Interactions between oral commensal Candida and oral bacterial communities in immunocompromised and healthy children

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Interactions between oral commensal *Candida* and oral bacterial communities in immunocompromised and healthy children

Short title: Commensal *Candida* and oral bacterial communities

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Keywords

Candida albicans; oral bacteria; coaggregation, biofilm; *ALS* genes

Abstract

Candida species are usually found as commensal microorganisms in the oral cavity of healthy people. During chemotherapy, cytostatic drugs lead to depletion of the oral flora with the emergence of a dominant bacterial species. The transition from commensal to pathogenic state, further associated with yeast colonization and oral mucositis implies a replacement of the dominant microorganism by *Candida albicans*. This process goes plausibly through cooperation between *C. albicans* and bacteria. This study focused on the first step of cooperation between microorganisms isolated from the same oral flora either of leukemic or healthy children. *C. albicans* isolated from 8/20 children were cultured to display their noninvasive blastosporic yeast form and mixed with their dominant bacteria to study the capacity of planktonic aggregation and the early state of biofilm formation. None of the dominant bacteria opposed the presence of yeast, on the contrary, an interesting cooperation was observed. This behavior is apparently different from that observed when mixing the type strains. In fact, three mutated *C. albicans* strains display, by their spontaneous ability to form filament, enhanced risks of virulence for leukemic ill carriers. Despite such risks, neither oral nor systemic pathology were observed in ill patients probably because the study was conducted during the first course of chemotherapy and *Candida* colonization is related to the number of chemotherapeutic cycles.

The presence of *C. albicans* during the initial cycle represents, by its ability to interact with oral bacteria, an actual threat for further cures.

Introduction

So far, more than 600 bacterial species have been found amongst the human oral flora forming therefore a well-balanced system which behaves in a commensal manner most of the time [1]. Moreover numerous bacterial species are also able to form commensal relationship with yeasts such as *Candida* [2]. This relation must be noticed as it occurs in case of both sick and healthy patients. Actually the presence of *Candida* does not usually break the balance, but may contribute to the colonization of oral and denture surfaces thus causing the formation of local dysbiosis [3,4]. As a consequence, oral cavity would then turn itself into a haven for pathogens and a potential entrance point for opportunistic infections.

Fungal infections remain the major complications in the treatment of hematological diseases [5-7]. Main risk factors for candidemia are known to be neutropenia, mucositis, and the presence of a central venous catheter [8-11]. Administration of cytostatic drugs during chemotherapy in case of leukemia involves oral mucositis as a primary side effect[12]. It frequently involves ulceration, bleeding and severe pain which may prevent a proper diet and result in poor patient compliance to treatment [13]. Moreover, the use of cytostatic drugs leads to the depletion of the oral flora with the emergence of a dominant bacterial species [14] further associated with yeast colonization and oral mucositis. *Candida albicans* is the most frequently encountered species [5-7].

Commensal to pathogenic state transition implies a replacement of the dominant microorganism by *C. albicans* which is a plausible process going through cooperation between yeasts and bacteria. In fact, interactions between bacteria and *Candida* have already been studied [3,15-18]. Such interactions play a key role in biofilm formation and protect the potential pathogens from targeted treatments. Some bacteria such as *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* or *Lactobacillus* spp prevent *Candida* from colonizing their biofilm [19-24]. Considering oral cavity, *Streptococcus or Actinomyces* spp, the main constituents of bacterial flora, are known for being more permissible concerning *Candida* proliferation into the biofilm [2,3,17,18]. These previous studies [3,15-18] have been conducted on ATCC type strains.

To our knowledge, this is the first study carried out on the behavior of *C. albicans* and bacterial strains collected from orally healthy volunteers and patients with hematological diseases. Interactions between the dominant bacterial strain and *C. albicans* were intended to identify any evaluable cooperation that occurs when yeast and bacteria are isolated from the same patient.

As adherence constitutes a crucial step in the initiation and propagation of oral candidiasis, we decided to focus on the ability of *C. albicans* to form biofilm either alone or with dominant bacteria from the same oral flora. In order to observe the transition from commensal form into a pathogenic one, culture conditions were aimed at maintaining *C. albicans* under the yeast state. By this way, *C. albicans* remained commensal, therefore displaying a noninvasive blastosporic yeast form during the whole study [25,26].

The first state of adherence runs under the control of *ALS* type genes. *ALS3* gene is directly involved in germ tube and biofilm formation [27,28] while the deletion of *ALS1* gene leads to nonfunctional biofilm formation [29]. *ALS4* gene is induced early in biofilm formation and upregulated compared to the non-adherent planktonic reference [30]. Germ tube formation and cell adhesion decrease when *ALS4* gene is deleted [31]. Als4p covers the surface of yeast cells with a greater abundance on cells grown at 30°C compared to 37°C. On germ tubes, Als4p is localized in a restricted area proximal to the mother yeast [32]. At last, *ALS*6 gene expression increases yeast adhesion to oral epithelial cells [33], while its expression corrects effects of *ALS1* and *ALS3* gene deletions [29]. The study of *ALS* genes with their contribution to cell adhesion and germ tubes formation appeared to be a relevant way to explain the differences in the behavior of the wild strains compared to the type ones.

The goal of this study was to evaluate the cooperation between *C. albicans* and the dominant bacterial strain isolated from the mouth in leukemic and/ or healthy children. This point was explored through the microbial aggregate properties and the early state of biofilm formation capacities in wild compared to type strains and/or in ill compared to healthy patients.

Patients and methods

Patients

Two groups of 10 patients (aged 3 to 13 years) were enrolled during 4 weeks in the study. The parents gave their informed consent (contrat CCPPRB 95 /12.92). The first group gathered children suffering from acute lymphoblastic or myeloblastic leukemia treated by chemotherapy for the first time. The control group included healthy children paired by age and dental formula. The study began by an oral hygiene appointment assessing initial oral health, excluding children showing oral lesions. Patients requiring antibiotics during the study were excluded.

Samples

The study enclosed 20 children for a total of 80 samples collected once a week during 4 weeks (at day 0, 7, 14 and 21), before any meal and brushing of teeth. Sample collection was performed by swabbing the supra-gingival plaque of the last three teeth of the upper right quadrant and the last three teeth of the lower left quadrant. The samples were carefully taken by passing the swab onto the gingivodental line [14].

The samples (labeled L for leukemia group, T for control group) were transferred to 1 ml of pre-reduced transport fluid (RTF) for transport to the laboratory. All specimens were processed within the following 2 h. Briefly, they were diluted in series and the dilution of 10^{-5} was cultured at 37°C on Columbia agar containing 5% blood under aerobic and anaerobic (80%N_2, 10%H_2, 10%CO_2) condition to select the dominant bacterial strains. To isolate fungi, the undiluted and samples diluted to 10^{-1} were grown simultaneously on Sabouraud agar at 30°C and at 37°C under aerobic conditions.

Identification

The dominant bacterial strains were selected after 48 hours in aerobic and after 5 days under anaerobic conditions. The characterization of the dominant bacterial strains included morphological assessment as well as identification by strips (Rapid ID 32 STREP®, Rapid ID 32A®, API NH® Biomérieux, France). Yeasts were assessed by examining Sabouraud agar after 48 hours of incubation and identified using Rapid Yeast plus® (AES Laboratoires, France).

Aggregation of planktonic cells

Aggregation protocol was performed according to Hsu et al. [34]. The cultures were performed in enriched Todd Hewitt broth for bacteria and Sabouraud broth for yeast strains. Both culture were incubated individually for 24 hours. They were then washed twice in PBS and buffered using potassium phosphate buffer (pH 7.4). One equal volume of 10^8 cfu mL^{-1} bacterial suspension (OD_{600} 0.02 for *Streptococcus* to 0.05 for other bacteria) were mixed to the 10^7 mL^{-1} yeast suspension (OD_{600} 0.4) in the 24-well plate. These concentrations are near the end of the exponential phase of growth. In the same way, the self-aggregation was carried out for a well contained bacteria or yeast strains alone using *C. albicans* ATCC 26555 *and S. mutans* ATCC 25175 as control. The plates were shaken at 33 rpm during 16 hours. The aggregation was then evaluated using a scale from 0 to 3 after the observation of the entire well by inverted photonic microscope.

While 0 meant an absence of any aggregate, 1 assessed an observation of less than one aggregate on a field of view at 200 magnification, 2 was attributed when one aggregate was observed on a single field of view, 3 was attributed when several aggregates were observed on a single field of view. The entire well, almost 20 fields, was always scored by the same biologist. *Candida albicans* and *Streptococcus mutans* are two species known to show strong synergism and high cells aggregation in dual species biofilm [35,36]. We used *Candida albicans* ATCC 26555 and *Streptococcus mutans* ATCC 25175 as control.

Characterization of planktonic cells aggregates by electronic scanning microscopy

After scoring the planktonic cell aggregates by photonic microscopic analysis, the yeast and bacterial coaggregates in the suspension were collected by centrifugation. The supernatant was removed and discarded. Pellets were then fixed using a 2.5 % glutaraldehyde solution in a cacodylate buffer at pH 7.2 for 1 hour, rinsed and dehydrated using ethanol [37]. Specimens were then coated with a thin film of gold-palladium. New observations were performed using a scanning tunneling microscope JEOL IT 300 with JEOL image analysis piloted by JEAOL software.

Evaluation of the initial phase of biofilm formation using Biofilm Ring Test®

Early state of biofilm formation was assessed using the Biofilm Ring Test method (BioFilm Control, Saint Beauzire, France) as described by the manufacturer [38]. This assay is based on the immobilization of magnetic beads (TON 006) while they get embedded in microbial aggregates. Microbial inoculum (fungi and /or bacteria) were prepared from exponential cultures in filtered BHI medium and adjusted for an initial concentration corresponding to OD_{600} ranging between 0.4 for fungi strains (10^7 mL^{-1}) to 0.04 for the three spontaneous filamentous fungi determined by direct microscopic examination (L6 J7, L7 J14 and L7 J21) and 0.02 for *Streptococcus* to 0.05 for other bacteria (10^8cfu mL^{-1}). The microbial suspension supplemented with 1% magnetic beads solution were distributed in sterile wells of polystyrene strips (Strip Well MSW002B) and incubated at 37°C for 0 hour (t_{0h}), 4 hours (t_{4h}) and 22 hours (t_{22h}). One well with TON and without microorganism was used as control for bead efficiency. At any time point, wells were scanned by a dedicated scanner before and after magnetization. Biofilm formation was expressed as a Biofilm Formation Index (BFI) calculated by the Biofilm Control Software. A low BFI value ≈ 2 corresponds to complete immobilization of beads by cells forming a biofilm whereas a high BFI value ≥ 7 indicates that the microbial population did not form biofilm [38]. The results are expressed as the mean of triplicate BFI values from

three independent experiments per isolate and incubation time. Controls were performed using strains of *C. albicans* ATCC 26555 and *Streptococcus gordonii* DL1 as initial colonizing bacteria in oral biofilm.

Visualization of microbial communities in biofilm by confocal scanning microscopy

The study focused on the three couples of spontaneous filamentous fungi isolated from ill patients and their dominant bacterial strain: L6 J7, L7 J0 and L7 J21. They were monitored using confocal scanning microscopy. Strains were prepared according to the Biofilm Ring Test protocol. Biofilm were then grown in sterile Ludin® chambers (Life Imaging Services, Switzerland) connected to a peristaltic pump (Minipuls 3, Gilson, Middleton, WI) allowing a flow rate of 7 $mL.h^{-1}$ through silicone tubing in aerobic conditions. Flow cells were coated with 0.22-µm filtrated sterile human saliva (collected from six healthy stimulated volunteers, treated with 2.5mM dithiothreitol and diluted in distilled water to obtain a 25% (v/v) solution) for 30 min before bacterial inoculation. All steps (inoculation, washing, staining) were performed with a flow rate of $7ml.h^{-1}$ [39].

Assays of mono or dual-species biofilm formation of fungi with or without bacteria were performed by inoculation for 30 minutes in the flowing system. Biofilms were then incubated for 24 hours at 37°C in a humidified chamber. After 15 minutes of washing with PBS, biofilms were stained with 5µM of Syto® 9 nucleic acid dye (Molecular Probes, Lieden, The Netherlands) diluted in PBS and a ½ dilution of Calcofluor White stain (Fluka, Sigma Aldrich, Buchs, Switzerland) diluted in KOH (1:10) for 15 minutes [40].

Flow cells were then observed *in situ* with a Leica TCS-SP8 confocal laser scanning microscope (Leica Microsystems, Wezlar, Germany). An HC PL Apo 63X, 1.4 NA, oil immersion objective lens was used for image capture and a numerical zoom of 1.5 was applied. The 488-nm Ar laser and a 500 to 570-nm band-pass emission filter were used to detect Syto®9-assocciated fluorescence. The 405-nm UV diode and a 450 to 500-nm band-pass emission filter were used to detect *C. albicans* stained with Calcofluor® [40].

Biofilm stacks (123 X 123 µm) acquired at 1 µm intervals were scanned with a line average of 2. Leica software (LAS AF V.2.2.1) was used for microscope piloting and image acquisition before image analysis by ImageJ V1.43m (National Institute of Health).

C. albicans ATCC 26555, *S. gordonii* DL1 and *S. mutans* ATCC 25175 were used as controls.

Amplification and sequencing of Agglutinin-Like Sequences ALS1, ALS3, ALS4 and ALS6 genes from Candida albicans isolates

Total genomic DNA was obtained using the chelex extraction protocol [41]. Using specific primers [42] (Table 1), a segment of 318bp for Als1p, 342bp for Als3p, 356bp for Als4p and 152bp for Als6p from *ALS* gene family of *C. albicans* were amplified from each genomic DNA extract.

PCR was performed using 1 µl of template DNA, 0.5 µl of each forward and reverse primer (both 5 µM). Total reaction volume was 15 µl made up using 8µl of MyTaq Mix (Bioline®) and 5µl of distilled water. Thermocycling conditions were; one cycle of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 15 s to 30 s at the annealing temperature of 45°C to 62°C, and 30 s at 72°C, and a final 7 min extension at 72°C [42]. Double strand sequences were obtained using an automated sequencer (PE Applied Biosystems 3730 Genetic Analyser; plate-forme de séquençage Biogenouest, Nantes). Sequences were aligned using the CodonCodeAligner® software v3.5 (CodonCode Corporation, Dedham, Massachusetts). Analyses of sequence polymorphism were carried out using DNASP v4.10.9 [43].

Statistical analysis

The quantitative results from the biofilm Ring Test were analysed using Statview V, one-way ANOVA followed by PLSD Fisher test to determine the significant differences layout. Fisher's exact tests were done to compare ill and healthy patients.

Results

Oral carriage of Candida and associated dominant bacteria isolates

C. albicans were found in 40% of patients both in the ill and control groups. Eight different isolates of *C. albicans* in ill patients and nine in the control groups were isolated from 40 samples during the four weeks of survey. However, carriers did not show any symptoms of *C. albicans* infection whether they belonged to the control group or not. The dominant bacterial strain was quite common for every *C. albicans* carriage in the leukemia group; 5 of them were colonized by *Streptoccocus spp.* and 4 others by *Actinomyces* except for subject L1 who showed a co-existence of two dominant strains at day 7 (Table 2). In the control group, the range of bacterial strains associated to *Candida* was more relevant: six *Streptoccocus*, one *Actinomyces*, one *Fusobacterium* and one *Neisseria* species were identified.

Aggregation of planktonic cells

Although experimental conditions were known to decrease mycelium formation, five *C. albicans* strains were able to auto-aggregate in planktonic conditions after 16 hours (aggregation score ≥ 2). The auto-aggregation was assessed by 84% (15 /18) of the oral bacterial strains while 72% (13/18) of them were qualified to co-aggregate with *C. albicans*. The co-aggregation intensity varied from a species to another (Table 2).

Characterization of the aggregation of planktonic cells by electronic scanning microscopy

Results obtained from scanning electron microscopy showed that all *C. albicans* strains studied here built a layer style organization with extra-cellular material (Figure 1) even if a low aggregation score was assessed by optical microscopy. Efficient filamentation in *C. albicans* requires, *in vitro*, nutriments as fetal calf serum or glucose which were not provided here. Every isolates observed remained in a yeast form except the samples of 3 ill patients; L6 J7, L7 J0 and L7 J21.

Regardless of isolated genus, as shown in Figure 2, *Streptococcus*, *Actinomyces*, *Fusobacterium* or *Neisseria* aggregated on their own in a planktonic medium forming layers of adherent bacteria.

The planktonic aggregate formed when *C. albicans* and bacteria were incubated together showed that the bacteria were inserted between the yeast cells of *C. albicans* and attached to the hyphal form when present (Figure 3). Bacteria were clearly adherent to both morphological forms of the fungus.

Evaluation of the initial phase of biofilm formation using Biofilm Ring Test®

In biofilm formation, the first step is the association of planktonic cells with surfaces constituting the reversible attachment stage. This step contributes to the irreversible attachment stage called "early stage biofilm formation" where adhered cells develop biofilm. At T0, there is no adhesion of microorganisms on the surface (BHI > 7). When present, early stage biofilm formation (or irreversible attachment stage) appears at T 4h (BFI ≈ 2) and remains stable after 22 h. Biofilm Ring Test methodology permitted to detect the early state of biofilm formation of three *C. albicans* strains from L6 J7, L7 J0 and L7 J21 samples, isolated from two ill patients, in less than 4 hours with BFI value ≈ 2. This adherent community partner, crucial element in biofilm formation, remained stable after 22 hours as shown in Figure 4. None of other *C.*

albicans isolates either in control or ill groups (Table 2 and Figure 4), were able to form biofilm (BFI value ≥12) even at the end of incubation time (22 hours).

On studying dominant bacterium and *C. albicans* pairs, we observed that biofilm formation also occurred within 4 hours and lasted at least 22 hours (BFI value ≈2), noticing that dominant bacteria, *S. oralis* (shown in Figure 5), *S. gordonii* and *A. odontolyticus* (data not shown) were showing biofilm formation capabilities themselves (BFI value ≈2).

Visualization of microbial communities in biofilm by confocal laser scanning microscopy (CLSM)

The experimental conditions used were not favorable for the filamentation of *C. albicans*. This fact was confirmed by the evaluation of the development of the yeast form of *C. albicans* ATCC 26555 stained with calcofluor as control. Controls using *Streptococcus gordonii DL1* and *Streptococcus mutans* ATCC 25175 stained with Syto®9 formed a biofilm alone within 24 hours. The three studied couples L6 J7 + *Streptococcus oralis*, L7 J0 + *Streptococcus gordonii* and L7 J21 + *Actinomyces odontolyticus* developed an adherent microbial community (Figure 6).

Amplification and sequencing of Agglutinin-Like Sequences ALS1, ALS3, ALS4 and ALS6 genes from Candida albicans isolates

As shown in Table 3, sequence analysis demonstrated a homozygous thymidin-cytosin mutation on the 263rd position of *ALS4* gene. This mutation concerned the strains of *C. albicans* in sample L6 J7, L7 J0 and L7 J21. *C. albicans* from T4 J7 and L9 J0 samples displayed the same mutation but only in a heterozygous manner. These strains were also homozygous at position 122 whereas others were heterozygous. A part of these five heterozygous strains regarding to position 77, 156 and 209 of *ALS3* gene remained monomorphic for all of the others. No genetic variation was observed in *ALS1* and *ALS6* gene sequences of the 18 isolated strains studied. These results attest a greater variability of the previously cited strains.

Discussion

The structure of oral microbiota can differ considerably from one age group to another; i.e., between toothless infants, preschoolers with milk teeth, children with mixed teeth and

adolescents with permanent teeth [44-46]. This study was designed to include a paired-control group in order to provide reliable information with no age bar.

Candida species can be found as commensal microorganisms in the oral cavity in approximately 10% to 70% of the total population, increasing with age [47,48]. In children however, occurrence of oral *Candida* carriage is not well known. Colonization in the mouth, influenced by lifestyle, [7,49] occurs in the first years of life [50] and may be involved in childhood caries [51]. In our work, 40 % of ill and healthy children showed at least one positive sample. These observed data fit theoretical distribution when carriage of *Candida* is independent from the healthy status (p Fisher's exact test > 0.99). Furthermore *C. albicans* was isolated persistently over the course of the study for 2 /8 patients (one in each group).The species *C. albicans* was identified in all our samples. In the most part, this was a consistent result given the spread of this species. Westbrook et al [7] noted that colonization by a single *Candida* species is common and the rate of colonization by multiple yeasts is known to be low.

Like other studies [1,44,52], in our case the oral samples were dominated by *Streptococcus*, *Actinomyces* and *Fusobacterium* genera. These strains belonging to the oral flora have been identified by other studies as common genera in dental plaque using pyrosequensing [6,52-54]. The Mitis group streptococci, mainly represented by *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus sanguinis*, and *Streptococcus mitis* colonize both teeth and oral mucosal surfaces. In accordance with our results, it is the most numerically dominant [1,18,55]. Two patients (one ill and one healthy) showed a shift in dominant bacterial strain going from *Streptococcus* to *Actinomyces*. For the ill patient, this occurred at day 7 supposedly caused by the administration of cytostatic drugs. For one child the predominant bacteria was *Neisseria cinerea*; a taxa regarded as one of the most variable of its genera. *Neisseria* may hereby fill a functional role generally carried out by facultative aerobic *Streptococcus* bacteria [56].

In planktonic conditions, the aggregation score for *C. albicans*, appeared to be low for most of the studied fungi isolates. However, five of them showed a good cell auto-aggregation (aggregation score ≥ 2). Using Scanning electron microscopy, we observed a layer organization embedded in extra cellular material for all strains even under non-permissive condition for filament formation. These strains self-aggregate to form the basal community layer from which the filaments with a role in the forming of biofilm could be developed (Figure 1). This observation corroborated the study of Pereira et al. [57] who found that all *Candida* strains from the oral cavities of patients with denture stomatitis had a high capacity to produce a biofilm.

This basal substrate-bound layer of yeast cells is usually described as the first step of colonization [58].

As shown in Figure 2, studied bacteria are more likely to aggregate using either pili or long fibrillar-like structures to promote intercellular binding. This phenomenon occurs in spite of growth conditions.

We have showed here that the *C. albicans* and dominant bacteria from same patient cultured together produced a co-aggregate in planktonic conditions. Analysis of these co-aggregates by SEM microscopy revealed the presence of the dense clumps containing both bacteria and blastosporic yeast that resembled to a miniature biofilm on solid surfaces as described by Fox et al. [59]. Similar observations have been founded by Chassot et al. [60] and Fox et al. [59] by revealing this kind of relationship between *Candida* and either *Lactobacillus* or *Clostridium* respectively under planktonic conditions. These observations indicate that bacteria strains and *C. albicans* from the same oral cavity do not require a solid surface to initiate the formation of a microbial community. This property confirms the different behaviors described between clinical isolates and reference strains [5,15]. Three *Candida* isolated from ill patients; L6 J7, L7 J0, and L7 J21 showed spontaneous filament formation. The dominant bacterial strains from these couples adhered to the yeast as well as the hyphal forms (Figure 3 C and D). This observation supports the thesis that these strains are probably more virulent and present a real danger for their immunocompromised carriers [25,26]. They could be 'high invaders' [61] even if during this study, either diagnosed subjects undergoing myelosuppressing chemotherapy or healthy ones were not affected by any oral infection.

The SEM analysis has led us to evaluate the biofilm formation capacity of microbial strains through the study of the initial adhesion phase onto a polystyrene support by Biofilm Ring Test technology [38,62]. Our results showed that 15 pairs /18 were not able to initiate the biofilm formation in 24 hours. It can be explained by the use of the brain heart infusion medium that allows yeast growth under a blastosporic shape.

The three spontaneous filamentous isolates L6J7, L7J0, L7J21 were adhering to the surface of polystyrene support after 4 hours and were maintained until 22 hours, even with a ten-fold diluted inoculum. The filamentation ability of strains in non-permissive conditions might explain such observation, thus *C. albicans* is known to constitute biofilm *via* its filament form [16,63-65].

Nonetheless, dominant bacterial strains; *S. oralis*, *S. gordonii* and *A. odontolyticus*, were able to initiate a mono-biofilm and /or duo-biofilm formation with *C. albicans* after 4 hours. The

latter remains stable for at least 22 hours. The early stage of biofilm formation by these couples; L6 J7, L7J0 and L7 J21 was visualized using CLSM. This microscopy method has been recognized as the most powerful microscopic technique to analyze the biofilm structure [66]. Our results showed that each of the two partners was involved in the biofilm construction (Figure 6). It suggested that no antagonism effect occurred between the two associated partners after 24 hours of co-habitation. This positive interaction has already been described concerning *S. gordonii* and *C. albicans* with reference strains [4,18]. In a similar way, it was demonstrated that *C. albicans* can attach to surface bound from *S. gordonii* by protein interactions (Alsp and SspA and SspB; antigen I /II family polypeptide) or by direct recognition of salivary proteins previously adsorbed by *S. gordonii* [22,67-69]. Unlike us, Diaz et al. [18] and Xu et al., [4] have shown that *S. oralis* 34 lack the ability to form robust mucosal biofilm [4,18]. In the present study *S. oralis* as *C. albicans* in L6 J7 couple strain clearly formed a mono and duo-biofilm (Figure 6). As Cavalcanti et al. [17], we did not observe inhibition of *C. albicans* by *A. odontolyticus* in any case of dual species biofilms; despite the observation of Guo et al. [70].

This interesting relationship suggests intergeneric communication that may involve adhesin-receptor interactions such as bacterial adhesins with hyphal cell wall receptor Als. Als family proteins are known to mediate aggregation between bacteria and yeasts as demonstrated by Klotz et al. [71]. In our study, sequencing of *ALS3* and *ALS*4 genes unveiled a versatility for 5 /17 *C. albicans* isolates from 4 ill patients; L6 J7, L7 J0, L7 J21, L9 J0 and one control T4 J7 whereas all strains appeared to be homogenous for *ALS1* and *ALS6* genes. Meanwhile 3 /5 versatile strains display a spontaneous filamentation without any serum or glucose supplementation. This property could be related to the thymidine /cytosine mutation at the 263rd position observed in the isolates of *C. albicans* from couples L6 J7, L7 J0 and L7 J21 strains during this study as to another mutation outside the sequenced fragments of *ALS* genes (Table 3). Regarding heterozygous mutation (L9 J0 and T4 J7), filaments would be inexistent in non-permissive conditions. This could corroborate the observations of Garcia et al. [72] that the mutation Als5p-V326N decreases significantly the Als5p aggregation capacity. Supposing that the corollary must be true: a different mutation might induce an increase of aggregation capability hence explaining the behavior discrepancies observed on strains L6 J7, L7 J0 and L7 J21.

These results seem to comfort the idea that wild strains can behave differently while belonging to the same flora [5].

In conclusion, our study focused on the cooperation between the dominant bacterial strain and *C. albicans* from the same oral flora isolated either from ill or healthy children. Considering the

two populations, *C. albicans* was always found in small amounts regardless of any infection symptoms. Regarding commensalism, dominant bacterial strains generally belonged to the expected genus. In fact, none of the dominant bacteria tested here antagonized the yeast presence in microbial community. Instead, the results clearly suggested the cooperation between cells. This behavior is seemingly different to the one observed while mixing the type strains. In the wild oral flora, *C. albicans* seems to be helped by endogenous bacteria agreeing with the hypothesis of a more "sinister" role for the cocci as accessories to primary pathogens in mucosal infections [3,55,73]. On the other hand, 3 mutated *C. albicans* strains display, by their spontaneous ability to form filaments, enhanced risks of virulence for leukemia affected carriers. Despite such risks, we have observed neither oral nor systemic pathology in the ill patients studied. It could be suggested that immunosuppression resulting from the first antineoplastic cure may not be severe enough. Indeed, oral colonization of *Candida sp.* is directly related to number of chemotherapy cycles [74].

Declaration of interest statement

The authors declare no conflict of interest.

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References

1 Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH et al. The human oral microbiome. J Bacteriol 2010;192(19):5002-17.

2 Shirtliff ME, Peters BM, Jabra-Rizk MA. Cross-kingdom interactions: *Candida albicans* and bacteria. FEMS Microbiol Lett 2009;299(1):1-8.

3 Xu H, Jenkinson HF, Dongari-Bagtzoglou A. Innocent until proven guilty: mechanisms and roles of *Streptococcus-Candida* interactions in oral health and disease. Mol Oral Microbiol 2014b;29(3):99-116.

4 Xu H, Sobue T, Thompson A, Xie Z, Poon K, Ricker A et al. Streptococcal co-infection augments *Candida* pathogenicity by amplifying the mucosal inflammatory response. Cell Microbiol 2014a;16(2):214-31.

5 Alnuaimi AD, O'Brien-Simpson NM, Reynolds EC, McCullough MJ. Clinical isolates and laboratory reference *Candida* species and strains have varying abilities to form biofilms. FEMS Yeast Res 2013;13(7):689-99.

6 Wang Y, Xue J, Zhou X, You M, Du Q, Yang X et al. Oral microbiota distinguishes acute lymphoblastic leukemia pediatric hosts from healthy populations. PLoS One 2014;9(7):e102116.

7 Westbrook SD, Kirkpatrick WR, Wiederhold NP, Freytes CO, Toro JJ, Patterson TF et al. Microbiology and epidemiology of oral yeast colonization in hemopoietic progenitor cell transplant recipients. Oral Surg Oral Med Oral Pathol Oral Radiol 2013;115(3):354-8.

8 Bergamasco MD, Garnica M, Colombo AL, Nucci M. Epidemiology of candidemia in patients with hematologic malignancies and solid tumours in Brazil. Mycoses 2013;56(3):256-63.

9 Montagna MT, Lovero G, Borghi E, Amato G, Andreoni S, Campion L et al. Candidemia in intensive care unit: a nationwide prospective observational survey (GISIA-3 study) and review of the European literature from 2000 through 2013. Eur Rev Med Pharmacol Sci 2014;18(5):661-74.

10 Sahbudak Bal Z, Yilmaz Karapinar D, Karadas N, Sen S, Onder Sivis Z, Akinci AB et al. Proven and probable invasive fungal infections in children with acute lymphoblastic leukaemia: results from an university hospital, 2005-2013. Mycoses 2015;58(4):225-32.

11 Tadec L, Talarmin JP, Gastinne T, Bretonniere C, Miegeville M, Le Pape P et al. Epidemiology, risk factor, species distribution, antifungal resistance and outcome of Candidemia at a single French hospital: a 7-year study. Mycoses 2016;59(5):296-303.

12 Epstein JB, Tsang AH, Warkentin D, Ship JA. The role of salivary function in modulating chemotherapy-induced oropharyngeal mucositis: a review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94(1):39-44.

13 Bonnaure-Mallet M, Bunetel L, Tricot-Doleux S, Guerin J, Bergeron C, LeGall E. Oral complications during treatment of malignant diseases in childhood: effects of tooth brushing. Eur J Cancer 1998;34(10):1588-91.

14 Sixou JL, De Medeiros-Batista O, Gandemer V, Bonnaure-Mallet M. The effect of chemotherapy on the supragingival plaque of pediatric cancer patients. Oral Oncol 1998;34(6):476-83.

15 Arzmi MH, Dashper S, Catmull D, Cirillo N, Reynolds EC, McCullough M. Coaggregation of *Candida albicans*, *Actinomyces naeslundii* and *Streptococcus mutans* is *Candida albicans* strain dependent. FEMS Yeast Res 2015;15(5):fov038.

16 Bertolini MM, Xu H, Sobue T, Nobile CJ, Del Bel Cury AA, Dongari-Bagtzoglou A. *Candida*-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotypes. Mol Oral Microbiol 2015;30(4):307-22.
17 Cavalcanti IM, Del Bel Cury AA, Jenkinson HF, Nobbs AH. Interactions between *Streptococcus oralis*, *Actinomyces oris*, and *Candida albicans* in the development of multispecies oral microbial biofilms on salivary pellicle. Mol Oral Microbiol 2016;
18 Diaz PI, Xie Z, Sobue T, Thompson A, Biyikoglu B, Ricker A et al. Synergistic interaction between *Candida albicans* and commensal oral streptococci in a novel *in vitro* mucosal model. Infect Immun 2012;80(2):620-32.
19 Hogan DA, Kolter R. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. Science 2002;296(5576):2229-32.
20 Kim Y, Mylonakis E. Killing of *Candida albicans* filaments by *Salmonella enterica serovar Typhimurium* is mediated by sopB effectors, parts of a type III secretion system. Eukaryot Cell 2011;10(6):782-90.
21 Kolenbrander PE, Palmer RJ, Jr., Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. Nat Rev Microbiol 2010;8(7):471-80.
22 Morales DK, Hogan DA. *Candida albicans* interactions with bacteria in the context of human health and disease. PLoS Pathog 2010;6(4):e1000886.
23 Park SJ, Han KH, Park JY, Choi SJ, Lee KH. Influence of bacterial presence on biofilm formation of *Candida albicans*. Yonsei Med J 2014;55(2):449-58.
24 Tampakakis E, Peleg AY, Mylonakis E. Interaction of *Candida albicans* with an intestinal pathogen, *Salmonella enterica serovar Typhimurium*. Eukaryot Cell 2009;8(5):732-7.
25 Gow NA, van de Veerdonk FL, Brown AJ, Netea MG. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol 2011;10(2):112-22.
26 Sudbery PE. Growth of *Candida albicans* hyphae. Nat Rev Microbiol 2011;9(10):737-48.
27 Beucher B, Marot-Leblond A, Billaud-Nail S, Oh SH, Hoyer LL, Robert R. Recognition of *Candida albicans* Als3 by the germ tube-specific monoclonal antibody 3D9.3. FEMS Immunol Med Microbiol 2009;55(3):314-23.
28 Liu Y, Filler SG. *Candida albicans* Als3, a multifunctional adhesin and invasin. Eukaryot Cell 2011;10(2):168-73.
29 Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR et al. Complementary adhesin function in *C. albicans* biofilm formation. Curr Biol 2008;18(14):1017-24.
30 Fox EP, Bui CK, Nett JE, Hartooni N, Mui MC, Andes DR et al. An expanded regulatory network temporally controls *Candida albicans* biofilm formation. Mol Microbiol 2015;96(6):1226-39.
31 Oh SH, Cheng G, Nuessen JA, Jajko R, Yeater KM, Zhao X et al. Functional specificity of *Candida albicans* Als3p proteins and clade specificity of ALS3 alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. Microbiology 2005;151(Pt 3):673-81.
32 Coleman DA, Oh SH, Manfra-Maretta SL, Hoyer LL. A monoclonal antibody specific for *Candida albicans* Als4 demonstrates overlapping localization of Als family proteins on the fungal cell surface and highlights differences between Als localization *in vitro* and *in vivo*. FEMS Immunol Med Microbiol 2012;64(3):321-33.

33 Hoyer LL, Green CB, Oh SH, Zhao X. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family--a sticky pursuit. Med Mycol 2008;46(1):1-15.
34 Hsu LY, Minah GE, Peterson DE, Wingard JR, Merz WG, Altomonte V et al. Coaggregation of oral *Candida* isolates with bacteria from bone marrow transplant recipients. J Clin Microbiol 1990;28(12):2621-6.
35 Brusca MI, Irastorza RM, Cattoni DI, Ozu M, Chara O. Mechanisms of interaction between *Candida albicans* and *Streptococcus mutans*: an experimental and mathematical modelling study. Acta Odontol Scand 2013;71(3-4):416-23.
36 Sztajer H, Szafranski SP, Tomasch J, Reck M, Nimtz M, Rohde M et al. Cross-feeding and interkingdom communication in dual-species biofilms of *Streptococcus mutans* and *Candida albicans*. ISME J 2014;8(11):2256-71.
37 Holmes AR, Gopal PK, Jenkinson HF. Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. Infect Immun 1995;63(5):1827-34.
38 Chavant P, Gaillard-Martinie B, Talon R, Hebraud M, Bernardi T. A new device for rapid evaluation of biofilm formation potential by bacteria. J Microbiol Methods 2007;68(3):605-12.
39 Meuric V, Martin B, Guyodo H, Rouillon A, Tamanai-Shacoori Z, Barloy-Hubler F et al. *Treponema denticola* improves adhesive capacities of *Porphyromonas gingivalis*. Mol Oral Microbiol 2013;28(1):40-53.
40 Jackson S, Coulthwaite L, Loewy Z, Scallan A, Verran J. Biofilm development by blastospores and hyphae of *Candida albicans* on abraded denture acrylic resin surfaces. J Prosthet Dent 2014;112(4):988-93.
41 Estoup A, Largiader CR, Perrot E, Chourrot D. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphix markers and transgenes. Mol Mar Biol Biotechnol 1996;5:295-8.
42 Green CB, Cheng G, Chandra J, Mukherjee P, Ghannoum MA, Hoyer LL. RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology 2004;150(Pt 2):267-75.
43 Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 2003;19(18):2496-7.
44 Crielaard W, Zaura E, Schuller AA, Huse SM, Montijn RC, Keijser BJ. Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. BMC Med Genomics 2011;4:22.
45 Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D et al. Cohabiting family members share microbiota with one another and with their dogs. Elife 2013;2:e00458.
46 Xu X, He J, Xue J, Wang Y, Li K, Zhang K et al. Oral cavity contains distinct niches with dynamic microbial communities. Environ Microbiol 2015;17(3):699-710.
47 Kamat MS, Vanaki SS, Puranik RS, Puranik SR, Kaur R. Oral *Candida* carriage, quantification, and species characterization in oral submucous fibrosis patients and healthy individuals. J Investig Clin Dent 2011;2(4):275-9.
48 Ribeiro DG, Pavarina AC, Dovigo LN, Machado AL, Giampaolo ET, Vergani CE. Prevalence of *Candida* spp. associated with bacteria species on complete dentures. Gerodontology 2012;29(3):203-8.
49 Gaitan-Cepeda LA, Sanchez-Vargas LO, Pavia-Ruz N, Munoz-Hernandez R, Villegas-Ham J, Caballos-Salobrena A. [Oral *Candida* in Mexican children with malnutrition, social marginalization, or HIV/AIDS]. Rev Panam Salud Publica 2012;31(1):48-53.

50 Stecksen-Blicks C, Granstrom E, Silfverdal SA, West CE. Prevalence of oral *Candida* in the first year of life. Mycoses 2015;58(9):550-6.
51 Xiao J, Grier A, Faustoferri RC, Alzoubi S, Gill AL, Feng C et al. Association between Oral Candida and Bacteriome in Children with Severe ECC. J Dent Res 2018:22034518790941.
52 Lee SE, Nam OH, Lee HS, Choi SC. Diversity and homogeneity of oral microbiota in healthy Korean pre-school children using pyrosequencing. Acta Odontol Scand 2016;74(5):335-6.
53 Ye Y, Carlsson G, Agholme MB, Wilson JA, Roos A, Henriques-Normark B et al. Oral bacterial community dynamics in paediatric patients with malignancies in relation to chemotherapy-related oral mucositis: a prospective study. Clin Microbiol Infect 2013;19(12):E559-67.
54 Zijnge V, van Leeuwen MB, Degener JE, Abbas F, Thurnheer T, Gmur R et al. Oral biofilm architecture on natural teeth. PLoS One 2010;5(2):e9321.
55 Diaz PI, Strausbaugh LD, Dongari-Bagtzoglou A. Fungal-bacterial interactions and their relevance to oral health: linking the clinic and the bench. Front Cell Infect Microbiol 2014;4:101.
56 Utter DR, Mark Welch JL, Borisy GG. Individuality, stability, and variability of the plaque microbiome. Front Microbiol 2016;7:564.
57 Pereira CA, Domingues N, Araujo MI, Junqueira JC, Back-Brito GN, Jorge AO. Production of virulence factors in *Candida* strains isolated from patients with denture stomatitis and control individuals. Diagn Microbiol Infect Dis 2016;85(1):66-72.
58 Desai JV, Mitchell AP. *Candida albicans* biofilm development and its genetic control. Microbiol Spectr 2015;3(3)
59 Fox EP, Cowley ES, Nobile CJ, Hartooni N, Newman DK, Johnson AD. Anaerobic bacteria grow within *Candida albicans* biofilms and induce biofilm formation in suspension cultures. Curr Biol 2014;24(20):2411-6.
60 Chassot F, Camacho DP, Patussi EV, Donatti L, Svidzinski TI, Consolaro ME. Can *Lactobacillus acidophilus* influence the adhesion capacity of *Candida albicans* on the combined contraceptive vaginal ring? Contraception 2010;81(4):331-5.
61 Malic S, Hill KE, Ralphs JR, Hayes A, Thomas DW, Potts AJ et al. Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy. Oral Microbiol Immunol 2007;22(3):188-94.
62 Nagant C, Tre-Hardy M, Devleeschouwer M, Dehaye JP. Study of the initial phase of biofilm formation using a biofomic approach. J Microbiol Methods 2010;82(3):243-8.
63 Pathak AK, Sharma S, Shrivastva P. Multi-species biofilm of *Candida albicans* and non-*Candida albicans Candida* species on acrylic substrate. J Appl Oral Sci 2012;20(1):70-5.
64 Pereira-Cenci T, Deng DM, Kraneveld EA, Manders EM, Del Bel Cury AA, Ten Cate JM et al. The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. Arch Oral Biol 2008;53(8):755-64.
65 Silva-Dias A, Miranda IM, Branco J, Monteiro-Soares M, Pina-Vaz C, Rodrigues AG. Adhesion, biofilm formation, cell surface hydrophobicity, and antifungal planktonic susceptibility: relationship among *Candida* spp. Front Microbiol 2015;6:205.
66 Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR et al. Critical review on biofilm methods. Crit Rev Microbiol 2017;43(3):313-51.
67 Bamford CV, d'Mello A, Nobbs AH, Dutton LC, Vickerman MM, Jenkinson HF. *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. Infect Immun 2009;77(9):3696-704.

68 Silverman RJ, Nobbs AH, Vickerman MM, Barbour ME, Jenkinson HF. Interaction of *Candida albicans* cell wall Als3 protein with *Streptococcus gordonii* SspB adhesin promotes development of mixed-species communities. Infect Immun 2010;78(11):4644-52.
69 Wright CJ, Burns LH, Jack AA, Back CR, Dutton LC, Nobbs AH et al. Microbial interactions in building of communities. Mol Oral Microbiol 2013;28(2):83-101.
70 Guo Y, Wei C, Liu C, Li D, Sun J, Huang H et al. Inhibitory effects of oral *Actinomyces* on the proliferation, virulence and biofilm formation of *Candida albicans*. Arch Oral Biol 2015;60(9):1368-74.
71 Klotz SA, Gaur NK, De Armond R, Sheppard D, Khardori N, Edwards JE, Jr. et al. *Candida albicans* Als proteins mediate aggregation with bacteria and yeasts. Med Mycol 2007;45(4):363-70.
72 Garcia MC, Lee JT, Ramsook CB, Alsteens D, Dufrene YF, Lipke PN. A role for amyloid in cell aggregation and biofilm formation. PLoS One 2011;6(3):e17632.
73 Janus MM, Crielaard W, Volgenant CM, van der Veen MH, Brandt BW, Krom BP. *Candida albicans* alters the bacterial microbiome of early *in vitro* oral biofilms. J Oral Microbiol 2017;9(1):1270613.
74 Soni P, Parihar RS, Soni LK. Opportunistic microorganisms in oral cavity according to treatment status in head and neck cancer patients. J Clin Diagn Res 2017;11(9):DC14-DC7.

Figure captions

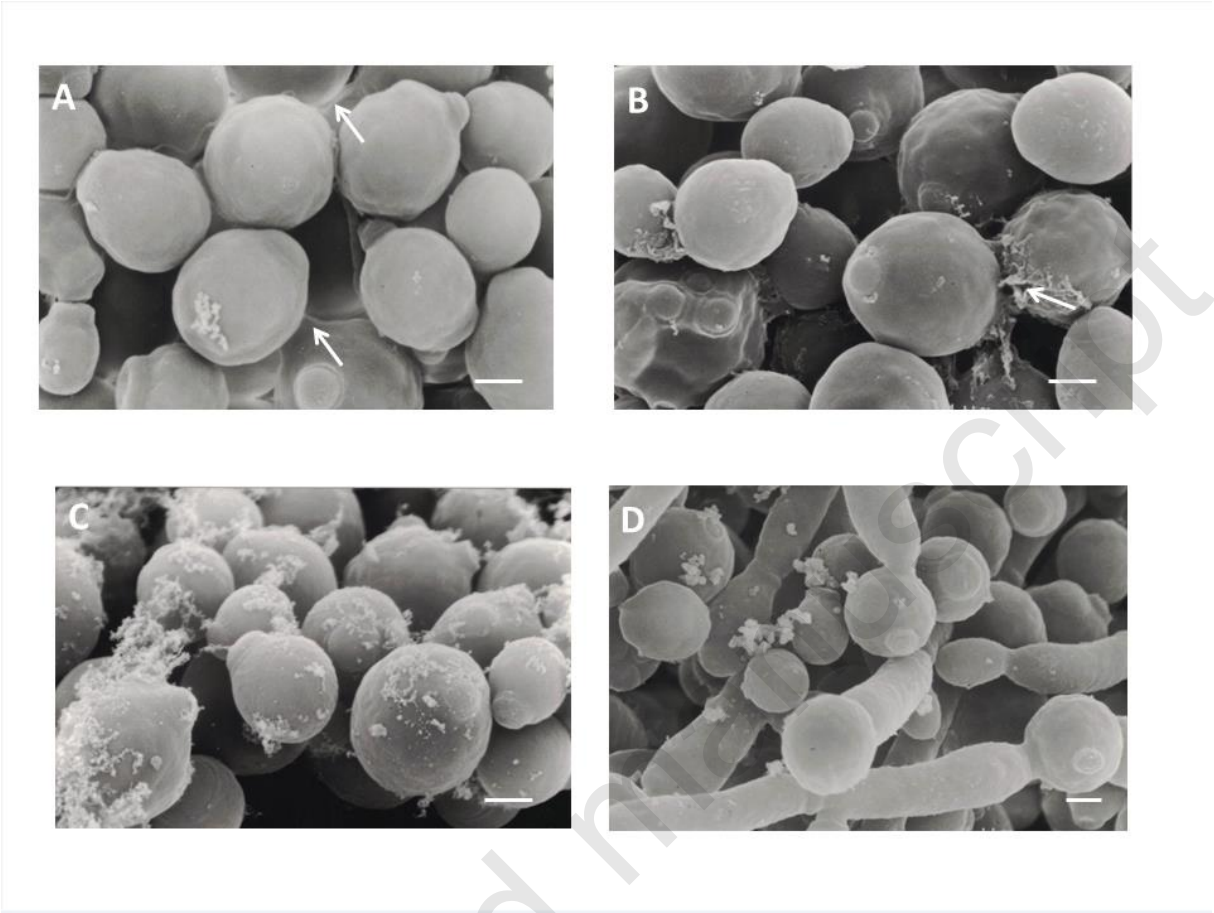

Figure 1. Scanning electron micrographs of the clinical strains of *Candida albicans* showing layer style organization in planktonic conditions with extra-cellular material sediment (white arrows). A: strain from the control group, B and C: strains from the leukemia group, D: strain from L6 leukemia patient at day 7 with spontaneous pseudohyphae formation under unfavorable conditions. Bars in all images, 1µm.

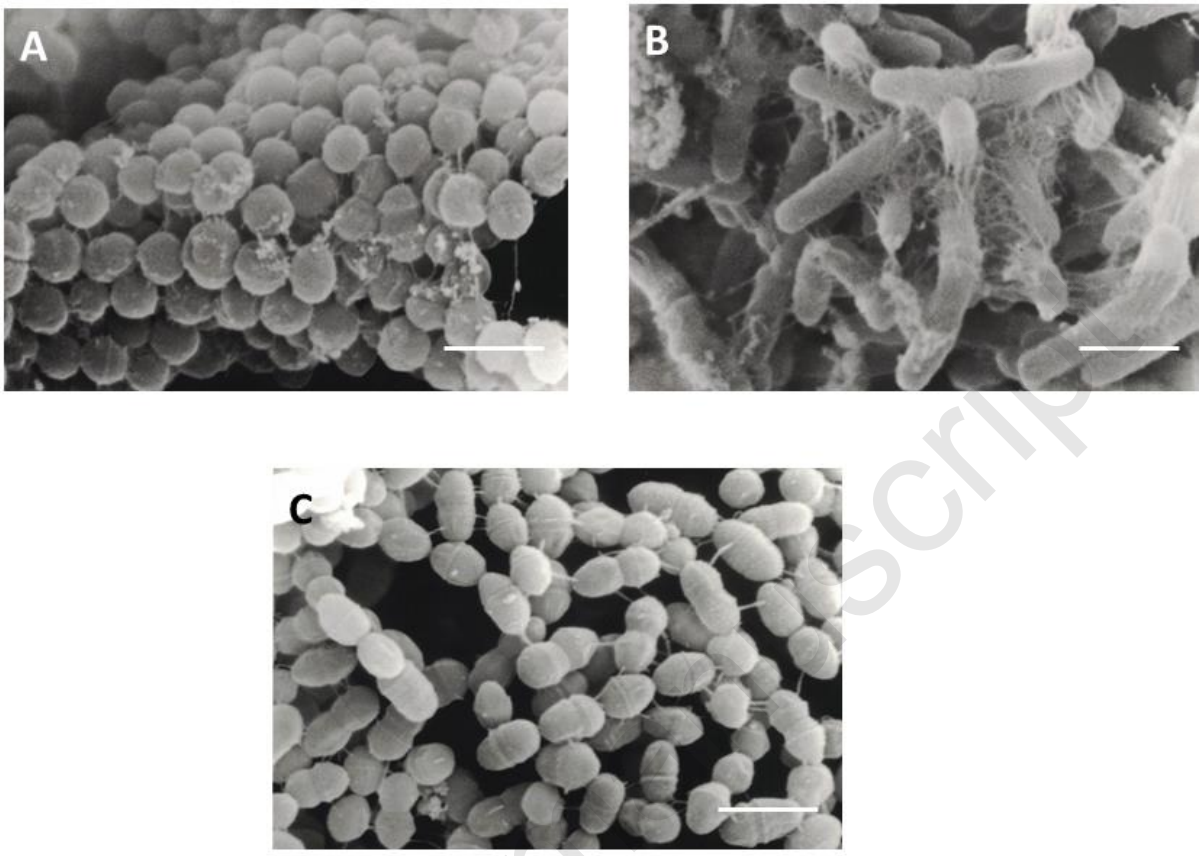

Figure 2. Scanning electron micrographs of bacterial strains. A: *Streptococcus sanguinis*, B: *Actinomyces viscosus*, C: *Streptococcus gordinii* bacteria alone aggregated in a planktonic medium by forming layers. A and B were isolated from leukemia group, C was isolated from control group. Bars in all images, 1µm.

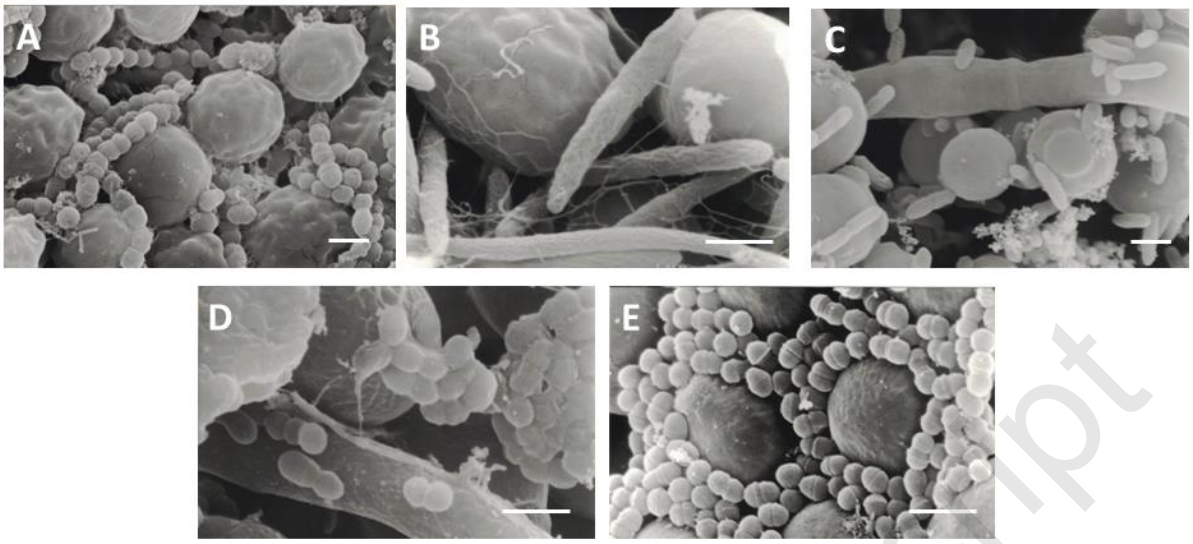

Figure 3. Scanning electron micrographs of dual-species planktonic aggregate of *Candida albicans* and dominant bacteria from the same oral flora. A: with *Streptococcus sanguinis* and B: with *Fusobacterium nucleatum* from two different patients from the control group. Bacteria are inserted between the *C. albicans* cells. C and D: L7 leukemia patient with spontaneous *Candida* pseudohyphae formation at day 0 (C) and day 21 (D). Note that whatever is the dominant strain: *Streptococcus gordonii* (Day 0) or *Actinomyces odontolyticus* (Day 21) bacteria are clearly adherent to both morphological forms of the fungus. E: Control strains: *C. albicans* ATCC 26555 and *Streptococcus mutans* ATCC 25175. Bars in all images, 1µm.

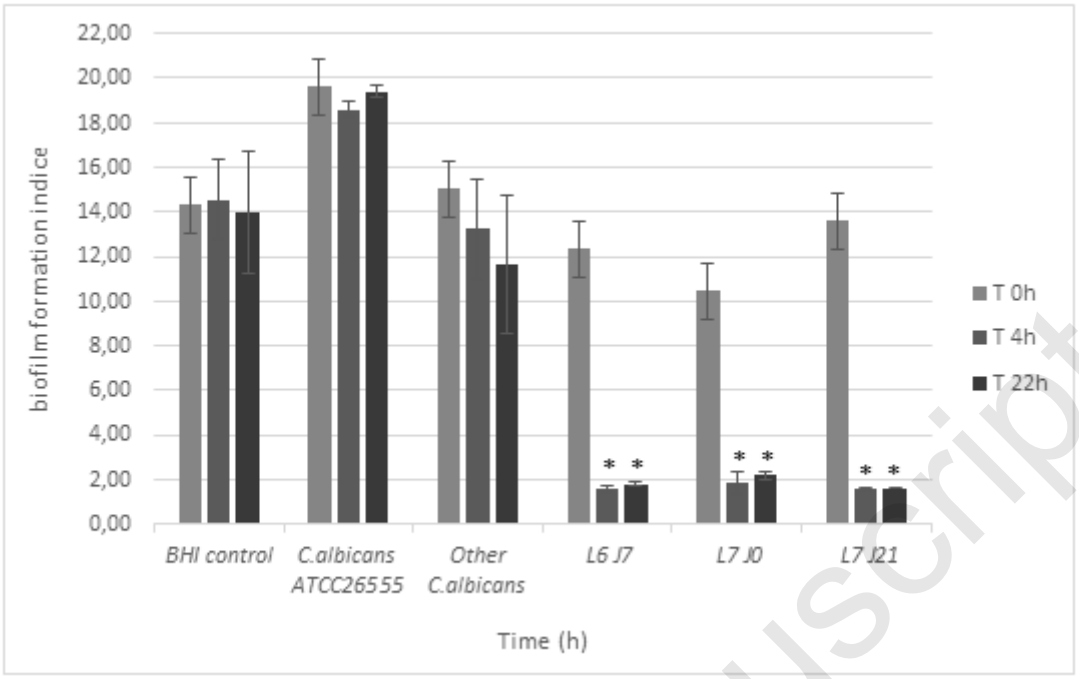

Figure 4. Biofilm formation by *C. albicans* strains

Numerically evaluated biofilm using the "biofilm formation indice" (BFI). Low BFI (≈2) reflects full immobilization of beads and formation of a biofilm observed for patients L6 at day 7 (see below) and L7 day 0 and 21. Other *C. albicans* isolates remain blastosporic and do not form biofilm. Asterisks (*) indicate significant differences between these 3 isolates and other *C. albicans* ($p<0.05$).

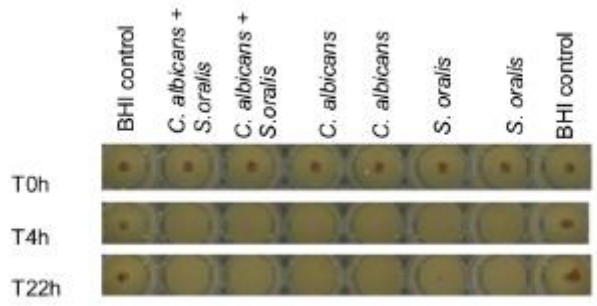

Figure 5. Biofilm formation by *C. albicans* from patient L6 at day 7

A central point indicates the absence of biofilm formation. For this variable strain, *C. albicans* and dominant bacteria *S. oralis*, were showing biofilm formation capabilities by themselves and together.

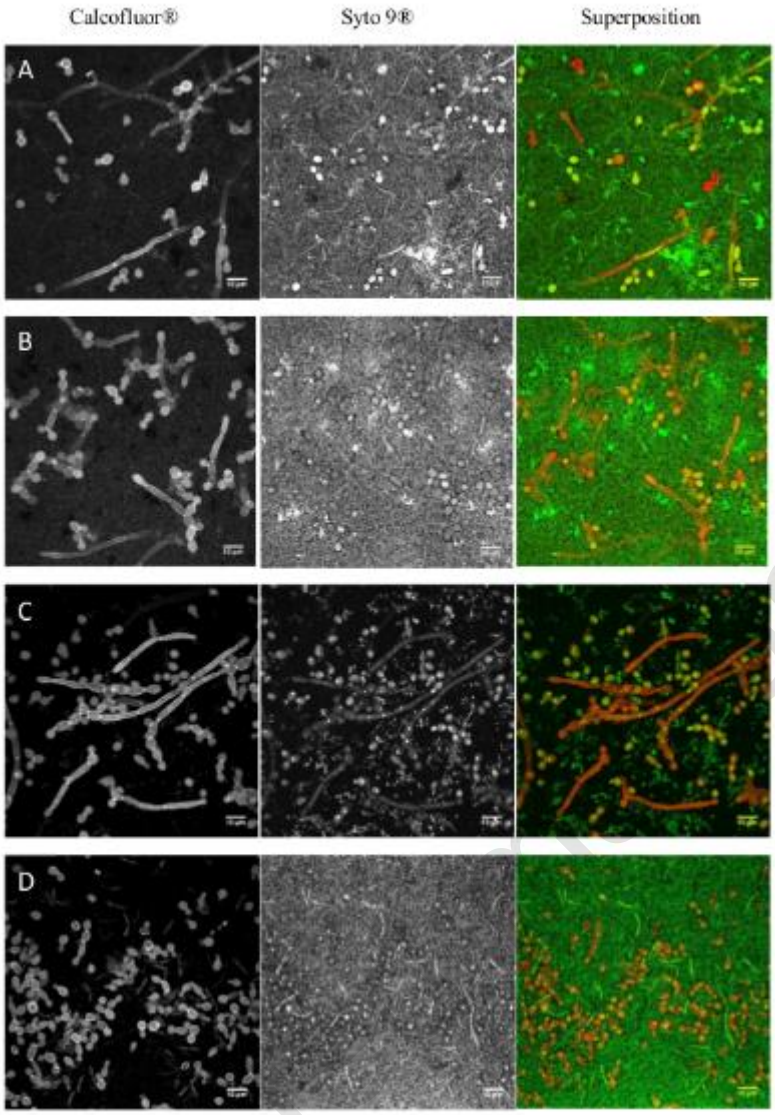

Figure 6. Confocal laser scanning microscopy of dual-species biofilms formed on salivary pellicle after 24 h at 37°C. *C. albicans* was stained with Calcofluor® white (shown in red), bacteria were stained with Syto® 9 (shown in green). A, B and C are respectively strains from patient L6J7 (*C. albicans* and *S. oralis*), patient L7J0 (*C. albicans* and *S. gordonii*) and patient L7J21 (*C. albicans* and *A. odontolyticus*). D: control strains *C. albicans* ATCC 26555 and *S. gordonii* DL1.

Table 1. PCR primers for analysis of *ALS* genes

Gene	Primer name	Sequence (5'→ 3')	PCR product size (bp)
ALS1	ALS1F	GAC TAG TGA ACC AAC AAA TAC CAG A	318
	ALS1R	CCA GAA GAA ACA GCA GGT GA	
ALS3	ALS3F	CCA CTT CAC AAT CCC CAT C	342
	ALS3R	CAG CAG TAG TAG TAA CAG TAG TAG TTT CAT C	
ALS4	ALS4F	CCC AGT CTT TCA CAA GCA GTA AAT	356
	ALS4R	GTA AAT GAG TCA TCA ACA GAA GCC	
ALS6	ALS6F	GAC TCC ACA ATC ATC TAG TAG CTT GGT TT	152
	ALS6R	CAA TTG TCA CAT CAT CTT TTG TTG C	

F, forward; R, reverse

Table 2. Oral *Candida albicans* carriage, associated dominant bacteria, planktonic aggregation and Biofilm ring test from immunocompromised and control group patients.

Immunocompromised patients		**Planktonic aggregation score**			**Biofilm formation (BRT) *Candida albicans* alone**	
***Candida albicans* carriage sample**	**Associated dominant bacteria**	**Bacteria alone**	***Candida albicans* alone**	**Bacteria + *Candida albicans***	**4 hours**	**22 hours**
L1 J0	*Streptococcus sanguinis*	2	1	3	-	-
L1 J7 1	*Streptococcus sanguinis*	2	1	3	-	-
L1 J7 2	*Actinomyces viscosus*	1	1	1	-	-
L1 J14	*Actinomyces viscosus*	2	0	1	-	-
L1 J21	*Actinomyces viscosus*	2	0	2	-	-
L6 J7	*Streptococcus oralis*	3	2	2	+	+
L7 J0	*Streptococcus gordonii*	3	3	2	+	+
L7 J21	*Actinomyces odontolyticus*	1	3	2	+	+
L9 J0	*Streptococcus oralis*	3	1	1	-	-

Control group patients		**Planktonic aggregation score**			**Biofilm formation (BRT) *Candida albicans* alone**	
***Candida albicans* carriage sample**	**Associated dominant bacteria**	**Bacteria alone**	***Candida albicans* alone**	**Bacteria + *Candida albicans***	**4 hours**	**22 hours**
T1 J7	*Streptococcus mitis*	3	0	1	-	-
T3 J21	*Streptococcus oralis*	1	0	2	-	-
T4 J7	*Streptococcus salivarius*	3	2	3	-	-
T4 J14	*Streptococcus oralis*	2	2	2	-	-
T4 J21	*Streptococcus sanguinis*	3	1	3	-	-
T5 J0	*Fusobacterium nucleatum*	3	1	3	-	-
T5 J7	*Neisseria cinerea*	3	1	3	-	-
T5 J14	*Actinomyces naeslundi*	3	1	3	-	-
T5 J21	*Streptococcus oralis*	2	0	1	-	-
Candida albicans ATCC2655	*Streptococcus mutans* ATCC 25175	3	1	3	-	-

L: leukemic ill patients; T: control group

BRT: Biofilm Ring Test

Table 3. DNA sequences from *ALS3* and *ALS4* genes of *Candida albicans* strains isolated from immunocompromised and control group patients

ALS3

Sample	Position			
5’	1	77	156	209 316
T1 J7		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T3 J21		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T4 J7		GGT TKC ATT	CAC ATK AGT	AAA TWC AAC
T4 J14		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T4 J21		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T5 J0		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T5 J7		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T5 J14		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
T5 J21		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
L1 J0		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
L1 J7		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
L1 J14		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
L1 J21		GGT TTC ATT	CAC ATT AGT	AAA TAC AAC
L6 J7		GGT TKC ATT	CAC ATK AGT	AAA TWC AAC
L7 J0		GGT TKC ATT	CAC ATK AGT	AAA TWC AAC
L7 J21		GGT TKC ATT	CAC ATK AGT	AAA TWC AAC
L9 J0		GGT TKC ATT	CAC ATK AGT	AAA TWC AAC

ALS4

Sample	Position			
5’	1	122	263	330
T1 J7		GAT AWG TTA	CAG TTG AAA	
T3 J21		GAT AWG TTA	CAG TTG AAA	
T4 J7		GAT AAG TTA	CAG TMG AAA	
T4 J14		GAT AWG TTA	CAG TTG AAA	
T4 J21		GAT AWG TTA	CAG TTG AAA	
T5 J0		GAT AWG TTA	CAG TTG AAA	
T5 J7		GAT AWG TTA	CAG TTG AAA	
T5 J14		GAT AWG TTA	CAG TTG AAA	
T5 J21		GAT AWG TTA	CAG TTG AAA	
L1 J0		GAT AWG TTA	CAG TTG AAA	
L1 J7		GAT AWG TTA	CAG TTG AAA	
L1 J14		GAT AWG TTA	CAG TTG AAA	
L1J 21		GAT AWG TTA	CAG TTG AAA	
L6 J7		GAT AAG TTA	CAG TCG AAA	
L7 J0		GAT AAG TTA	CAG TCG AAA	
L7 J21		GAT AAG TTA	CAG TCG AAA	
L9 J0		GAT AAG TTA	CAG TMG AAA	

K = G or T, M = A or C, W = A or T