albicans* is able to modulate oxygen concentrations in these microniches. In *C. albicans*, the main site of oxygen consumption is the electron transport chain (ETC) in the mitochondria, which is responsible for 90% of the total oxygen consumption (Hassan and Bilitewski, 2013). The function of the ETC is to transport protons over the mitochondrial membrane by oxidizing NADH and transferring the H\(^+\) ions to molecular O\(_2\). The ETC of *C. albicans* consists of three different pathways: the classical respiratory pathway (CRC), the parallel pathway (PAR) and the alternative oxidative pathway (AOX) (Helmerhorst et al., 2002; Ruy et al., 2006). All three pathways require the oxidation of NADH by NADH-dehydrogenase leading to a reduced form of coenzyme-Q. From there on, the three pathways differ in their function. Coenzyme-Q is further oxidized, either directly by oxygen via the AOX or by cytochrome c in the cytochrome *bc\(_r\)*-complex in the CRC and PAR. The CRC is the main site of oxygen consumption in *C. albicans* and is active under normal circumstances. However, when the CRC is blocked downstream (*e.g.* with Antimycin A), the AOX takes over part of the oxygen consumption (Helmerhorst et al., 2002). The PAR only becomes active once both the CRC and AOX are blocked (Ruy et al., 2006).
The aim of this study was to determine if the rate of oxygen consumption by *C. albicans*, mediated by mitochondrial oxygen consumption, is involved in the creation of microaerophilic niches thereby influencing the bacterial species composition of early *in vitro* oral biofilms.
Materials and Methods

Saliva inoculum and strains used.

Stimulated saliva was collected from three self-reported orally and systemically healthy individuals as described previously (Exterkate et al., 2010). Subsequently, the saliva was diluted 1:1 in 60% glycerol, divided into 500 µl portions and stored at -80°C (Janus et al., 2015). The saliva was pooled before use and diluted five times during the measurements unless indicated otherwise.

Wild-type strains of five different Candida species; C. albicans SC5314, C. glabrata CBS 138, C. tropicalis HG1936, C. parapsilosis ATCC 90030 and C. dubliniensis HG1932 were used during the experiments. Prior to all experiments, the Candida spp were grown aerobically overnight in Brain Heart Infusion (BHI) at 30°C while shaking at 150 rpm to maintain the yeast morphology. These cultures were centrifuged (5 min, 5000 x g) and concentrated in fresh medium to a final optical density measured at 600 nm (OD₆₀₀) of 5.0.

Development of a dissolved oxygen consumption assay

To determine the rate of oxygen consumption, a dissolved oxygen consumption (DOC) assay was developed. To validate the methodology an OD₆₀₀ ≈ 5.0 suspension of C. albicans was diluted to different OD₆₀₀ values (0.01, 0.05, 0.1, 0.2, 0.5 and 1.0) in fresh BHI to a final volume of 2 ml in an 8 ml wide-mouth tube (Fisher Scientific, Landsmeer, The Netherlands) and pre-incubated for 30 min. at 37°C. Prior to the start of the measurement, the suspension was vortexed vigorously to achieve full aeration. Subsequently, a dissolved oxygen sensor (Applikon Biotechnology B.V. Delft, The Netherlands), housed in an Incucell incubator (37°C, MMM Medcenter Einrichtungen GmbH, Munich, Germany), coupled to a Bio Controller ADI 1030
(Applikon Biotechnology B.V.) was positioned at a fixed height in the suspension. Two individual electrodes were used simultaneously with good agreement (data not shown). Before each experiment, the electrodes were calibrated, in fully aerated BHI medium, using the default mode of the ADI 1030 controller. Blank BHI medium and heat inactivated *C. albicans* (10 min. at 80°C) served as negative control groups. Heat inactivation was validated by plating 100 µl of the treated inoculum on BHI agar plates; no colonies appeared after 24 h incubation at 30°C (not shown). The DOC of *C. albicans* was determined in time and expressed as either the dissolved oxygen concentration (% dO\textsubscript{2}) or the maximum rate of DOC (% dO\textsubscript{2}/min), based on the initial linear range of the DOC plotted over time. Experiments were performed at least in triplicate for each sensor.

*Dissolved oxygen consumption of Candida spp. in buffered artificial saliva medium.*

To study whether the oxygen consumption is a unique trait of *C. albicans* and to mimic conditions better suited for the growth of oral microcosms, DOC of *C. albicans* and four additional *Candida* species; *C. glabrata, C. dubliniensis, C. parapsilosis* and *C. tropicalis* were measured in buffered artificial saliva medium McBain (McBain et al., 2003) without sucrose (Janus et al., 2015; Janus et al., 2017). For all *Candida* species, suspensions were prepared at OD\textsubscript{600} ≈ 0.5. in buffered artificial saliva medium without sucrose. The DOC and maximum rate of DOC were determined as described above. Experiments were performed at least in triplicate for each sensor and each *Candida* species.

*Dissolved Oxygen Consumption by Candida – saliva mixtures*
To investigate a possible role of *Candida* species induced oxygen depletion in a microcosm environment, *C. albicans* was added to buffered artificial saliva medium, with or without pooled saliva in a 1:1:50 ratio (See Table 1 for all combinations). Fully aerated, sterile buffered artificial saliva medium served as a negative control group. To inhibit the major oxygen consuming pathways in the fungal mitochondria, Antimycin A (Sigma, St Louis, MO, USA) was added to a final concentration of 10 µM (Helmerhorst et al., 2002). The DOC and maximum rate of DOC were determined as described above.

**Growth and microbiome analysis of initial biofilms**

The effect of mitochondrial oxygen consumption on initial *in vitro* oral biofilms was studied as described previously (Janus et al., 2017). Briefly, sterilized microscope slides (Menzel-Gläser, 7.6 x 2.6 cm, Fisher Scientific) were placed in a 50 ml Greiner tube (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), filled with 50 ml buffered artificial saliva medium, containing various types of inocula (see Table 1 for more details). All tubes were incubated under aerobic conditions at 37°C for 5 h, after which the slides were aseptically transferred to a petri dish filled with 25 ml buffered peptone water (BPW, Oxoid Ltd, Basingstoke, United Kingdom). Attached microbes were dislodged using a cell scraper (Sarstedt, Nümbrecht, Germany), harvested by centrifugation for 5 min at 5000 x g and immediately frozen at -20°C for subsequent DNA isolation.

**Microbiome analysis**

The bacterial microbiome of initial oral biofilms was analyzed as described previously (Janus et al., 2017). Briefly, after purification bacterial, the DNA concentration was
determined by qPCR and the V4 hypervariable region of the 16S rRNA gene was amplified. The generated amplicons were pooled in equimolar quantities, purified from agarose gels (Qiagen, Roermond, the Netherlands), and sequenced (2x 251 nt) using the Illumina MiSeq platform and Illumina MiSeq reagent kit V2 (Illumina Inc., San Diego, CA, USA) at the VUmc Cancer Centre, Amsterdam, the Netherlands.

Sequence processing and analysis

The sequence data was processed as described previously (Volgenant et al., 2017). After processing, 10 OTUs were removed from the OTU table based on their abundance in the blank isolations and (non-inoculated) medium control samples.

Statistical analysis

Results of the oxygen consumption measurements were analyzed using an independent samples t-test. Differences between groups were considered statistically significant if $p < 0.05$.

The OTU table was randomly subsampled at 9100 reads/sample. The Shannon Diversity index was calculated using PAST software v3.01 (Hammer Ø, 2001). To calculate the significance of the diversity differences between the groups a Friedman test followed by a post-hoc Wilcoxon was performed using SPSS version 23.0.0.2.

The OTU-dataset was $\log_2$ transformed and ordinated by Principal Component Analysis (PCA) into two dimensions using PAST. One-way PERMANOVA (permutational multivariate analysis of variance) were performed on the Bray-Curtis Similarity index to calculate the significance of the compositional differences between the saliva biofilms and mixed biofilms. Groups were considered statistically different if $p < 0.05$. 
Results & Discussion

*C. albicans dissolved oxygen consumption is fast and cell density dependent.*

To validate the DOC assay, the oxygen consumption of *C. albicans* was measured using six different cell densities (Figure 1). Uninoculated sterile BHI and BHI inoculated with heat inactivated *C. albicans* served as negative controls. Neither controls showed measurable decreases in dissolved oxygen concentration during the course of the experiment (Figure 1A). In contrast, BHI inoculated with viable *C. albicans* showed a decrease in dissolved oxygen, even at the lowest cell density (Figure 1B). Maximum DOC rates increased with increasing cell densities. At an inoculation density of OD\textsubscript{600}=0.5 and OD\textsubscript{600}=1.0, DOC was fast resulting in a reduction of 80% or more in the dissolved oxygen concentration within the first 15 min of incubation. Based on the results shown in Figure 1, all other measurements during this study were conducted at an OD\textsubscript{600} of 0.5.

*DOC by C. albicans and other Candida species occurs in artificial saliva medium*

Oral biofilms form in the presence of saliva which is significantly different in composition compared to BHI. Fungal species other than *C. albicans* occur in the oral cavity. Therefore, the DOC of *C. albicans* and other Candida species was evaluated in artificial saliva medium (Figure 2). Dissolved oxygen was depleted from artificial saliva medium by *C. albicans* at a comparable rate to the DOC in BHI (Compare Figure 1A with Figure 2A). With the exception of *C. glabrata*, all included Candida species consumed oxygen at a comparable rate (Figure 2B). The significantly lower DOC rate observed for *C. glabrata* could be related to the previously described absence of certain metabolic pathways (reviewed in (Ene et al., 2014)).
DOC by C. albicans is not influenced by salivary bacteria

*In vivo*, bacteria are present in saliva and these bacteria were used to inoculate *in vitro* models for initial oral biofilms as described previously (Janus et al., 2017). The DOC of artificial saliva containing salivary bacteria was observed (Figure 3A), yet at a slow rate of approximately 0.5% dO₂/min (Figure 3C). When using a mixed inoculum of artificial saliva containing salivary bacteria and *C. albicans* DOC was faster and similar to the DOC of *C. albicans* alone. Addition of human salivary microbiome in combination with *C. albicans* to create a mixed inoculum did not affect the decrease in dissolved oxygen.

Mitochondrial respiration is involved in rapid DOC by C. albicans

To investigate the role of mitochondrial oxygen consumption in this decrease in dissolved oxygen Antimycin A was used as described previously (Helmerhorst et al., 2002). Artificial saliva medium in combination with Antimycin A served as a negative control group, showing no measurable decrease in dissolved oxygen concentration during the course of the experiment (Figure 3B). Antimycin A did not affect oxygen consumption of the human salivary microbiome. In contrast, addition of Antimycin A to *C. albicans* significantly affected the decrease in the rate of DOC (Figure 3C). The mixed inoculum showed a stronger decrease in dissolved oxygen concentration compared to *C. albicans* alone, yet the rate of oxygen consumption was significantly reduced by addition of Antimycin A. This difference could be explained by interaction of Antimycin A with bacteria reducing the effective concentration of Antimycin A for *C. albicans* in presence of saliva. It should also be noted that the effect of Antimycin A was more pronounced than observed by Helmerhorst and co-workers previously, yet it is important to note that in contrast to our study, they added Antimycin A during 16-
h growth providing time to induce the expression of alternative respiratory pathways (Helmerhorst et al., 2002).

To allow comparison with previous studies (Janus et al., 2017), consumption of oxygen, and the effect of Antimycin A thereon, was also measured using a mixed inoculum at a ratio of 1:1:50 (OD$_{600}$ 0.1) in artificial saliva medium. The consumption of oxygen followed the same pattern as seen in Figure 3A, be it with a lower rate (Supplementary figure S1).

**Effect of C. albicans oxygen consumption on bacteria in initial oral biofilms**

To evaluate the effect of mitochondrial oxygen consumption of *C. albicans* on the bacterial composition of initial oral biofilm, in vitro oral biofilms were grown for 5 h. The biofilms were inoculated using saliva, a mixed inoculum of saliva and *C. albicans* and mixed inocula of saliva with heat-killed *C. albicans*. To inhibit mitochondrial respiration, saliva and mixed saliva-*C. albicans* inocula were also treated with Antimycin A. Composition of initial oral biofilms was determined using 16S rDNA sequencing. The complete subsampled Operational Taxonomic Unit (OTU)-table is available as Supplementary Table S1.

The Shannon Diversity index of the biofilms was significantly lower when Antimycin A was present; 1.2 ± 0.04 for saliva alone, 1.3 ± 0.17 for saliva with *C. albicans* and 1.3 ± 0.12 for saliva with heat-killed *C. albicans* vs 1.1 ± 0.07 and 0.9 ± 0.08 for saliva with Antimycin A and saliva with *C. albicans* and Antimycin A respectively (Figure 4). The species composition of all groups was different (p < 0.001, F = 3.64) which is visualized in the principal component analysis (PCA) plot (Figure 5), where the different biofilms clustered based on their inoculum. The first component, explaining 24% variance, was responsible for separating metabolic
active *C. albicans* (top-right quadrant) and metabolic inactive or absence of *C. albicans* (top-left quadrant), while the second component, explaining 12% variance, separates the groups containing Antimycin A (bottom half) from the groups without Antimycin A (top half).

The differences in diversity and in species composition between the saliva only biofilms with and without Antimycin A indicates that this compound not only blocked mitochondrial oxygen consumption by *C. albicans*, but also influenced some bacterial species in the biofilm. Biofilms without Antimycin A, visible on the top half of the PCA plot (Figure 5) contained more *Streptococcus* (OTU 2 and OTU 41), *Granulicatella* (OTU 75) and *Gemella* (OTU 9), while the biofilms with Antimycin A contained more *Actinomyces* (OTU 16). Antimycin A is a selective inhibitor of the *bc*₁-complex, which is a part of the ETC (Helmerhorst et al., 2002). The *bc*₁-complex is not specific to *C. albicans* but can be found in the mitochondria of aerobic eukaryotes and is also common, but not universal, in prokaryotes (Rotsaert et al., 2008; Trumpower, 1990). It is likely that Antimycin A also inhibits the *bc*₁-complex in bacteria. Since not all bacteria harbor this complex, the more sensitive bacteria will become less competitive within the biofilm, explaining the decrease in bacterial diversity and shift in bacterial composition.

The pooled saliva biofilms clustered in close proximity of the pooled saliva with heat-killed candida (top-left quadrant of Figure 5), indicating that their bacterial species composition is fairly similar. In contrast, the biofilms with pooled saliva and actively respiring *C. albicans* clustered in the top-right quadrant of the PCA plot, indicating a different bacterial species composition. These biofilms contained relatively more of the anaerobic species *Prevotella* (OTU 5 and OTU 130) and *Veillonella* (OTU 4 and OTU 66), while the pooled saliva and saliva with heat killed *C. albicans* (bottom half).
albicans biofilms contained more of the aerobic species Rothia (OTU 12, OTU 18 and OTU 162) and Neisseria (OTU 7). These findings are in accordance with previous studies showing that C. albicans can induce growth of anaerobic species under aerobic conditions (Fox et al., 2014; Janus et al., 2017; van Leeuwen et al., 2016). It also shows that C. albicans needs to be alive to induce the growth of anaerobic species in aerobic conditions, supporting the hypothesis that mitochondrial oxygen consumption causes this micro-aerobic/anaerobic niche. Despite the vertical shift in the PCA plot caused by Antimycin A, the saliva and C. albicans biofilms with Antimycin A shifted to the left of the PCA plot compared to the saliva and C. albicans biofilms without Antimycin A. This indicates that inhibition of mitochondrial oxygen consumption is indeed crucial for creating environmental conditions where anaerobic bacteria can thrive. Good examples of this phenomenon are OTU 4 (Veillonella), OTU 5 (Prevotella) and OTU 66 (Veillonella) (Supplementary Figure 1b). For these anaerobic OTUs, the highest relative abundance is found in the mixed saliva and Candida inoculated biofilms, followed by the mixed saliva and Candida inoculated biofilms with Antimycin A, followed by the mixed saliva and heat-killed Candida, and the lowest relative abundance is found in the saliva and saliva with Antimycin A biofilms. The reversed trend is clearly visible for OTU 12 (the aerobic Rothia) (Supplementary Figure 1a).

Conclusion

The aim of the described study was to determine if the rate of oxygen consumption by C. albicans, mediated by mitochondrial oxygen consumption, is involved in creation of microaerophilic niches thus influencing the bacterial species composition of early in vitro oral biofilms. Considering the data, we can confirm that C. albicans is
able to consume a large amount of oxygen in a short amount of time and that this ability is not dependent on the formation of biofilms. Metabolic activity of mitochondria of *C. albicans* is required for oxygen depletion to occur. Furthermore, we concluded that this ability is not exclusive to *C. albicans* as other species of the *Candida* genus are able to rapidly consume oxygen. In addition, it is likely that many other aerobic fungi have this potential. A consequence of this action is that microaerophilic sub-niches develop directly influencing the metabolism and growth of oral bacteria as well as of other fungi in the oral cavity. The described mechanism of oxygen depletion might thus be a general mechanism by which fungi modify their environment and influence the oral ecosystem.
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Legends

Figure 1 The consumption of dissolved oxygen in time of various cell densities of *C. albicans*. *C. albicans* was prepared in various cell densities; OD\textsubscript{600} 0.1 (◆), 0.2 (▼), 0.5 (▲) and 1.0 (■) with heat inactivated *C. albicans* serving as a negative control (●). The dissolved oxygen (dO\textsubscript{2}) consumption was monitored using a dO\textsubscript{2} sensor and recorded in time. The data is represented as a consumption of dO\textsubscript{2} (%) in time (fig 1a) and the maximum rate of dissolved oxygen consumption, presented as dO\textsubscript{2} (%)/min. (1b) The data is presented as the mean, with standard deviations (N ≥ 6) for all groups.

Figure 2. Dissolved oxygen consumption of *Candida spp* in buffered artificial saliva medium. The relative removal of dissolved oxygen for five different *Candida* species was determined in buffered artificial saliva medium. All species were inoculated to an OD\textsubscript{600} of 0.5. *C. albicans* (●), *C. glabrata* (■), *C. tropicalis* (▲), *C. parapsilosis* (▼) and *C. dubliniensis* (◆). B: The calculated maximum rate of dissolved oxygen removal for the five different *Candida* species in percentage per minute. Standard deviation is included in both figures, N = 6 for all groups. * indicates statistical significant difference compared to *C. albicans* (p<0.01).

Figure 3. Effect of Antimycin A on dissolved oxygen consumption assay in artificial saliva medium. A: The relative removal of dO\textsubscript{2} in percentage over time in artificial saliva medium. (●) uninoculated control; (■) saliva inoculum; (▲) *C. albicans* inoculum; (◆) *C. albicans* + saliva inoculum. B: The relative removal of dO\textsubscript{2} in percentage over time in artificial saliva medium in combination with Antimycin A. (●) uninoculated control + Antimycin A; (■) saliva inoculum + Antimycin A; (▲) C. albicans + Antimycin A.
*albicans* inoculum + Antimycin A; (◆) *C. albicans* + saliva inoculum + Antimycin A.

The y-axis in figures A and B shows the relative amount of dissolved oxygen in percentages. C: The rate of O$_2$ removal in percentage per minute for all measurements. The effect of Antimycin A statistically significant in measurements were *C. albicans* was present. The effect of Antimycin A on saliva and control measurements was not statistically significant. Standard deviation is displayed for all groups. N ≥ 5 for all groups. * indicates statistical significant difference compared to no Antimycin (p<0.01).

**Figure 4.** Effect of *C. albicans* oxygen consumption on microbial diversity of initial oral biofilms. Shannon Diversity Index of the biofilms, where S represents saliva inoculum, SA represents saliva with Antimycin A, SC represents saliva and *C. albicans*, SCA represents saliva and *C. albicans* with Antimycin A, and SCHK represents saliva and heat killed *C. albicans*. Statistical significance is indicated with a and b (p<0.05 compared to a), where bars with the same letter are not significantly different.

**Figure 5.** Principal component analysis plot illustrating how *C. albicans* oxygen consumption influences initial oral biofilms. (White squares) Saliva, (light grey squares) saliva with Antimycin A, (grey squares) saliva and *C. albicans*, (dark grey squares) saliva and *C. albicans* with Antimycin A, (black squares) saliva with heat killed *C. albicans*. The data was randomly subsampled and log2-transformed.

**Supplementary figure S1.** Relative abundance of all Operational Taxonomic Units (OTUs) with Principal Component 1 (PC1) or Principal Component 2 (PC2) loadings
above 0.15, and a total relative abundance of above 0.05%. S represents saliva inoculum, SA represents saliva with Antimycin A, SC represents saliva and C. albicans, SCA represents saliva and C. albicans with Antimycin A, and SCHK represents saliva and heat killed C. albicans. A) OTUs with higher abundance in biofilms without live C. albicans, B) OTUs with higher abundance in biofilms with live C. albicans, C) OTUs with higher abundance in biofilms without Antimycin A, D) OTU with higher abundance in biofilms with Antimycin A.

Supplementary table S1. The total subsampled OTU counts per sample are presented with the taxonomy (assigned to the representative sequence of the OTU) as derived from the RDP-classifier/SILVA. S represents saliva inoculum, SA represents saliva with Antimycin A, SC represents saliva and C. albicans, SCA represents saliva and C. albicans with Antimycin A, and SCHK represents saliva and heat killed C. albicans, and the number indicates the replicate number of the biofilm.
| Inoculum                              | Ratio inoculum to medium |
|--------------------------------------|--------------------------|
| Pooled saliva : MilliQ               | 1:1:50                   |
| Pooled saliva : *C. albicans*        | 1:1:50                   |
| Pooled saliva : *C. albicans* (Heat Killed)* | 1:1:50                   |
| Pooled saliva : *C. albicans* + Antimycin A | 1:1:50                   |
| Pooled saliva : MilliQ + Antimycin A | 1:1:50                   |
| *C. albicans* : MilliQ**             | 1:1:50                   |

**Table 1.** Inoculation ratios of pooled saliva, *C. albicans* and buffered artificial saliva medium used for the DOC of *Candida* – saliva mixtures and for the growth and microbiome analysis of initial biofilms

* not used in the experiment to determine the DOC in *Candida* – saliva mixture experiment

** not used in the initial biofilm experiment
Figures

Figure 1

A

B

-- Control
- 0.1
- 0.2
- 0.5
- 1.0

Relative O₂ (%)

Time (min)

Maximum rate of O₂ consumption (%/min)

OD₆₀₀

0.0  0.2  0.4  0.6  0.8  1.0  1.2
Figure 2
Figure 3

(A) Relative O₂ (%)

(B) Control, Mixed, C. albicans, saliva

(C) Rate of O₂ removal (l/min)

No amrycin A

Antyrycin A

Control, Saliva, C. albicans, Mixed
Figure 4.
Figure 5
