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T H È S E

Validation d’un extrait innovant de Solidago virgaurea : inhibition de la conversion levure-filament et de la formation du biofilm à Candida albicans

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AVANT- PROPOS

Le format de présentation de cette thèse correspond à une recommandation de l’École Doctorale des Sciences de la Vie et de la Santé de l’université d’Aix Marseille. Ce format a paru plus en adéquation avec les exigences de la compétition internationale.

Ce type de thèse (thèse sur article, dite aussi thèse sur travaux) présente les principaux résultats de la recherche sous forme d’articles publiés dans des revues scientifiques, ou sous forme d’articles soumis ou acceptés pour publication, dans le domaine de recherche du doctorant.

La partie introduction et bibliographie est remplacée par une revue publiée dans un journal scientifique afin de permettre une évaluation extérieure de la qualité de la revue.

Les articles qui constituent le corps du document doivent être précédés d’une présentation substantielle et suivis d’une synthèse substantielle qui comprend une discussion générale des résultats, une conclusion et une bibliographie.
RÉSUMÉ

Le genre *Candida* est à l’origine de 70 à 90% des infections fongiques invasives chez l’Homme et cause environ une infection nosocomiale sur quatre. L’incidence des candidoses est en constante augmentation depuis une vingtaine d’années. Cette augmentation peut, en partie, être expliquée par une augmentation de l’utilisation de techniques de soin invasives comme cathéters, de sondes ou de prothèses ; des traitements médicamenteux (antibiotiques, corticoïdes, antidépresseurs...) ; et par l’allongement de la durée de vie. *Candida albicans* est l’espèce de levure la plus souvent isolée et la plus pathogène au niveau de la cavité orale. Cette levure présente des facteurs de virulence multiples, notamment une capacité à modifier sa morphologie (dimorphisme), des adhésines servant à la reconnaissance de l’hôte, la sécrétion de phospholipases et d’aspartyl protéases et une capacité à former des biofilms. Or selon les souches impliquées, les biofilms sont de 20 à 1000 fois moins sensibles aux antimicrobiens que la forme planctonique. L’augmentation constante des infections fongiques et des résistances aux antifongiques, des effets secondaires et du coût des traitements, justifie la recherche de nouvelles molécules actives contre les biofilms impliquant des levures. Pour cela, le développement de modèles de biofilms complexes, composés de plusieurs espèces bactériennes et/ou fongiques, se rapprochant des conditions *in vivo*, est nécessaire.

Dans la première partie de notre thèse, nous avons fait le point des connaissances sur les modèles de biofilms oraux contenant à la fois des champignons et des bactéries. Cette revue de la littérature a montré qu’il s’agit d’un sujet de recherche en plein essor, et que différents modèles étaient proposés en fonction de l’objectif de l’étude, de la composition microbienne du biofilm et des méthodes de culture.

Dans la deuxième partie, nous avons étudié l’effet d’un extrait végétal de *Solidago virgaurea* sur *C. albicans*. Cet extrait est composé de métabolites secondaires comme des flavonoïdes, des acides phénoliques, des terpénoides et des saponines. Une première étude a montré que l’extrait, par ailleurs dépourvu d’effet antibactérien, pouvait agir sur le champignon en : 1) empêchant la conversion de levures en hyphes, 2) inhibant la formation de biofilm, et 3) en dissociant le biofilm déjà formé. La deuxième étude visait à comprendre le mode d’action de cet extrait. Elle a montré que l’extrait de *S. virgaurea* agissait sur plusieurs facteurs de virulence de *C. albicans*. Nous avons tout d’abord confirmé que l’extrait réduisait l’adhérence du
champignon aux surfaces inertes et aux cellules épithéliales. Puis, nous avons montré qu’il inhibait l’expression de protéines impliquées dans l’adhésion et la filamentation.

Enfin, nous avons mené en parallèle une étude qui a montré que, contrairement à l’extrait de *S. virgaurea*, l’utilisation régulière, et pendant plus de deux semaines, de bains de bouche antiseptiques augmentait la sécheresse buccale chez les patients polymédiqués. La sécheresse buccale favorisant les candidoses orales, cette observation justifie le développement d’une formulation basée sur l’extrait de *S. virgaurea* d’un bain de bouche qui serait particulièrement adapté à une utilisation au long cours chez ces patients. De plus, nous avons observé que l’extrait de *S. virgaurea* agit en synergie avec certains antifongiques (ex : miconazole, nystatine) sur l’élimination du biofilm. L’ensemble de ces résultats positionne l’extrait de *S. virgaurea* en candidat prometteur pour une approche innovante du traitement topique des candidoses cutanéomuqueuses.

**Mots clefs:** *Candida*; Biofilm oral; *Solidago virgaurea*; Santé buccale
ABSTRACT

The genus *Candida* causes 70 to 90% of human invasive fungal infections and is involved in one in four health-care associated infections. Invasive candidiasis incidence has been constantly increasing for the last twenty years. This can in part be explained by the growing use of invasive procedures such as catheters, probes or prostheses; treatment regimens (antibiotics, corticoids, antidepressants); and a rising life expectancy. In the oral cavity, *Candida albicans* is the most frequently isolated the most pathogenic species. This yeast has multiple virulence factors, including the capacity to alter its morphology (dimorphism), adhesins for host-recognition, the secretion of phospholipases and aspartyl proteases, and the ability to form biofilms. Yet, according to the strain involved, biofilms are 20 to 1000 times less sensitive to antimicrobial than planktonic forms. The search for new anti-biofilms drugs and, in particular, those targeting biofilm involving yeast is justified by the constantly increasing incidence of fungal infections and antifungal resistance, treatments side effects and costs. In this respect, the development of complex biofilm models, containing several bacterial and/or fungal species, which are closer to *in vivo* conditions, is necessary.

In the first part of our thesis we reviewed the current state of scientific knowledge on oral biofilm models including both fungi and bacteria. This review of the literature showed that this research subject was emerging and that various models were proposed according to study objectives, biofilm microbial composition and culture methods. In the second part, we studied the effect of a *Solidago virgaurea* extract on *C. albicans*. This plant extract contains several secondary metabolites such as flavonoids, phenolic compounds, terpenoids and saponins. A first study showed that this extract, which has no antibacterial effect, could act on the fungus in several ways, including: 1) inhibiting yeast-to-hypha transition, 2) inhibiting biofilm formation, and 3) disrupting existing biofilm. The second study aimed to understand the mode of action of this extract. It showed that *S. virgaurea* extract acted against several virulence factors of *C. albicans*. We first confirmed that the extract reduced fungal adhesion to both inert surfaces and epithelial cells. Then, we demonstrated that it inhibited the expression of proteins involved in yeast adhesion and yeast-to-hypha transition.
Finally, we conducted a parallel study, which showed that, contrary to the extract of *S. virgaurea*, the use of antiseptic mouthwashes for more than two weeks increased oral dryness in polymedicated patients. Oral candidiasis being known to be facilitated by oral dryness, this observation justifies the further development of a mouthwash formulation based on *S. virgaurea* extract, which would be particularly adapted to a long-term use in these patient population. Furthermore, *S. virgaurea* extract acted in synergy with antifungals (*e.g.* miconazole, nystatin) on biofilm elimination. Overall, these findings indicate that this *S. virgaurea* extract is a promising candidate for an innovative approach of superficial candidiasis topical therapy.

**Keywords**: *Candida*; oral biofilm; *Solidago virgaurea*; oral health
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INTRODUCTION
La cavité buccale héberge une flore bactérienne variée et abondante. Chez l’homme, on considère qu’elle contient plus de 700 espèces bactériennes dont plus de la moitié ne sont pas cultivables. On estime qu’environ 98% de ces microorganismes se développent principalement sous forme de biofilm. Un biofilm est défini comme une communauté structurée de microorganismes englobés dans une matrice de polysaccharides et adhérant à une surface vivante ou inerte (Costerton et al., 1987). Les nombreuses études parues sur le sujet depuis cette première définition ont permis d’établir également la présence de champignons principalement de type Candida, de virus et de parasites (Ghannoum et al., 2010; Samaranayake & Matsubara, 2017). Ce mode de développement a des conséquences importantes sur la physiologie et la survie de ces microorganismes. Des études (Costerton et al., 1999; Peyyala & Ebersole, 2013; Tsui et al., 2016) ont démontré qu’en comparaison avec un mode de vie planctonique, les microorganismes sous forme de biofilms présentaient des différences au niveau de leur métabolisme, de leur résistance aux biocides et au niveau de leur virulence. Ce mode de vie favorise également la coopération inter et intra espèces afin d’améliorer leur survie (ex : utilisation des produits de dégradation d’autres espèces, quorum sensing, transfert de gènes de résistance...) (Han et al., 2011; Lazar, 2011; Lebeaux et al., 2014). Particulièrement résistants aux thérapeutiques usuelles, les biofilms sont une source potentielle d’échecs thérapeutiques et de récidives de l’infection (Lebeaux & Ghigo, 2012; Mathé & Van Dijck, 2013).

Dans la cavité orale, bien que les biofilms recouvrent toutes les surfaces, leur composition est différente en fonction des sites (langue, joues, dents...), des apports en nutriments et des contraintes qui y sont exercées (flux salivaire, fluide gingival, pH, température...). De plus, chez un même individu, la composition du biofilm peut varier en fonction de l’âge, de la sécheresse buccale, des habitudes alimentaires, de l’hygiène buccale, de la prise de médicaments ou de bains de bouche, du tabagisme et autres addictions, et enfin de l’état de santé générale. La bouche présente ainsi des profils de biofilms différents dont le plus connu est la plaque dentaire.

La formation d’un biofilm s’effectue en trois grandes étapes : l’adhésion, la phase intermédiaire et la phase de maturation (Figure 1).
**Figure 1: Principales étapes de formation d’un biofilm (d’après Hojo et al., 2009)**

L’étape préalable à l’adhésion est le conditionnement du support par un film de molécules adsorbées à la surface. Au niveau de la cavité orale ces molécules sont principalement constituées de protéines salivaires (Lopez-Nguyen & Badet, 2016).

L’adhésion est une étape dont la durée va dépendre du type de bactéries, de l’importance de sa population dans le milieu et de la durée de la phase de croissance. Elle correspond à l’adhésion des microorganismes à une surface puis au développement de micro colonies. Cette étape fait intervenir le métabolisme bactérien via la présence d’appendices (flagelles, pilis, curlis) ou de protéines de surface (adhésines) (McDougald et al., 2011).

La phase intermédiaire correspond à l’étape de colonisation du support. Les cellules se multipliant, forment des agrégats qui deviennent confluentes et donnent une structure tridimensionnelle variable selon les espèces bactériennes et les conditions environnementales. Chez *Candida*, cette phase est caractérisée par la synthèse d’une matrice extracellulaire qui vient recouvrir les colonies (Chandra et al., 2001). Selon les bactéries, la production de cette matrice est très variable (Flemming, 2016; Flemming et al., 2016; Mitchell et al., 2015).

La dernière étape est la phase de maturation qui correspond à l’épaississement de la matrice extracellulaire puis au détachement de cellules ou d’agrégats qui vont alors coloniser d’autres supports. Ce phénomène dépend de plusieurs facteurs tels que la nature de la surface, la composition de la communauté microbienne et son état physiologique, la disponibilité en
nutriments et les conditions hydrodynamiques et physiques de l’environnement (Tremblay et al., 2014).

D’après Chandra et al. (2001), chez *C. albicans* l’adhésion concernerait les onze premières heures, suivie de la phase intermédiaire (entre 12 et 30 h) puis de la phase de maturation (entre 38 et 72h environ).

De façon générale, à l’équilibre, les espèces présentes dans ces biofilms sont peu pathogènes et ces biofilms endogènes sont indispensables à l’état de santé bucco-dentaire. De plus, par sa composition riche en eau et polymères, le biofilm maintient une hydratation de surface agréable et confortable sur les muqueuses orales. Malheureusement cet équilibre est fragile et la rupture de cet équilibre est caractérisée par l’augmentation non-contrôlée de la biomasse des biofilms qui se fait souvent au bénéfice d’espèces pathogènes. Il a par exemple été montré que chez un hôte immunocompétent, la prise d’antibiotiques (en particulier ceux agissant contre les bactéries anaérobies) entraitait un déséquilibre de la flore digestive au profit des microorganismes insensibles à cet antibiotique. Parmi eux, les *Candida* peuvent proliférer jusqu’à provoquer une candidose intestinale (Chabasse et al., 2006).

Les *Candida* sont des levures fréquemment isolées dans l’environnement (air, sol, plantes et notamment les fruits...). Ces eucaryotes appartiennent au règne des Fungi. Chez l’Homme ils peuvent coloniser et vivre à l’état commensal au niveau des muqueuses des cavités naturelles et sur la peau. Ce sont des microorganismes opportunistes qui peuvent se comporter en pathogènes lors d’un déséquilibre de la flore endogène, d’une altération des barrières locales ou d’un déficit immunitaire. Cependant, même si le genre *Candida* regroupe environ 200 espèces, seulement une dizaine est potentiellement pathogène (Chabasse et al., 2006).

Sur le plan taxonomique, *C. albicans* appartient à la division des Ascomycètes, à la classe des Saccharomycètes, à l’ordre des Saccharomycetales et fait partie de la Famille des Saccharomycètes. C’est un champignon diploïde, dont le génome de 16 Mbases environ, comprend entre 6000 et 7000 gènes répartis sur 8 chromosomes. La majorité des espèces du genre *Candida* se reproduit selon un processus asexué. Bien que cela soit extrêmement rare, des études ont montré que *C. albicans* était également capable de se reproduire de manière sexuée (Ene & Bennett, 2014). Il s’agit d’une levure dite « dimorphique », en raison de sa
capacité à passer d’une forme de levure ovoïde à une forme branchée, dite pseudo-hyphe, ou/et à une forme filamentuse ou hyphe.

Les levures sont immobiles, non pigmentées, non capsulées et d’une taille variant de 3 à 6 μm (Figure 2a). Elles se multiplient par bourgeonnement asymétrique. Les pseudo-hyphes sont formés d’une longue chaîne ramifiée de levures allongées d’un diamètre d’environ 2,8 μm (Figure 2c). Seules quelques espèces de *Candida* (*C. albicans, C. dubliniensis, C. tropicalis*) produisent des hyphes. Leur diamètre mesure environ 2 μm. Ils sont formés d’une cellule mère qui produit par bourgeonnement une cellule allongée nommée tube germinatif (Figure 2b). La croissance du tube germinatif est apicale et un cloisonnement apparaît au fur et à mesure de son développement donnant naissance à un filament composé de cellules allongées mononucléées séparées par des septa. Comme pour les pseudo-hyphes, des blastosporées peuvent également se développer au niveau des septa permettant une ramification du mycélium. *C. albicans* est l’une des seules espèces de *Candida* capable de produire également des chlamydosporées qui sont des structures mesurant de 6 à 15 μm à paroi épaisse et réfringente. Elles se développent lorsque le champignon est cultivé sur des milieux pauvres, en micro-aérobiose et à basse température (Sudbery et al., 2004). *In vivo*, ces différentes formes sont souvent considérées comme des étapes de développement successives. Tandis qu’*in vitro*, ce sont les conditions de culture qui déterminent la morphologie préférentielle des *Candida*. Ainsi, un pH acide et une température proche de 30°C favorisent la forme levure. Alors qu’un pH neutre, une température de 37°C, l’ajout de sérum ou de N-acétylglucosamine favorisent la forme hyphes (Sudbery et al., 2004).

![Images de levures, hyphes et pseudo-hyphes](Figure 2: Dimorphisme chez *C. albicans* (d’après Sudbery, 2011))
Le genre *Candida* est responsable de 70 à 90% des infections fongiques invasives. Environ 80% des adultes possèdent des *Candida* qui restent à un statut de commensaux tant que l’équilibre hôte/Candida n’est pas perturbé. Dans le cas contraire, les levures peuvent se multiplier et devenir des pathogènes opportunistes responsables de candidoses superficielles ou profondes. Il est, de plus, considéré comme responsable d’une infection nosocomiale sur quatre (Tsang *et al.*, 2012). Depuis une vingtaine d’années, on observe une augmentation de la fréquence des candidoses. Cette augmentation peut en partie être expliquée par une augmentation des techniques de soin invasives comme l’utilisation de cathéters, de sondes ou de prothèses ; une augmentation des traitements médicamenteux (antibiotiques, corticoïdes, immunosuppresseurs...); et l’allongement de la durée de vie. Une étude conduite de 2000 à 2013 (Ng *et al.*, 2015) a permis de montrer que, au niveau oral, *Candida albicans* était l’espèce majoritairement isolée (73.17%). Les autres espèces isolées étaient : *C. parapsilosis* (45.45%), *C. tropicalis* (29.55%), *C. glabrata* (17.05%), *C. krusei* (4.55%), *C. dubliniensis* (2.27%) and *C. rugosa* (1.14%)(Ng *et al.*, 2015).

La pathogénicité de *C. albicans* est liée à ses multiples facteurs de virulence (Calderone & Fonzi, 2001; Hirota *et al.*, 2017; Tsui *et al.*, 2016; Williams & Lewis, 2011) dont les principaux sont :

- **le dimorphisme** : la forme hyphe est connue pour être plus virulente. Cette forme lui permet de se frayer un chemin entre les cellules et ainsi envahir les tissus sous-jacents. Puis, une fois la circulation sanguine atteinte, la réversion de la forme filamentueuse vers une forme levure va permettre une meilleure dissémination et l’implantation de nouveaux foyers infectieux.

- la sécrétion de protéases (Secreted Aspartyl Proteases (SAPs)) : cette famille de dix membres (SAP1 à SAP10) permet de dégrader la surface de l’épithélium et favorise la pénétration des filaments dans les tissus.

- la capacité à former des biofilms : les *Candida* organisés en biofilms sont plus résistants aux agressions physiques et chimiques (Douglas, 2003; Kumamoto, 2002; Modrzewska & Kurnatowski, 2015). Par ailleurs, des études ont montré que plusieurs centaines d’ARNm et de protéines sont exprimés de manière différente dans la forme biofilm comparée à la forme planctonique (Martinez-Gomariz *et al.*, 2009; Thomas *et al.*, 2006).

D’un point de vue moléculaire, le développement de biofilms chez *C. albicans* est contrôlé par un réseau de régulation transcriptionnelle complexe (Nobile *et al.*, 2012). Nobile & Johnson (2015) ont répertorié 50 régulateurs transcriptionnels et 101 gènes non régulateurs ayant un rôle fonctionnel dans la formation de biofilms. Parmi les régulateurs transcriptionnels, 6 masters gènes (*EFG1, TEC1, BCR1, NDT80, BRG1* et *ROB1*) sont indispensables au développement de biofilms à la fois *in vitro* et *in vivo*. Ensemble ils régulent l’expression d’environ 1000 gènes cibles avec, parmi eux, d’autres régulateurs transcriptionnels (Nobile *et al.*, 2012). Plusieurs de ces gènes ont été bien décrits cependant la majorité n’a pas encore été étudiée.

De nombreux gènes sont impliqués dans l’étape d’adhésion au support. Notamment le master gène Bcr1 et certains de ses gènes cibles comme *ALS1, ALS3* et *HWP1* sont absolument nécessaires pour la phase d’adhésion (Chandra *et al.*, 2001; Nobile *et al.*, 2006). Les hyphes contribuent à la stabilité de la structure du biofilm mais ils sont également un support à l’adhésion d’autres hyphes ou microorganismes. Certains masters gènes sont requis pour leur formation tels qu’*EFG1, TEC1, NDT80* et *ROB1* (Nobile & Johnson, 2015). Bien que *BCR1* ne soit pas directement nécessaire à la formation des hyphes, il est indispensable pour l’adhésion des hyphes entre eux et aux supports (Nobile & Mitchell, 2005).

Les biofilms matures de *C. albicans* sont englobés dans une matrice extracellulaire qui agit en tant que barrière physique contre les agressions et contribue à l’intégrité de la structure du biofilm. Les régulateurs transcriptionnels *RLM1* et *ZAP1* agissent sur la production de cette matrice (Nobile & Johnson, 2015). Tout au long du cycle de vie du biofilm, des cellules, essentiellement sous forme levure, se détachent et peuvent ainsi coloniser d’autres sites. Bien
qu’elles ressemblent morphologiquement aux levures de la phase planctonique elles présentent néanmoins des propriétés d’adhérence accrues, une capacité à former des biofilms et une virulence plus importante. Deux régulateurs transcriptionnels ont été identifiés dans ce processus (NRG1 et UME6) (Uppuluri et al., 2010).

Environ 80% des adultes sont porteurs de Candida qui conservent un statut de commensaux tant que l’équilibre hôte/Candida n’est pas perturbé. Dans le cas contraire, les levures peuvent se multiplier et devenir des pathogènes opportunistes responsables de candidoses superficielles ou profondes.

Devant l’augmentation constante des infections fongiques et de la résistance aux antifongiques, des effets secondaires et du coût des traitements antifongiques, il semble nécessaire de découvrir de nouvelles molécules actives contre les biofilms et en particulier ceux impliquant des levures. Pour cela, la biodiversité représente une importante source de composés naturels. Les composés actifs peuvent provenir d’organismes variés comme les plantes, les algues, les microorganismes (bactéries, archées et champignons) et les animaux. En particulier, le développement de traitements susceptibles d’être co-administré avec les antifongiques conventionnels (type amphotérique B) représente une option attractive dans la mesure où l’effet synergique attendu permettrait de potentialiser l’activité antifongique et de limiter l’émirgence de résistance (Acar & Davies, 2009).

Plusieurs composés naturels ont montré des propriétés antifongiques intéressantes comme des huiles essentielles (Tea tree, Géranium, Lavande...) (Manoharan et al., 2017; Ramage et al., 2012) ou des composés riches en polyphénols (Seleem et al., 2016; Shahzad et al., 2014). Cependant la plupart d’entre eux possède également des propriétés antibactériennes : leur utilisation participe donc à une aggravation du déséquilibre de la flore endogène. Nos travaux montrent qu’une plante commune dans les Alpes-Maritimes, Solidago virgaurea, est particulièrement prometteuse (Figure 3). Cette plante est couramment utilisée en médecine traditionnelle comme diurétique, anti inflammatoire ou cicatrisant et aucune toxicité n’a été relevée (European Medicines Agency. Assessment report on Solidago virgaurea l., herba.). Les études phytochimiques réalisées sur cette plante ont mis en évidence plusieurs familles de métabolites secondaires comme des flavonoïdes, des acides phénoliques et des
terpénoïdes (ex : saponines). Nous avons réalisé un travail préliminaire au laboratoire MICORALIS, qui a été suivi d'une thèse réalisée en codirection entre le laboratoire MICORALIS et l’Institut de chimie de Nice. Ces travaux ont permis de déterminer quelle partie de la plante possédait l'activité la plus importante contre \textit{C. albicans} et quel était le meilleur solvant d’extraction. Nous avons également travaillé sur les composés isolés et en particulier les saponines (Laurençon, 2013; Laurençon \textit{et al.}, 2013). Ces résultats ont mis en évidence d’une part que l’extrait global était plus efficace que les composés isolés et d’autre part que l’extraction aqueuse était la plus intéressante.

\textbf{Figure 3: \textit{S. virgaurea} L. subsp. \textit{Virgaurea}}

L’objectif de ce travail de thèse était donc de valider l’efficacité de l’extrait de \textit{S. virgaurea} sur les différents facteurs de virulence de \textit{C. albicans}, de comprendre son mode d’action et enfin de valider les étapes préliminaires au développement d’un traitement contre les candidoses à base de cet extrait.

Pour cela, après avoir fait le point sur les modèles \textit{in vitro} de biofilms mixtes champignons-bactéries dans le chapitre I, nous étudierons l’effet de l’extrait de \textit{S. virgaurea} sur \textit{C. albicans}. Dans le chapitre II, nous commencerons par évaluer son activité contre les principaux facteurs de virulence du champignon (dimorphisme et formation de biofilms) et contre les bactéries de la flore orale. Dans le chapitre III nous approfondirons l’étude de l’extrait
et de ses effets sur d’autres facteurs de virulence (adhésion, SAPs), afin d’en comprendre le mode d’action et de vérifier sa compatibilité avec des antifongiques utilisés en usage externe contre les candidoses cutanéo-muqueuses. Dans le chapitre IV, nous étudierons les conséquences d’une utilisation régulière de bains de bouche antiseptiques chez des patients polymédiqués. Ce facteur est connu comme un facteur aggravant de candidoses. Enfin, le chapitre V montre l’intérêt du développement de modèles de biofilms in vitro dans la mise au point de nouveaux traitements dans le domaine de la santé bucco-dentaire.
CHAPITRE I- Revue de la littérature : Les biofilms oraux mixtes bactériens-fongiques *in vitro*

La cavité orale héberge plusieurs milliers de bactéries et des centaines de champignons. Les progrès des techniques de biologie moléculaire (séquençage, puces génomiques,...) permettent d’aujourd’hui de mieux pouvoir préciser la diversité de ces espèces et leur évolution dans leur écosystème (Peterson et al., 2014; Zijenge et al., 2010). Il est bien établi aujourd’hui que les espèces microbiennes vivent en communauté principalement sous forme de biofilms. Malgré cela, l’étude des bactéries orales et de leurs moyens d’élimination a longtemps été menée avec des bactéries planctoniques. La prise en compte du fait que ce mode de vie était en fait très éloigné de la réalité a conduit les auteurs à vouloir modéliser les biofilms *in vitro* et les premiers modèles de biofilms constitués d’une seule espèce ont été développés (Hawser & Douglas, 1994; Nickel et al., 1985). Ces modèles présentent l’avantage d’être simples et de permettre d’étudier l’impact d’une seule variable à la fois sur le biofilm, tel que l’apport du saccharose ou l’efficacité d’un antiseptique (Periasamy et Kolenbrander, 2009). Cependant bien que plus proches de la réalité, les biofilms mono-espèce restent encore très éloignés des microcosmes formés à l’état naturel. Pour pallier à cela plusieurs modèles de biofilms multi-espèces sont proposés aujourd’hui avec des objectifs divers tels que l’étude de l’effet d’un composé ou d’une technique sur un type de flore, l’étude des interactions entre les microorganismes entre eux ou avec les cellules de l’hôte (Cavalcanti et al., 2015).

Cette revue de la littérature porte sur 105 articles publiés depuis 2005 et fait le point sur les modèles de biofilms oraux mixtes, constitués de champignons et de bactéries. Nous proposons une synthèse sur le choix des souches, les conditions de culture et sur les méthodes d’analyse du biofilm. Des tableaux répertoriant des modèles *in vitro* de biofilms représentatifs de candidoses orales, de caries et de parodontites ont pour objectif d’aider les chercheurs à choisir un modèle de biofilm reproductible. Afin d’aider les chercheurs souhaitant développer leur propre modèle de biofilm, nous proposons également un tableau présentant les avantages et les inconvénients des différentes techniques ainsi qu’un *flow chart* permettant de choisir la méthode d’analyse la plus appropriée.
CHAPITRE I

Oral fungal-bacterial biofilm models in vitro: a review

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Abstract:

Hundreds of fungal and thousands of bacterial phylotypes can colonise the oral cavity. Taxonomic profiling combined with functional expression analysis has revealed that <i>Candida albicans</i>, <i>Streptococcus mutans</i> and prominent periodontopathogens are not always present or numerically important in candidiasis, caries or periodontitis lesions. However, <i>C. albicans</i> combined with <i>Streptococcus spp.</i> co-increase their virulence in invasive candidiasis, early childhood caries or peri-implantitis. As <i>Candida</i> species and many other fungi are also members of oral microcosms in healthy individuals, mixed fungal-bacterial biofilm models are increasingly valuable investigative tools, and new fungal-bacterial species combinations need to be investigated. Here we review the key points and current methods for culturing in vitro mixed fungal-bacterial models of oral biofilms. According to ecosystem under study (health, candidiasis, caries, periodontitis), protocol design will select microbial strains, biofilm support (polystyrene plate, cell culture, denture, tooth, implant), pre-treatment support (human or artificial saliva) and culture conditions. Growing mixed fungal-bacterial biofilm models in vitro is a difficult challenge. But reproducible models are needed, because oral hygiene products, food and beverage, medication, licit and illicit drugs can influence oral ecosystems. So, even though most oral fungi and bacteria are not cultivable, in vitro microbiological models should still be instrumental in adapting oral care products, dietary products and care protocols to patients at higher risk of
oral diseases. Microbial biofilm models combined with oral epithelial cell cultures could also aid in understanding the inflammatory reaction.
Oral fungal-bacterial biofilm models in vitro: a review

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Declaration of interest

The authors have no conflicts of interest to report. The authors alone are responsible for the content and the writing of the paper.

Search strategy and selection criteria

References in English were identified through PubMed and Science Direct searches for articles published since Jan 1, 2005. At first, the search terms used were “bacteria OR bacterial OR Streptococcus OR anaerobic bacteria” AND “fungi OR Candida” AND “biofilms OR models OR in vitro techniques”. Then secondly, search terms used were “dental caries OR periodontitis OR gingivitis OR abscess OR peri-implantitis OR denture stomatitis OR candidiasis OR mouth diseases OR oral health” AND “biofilms OR models OR in vitro techniques”. Finally we retained only the studies with an in vitro model of bacterial and fungal cultures, in connection with the oral health or the diseases of the mouth.
ABSTRACT

Hundreds of fungal and thousands of bacterial phylotypes can colonise the oral cavity. Taxonomic profiling combined with functional expression analysis has revealed that *Candida albicans*, *Streptococcus mutans* and prominent periodontopathogens are not always present or numerically important in candidiasis, caries or periodontitis lesions. However, *C. albicans* combined with *Streptococcus* spp. co-increase their virulence in invasive candidiasis, early childhood caries or peri-implantitis. As *Candida* species and many other fungi are also members of oral microcosms in healthy individuals, mixed fungal-bacterial biofilm models are increasingly valuable investigative tools, and new fungal-bacterial species combinations need to be investigated. Here we review the key points and current methods for culturing *in vitro* mixed fungal-bacterial models of oral biofilms. According to ecosystem under study (health, candidiasis, caries, periodontitis), protocol design will select microbial strains, biofilm support (polystyrene plate, cell culture, denture, tooth, implant), pre-treatment support (human or artificial saliva) and culture conditions. Growing mixed fungal-bacterial biofilm models *in vitro* is a difficult challenge. But reproducible models are needed, because oral hygiene products, food and beverage, medication, licit and illicit drugs can influence oral ecosystems. So, even though most oral fungi and bacteria are not cultivable, *in vitro* microbiological models should still be instrumental in adapting oral care products, dietary products and care protocols to patients at higher risk of oral diseases. Microbial biofilm models combined with oral epithelial cell cultures could also aid in understanding the inflammatory reaction.
INTRODUCTION

Metagenomics and proteomics screening have revealed that hundreds of fungal and thousands of bacterial phylotypes can colonise the oral cavity. Microbial communities colonising the mouth grow in a biofilm with a protective extracellular matrix. Specific biofilms colonise soft and hard oral surfaces, referred to as the oral microbiome. In addition to bacteria and fungi, they also contain archaea, parasites, and viruses infecting oral epithelia or vectored via saliva and respiratory secretions [1–6]. In oral health, biofilms modulate the host immune system, which in turn tolerates them [7]. Commensal bacteria and fungi and their polymeric and hydrated matrix constitute a first-line defence against pathogenic microorganisms. Any breakdown favours local infections (gingivitis and periodontitis, dental caries and endodontic infections, oral candidiasis, mucositis, peri-implantitis) as well as aspiration pneumonia and blood-borne infections (infectious endocarditis, deep abscesses) [8–11]. Oral infections are often complicated by oral pain and tooth loss, increasing the risk of anorexia and malnutrition [12]. Evidence points to an association between oral infections, the resulting inflammation, and systemic diseases such as diabetes mellitus, rheumatoid arthritis, neurodegenerative diseases (Alzheimer’s disease), atherosclerosis, cardiovascular disease, and stroke [11; 13; 14]. Recent research has underlined the role of inter-kingdom microbial synergies or antagonisms in biofilms in health and oral diseases [15]. For instance, oral Actinomyces and Lactobacillus spp. can inhibit Candida albicans biofilm formation [15; 16]. Conversely, C. albicans combined with Streptococcus spp. can co-increase their virulence in invasive candidiasis, early childhood caries or peri-implantitis [17–22].
**In vivo,** the dental plaque typically presents a high microbial density, with approximately $10^{11}$ cells/g (wet weight). Molecular taxonomic studies, based on 16 RNA sequencing, have demonstrated that approximately 700 oral bacterial species could be isolated and grown in vitro, and that any given individual generally harbours 100 or more cultivable bacterial strains in its mouth [23]. However, screening of various oral ecosystems has revealed that more than 19,000 non-cultivable bacterial phylotypes could also colonise the human oral cavity [24–27]. Similarly, more than 100 fungal species have been identified in the oral microbiome, most of them non-cultivable [2; 28; 29]. Animal models have been developed using mice, rats and worms to mimic the human oral microcosm, but they have specific limitations [16; 18; 30–32]. In order to get closer to in vivo conditions, some biofilm models are grown in contact with human epithelial cell cultures [17; 33; 34].

It is very likely that many non-cultivable species play a role in health and disease [9; 23; 28; 35]. Among cultivable species, taxonomic profiling combined with functional expression analysis has recently revealed that *C. albicans,* *Streptococcus mutans* and prominent periodontopathogens were not always present or numerically important in candidiasis, caries or periodontitis lesions [2; 4; 9; 18; 29]. Thus, metagenomics and proteomics screening are driving new trends in oral disease prevention, diagnosis and treatment efficacy control based on individual follow-up [7; 26; 36–38]. Future therapies will aim to replace cariogenic and periodontopathogenic microbiomes with the initial healthy microbiome of each subject [5; 7; 26; 36].

However, there is a need for reproducible in vitro models of mixed fungal and bacterial biofilms whenever it is necessary to compare different growth conditions or inhibitory substances at different concentrations [39–45]. Multispecies culture is difficult because, in
contrast to oral Candida, most other oral bacteria are slow-growing, nutritionally fastidious, and oxygen-sensitive [46–48]. In mixed biofilm models, strains can have two origins: some biofilms are grown with a defined consortium of bacteria and fungi, while others, called microcosms, are grown from saliva samples from donors. Co-cultures in defined consortiums are reproducible and easier to study because all the strains are known [49; 50]. In contrast, saliva sampling is closer to real-world conditions in terms of number and respective proportions of microbial species, but suffers a lack of reproducibility due to the different saliva donors used [31; 51–53]. Moreover, only a limited number of bacterial and fungal species can be grown in vitro, and biofilms composition always differs from the initial inoculum [54; 55].

Choice of microbial strains or inoculum is pivotal. Co-culture conditions are critical too, as aerobic fungi are grown with strict anaerobic bacteria, hyphal formation is often required, and overgrowth of any one species is to be avoided. Here we review the key points and current methods for designing and culturing oral multispecies biofilms in vitro.

STAGES OF ORAL BIOFILM DEVELOPMENT

In vivo and in vitro, biofilm development follows five stages: 1) adhesion to hard or soft tissues (adhesins and extracellular polysaccharides); 2) growth (microbial co-adhesion and co-aggregation, matrix formation); 3) maturation characterised by metabolic and genetic microbial exchanges, growth control by quorum-sensing molecules (auto-inducers) and antimicrobial peptides (bacteriocins); 4) tissue invasion/destruction (toxic metabolites and enzymes); and 5) surface detachment (enzymes) [20; 36; 56–58].
Early biofilms

Early microbial colonisers specifically adhere to cellular and to salivary receptors, such as mucins, proline-rich protein, statherin, salivary agglutinin (gp-340) and α-amylase [59; 60]. Mineral and organic salivary compounds are adsorbed to epithelial surfaces or hard surfaces (enamel, dentin, calculus, and restorative or prosthetic materials), and then provide static receptors for early colonizers [61; 62]. The salivary film coating dental enamel is called acquired salivary pellicle [63]. The initial adhesion stage of oral bacteria lasts only a few seconds and is reversible. There are both non-specific surface forces and recognition between microbial adhesins and their receptors. Hydrodynamic forces create either repulsive or attractive non-specific connections via low-energy interactions (electrostatic, steric, hydrophobic, Van de Waals). Next step, the irreversible adhesion process is slower. Its duration depends on the microbial strains, its population density and the duration of its exponential growth phase [64; 65]. The taxonomic bacterial profile of early dental plaque, based on genomic data, has been recently detailed [23]. In vitro, these parameters influence the choice of biofilm support (polystyrene plate, cell culture, acrylic resin for denture, tooth hydroxyapatite crystals, titanium implant...) and pre-treatment support (human or artificial saliva).

Biofilm growth and maturation can take hours or days depending on the microbial species and environmental conditions involved. Bacteria and fungi multiply, colonise the support, and form aggregates (or microcolonies) that become confluent. The production of extracellular polymers varies according to microbial communities, local geochemical environment, and the biofilm maturity [66; 67].

Mature biofilms
Mature biofilms are aggregates of microorganisms growing within an extracellular matrix. *In vivo*, *Candida* and streptococci form corn-cob-like structures [24]. The matrix contains microbial metabolites, dead microbial and host cells (desquamated epithelial cells), other host components (mainly fibronectin, laminin, collagen and salivary constituents), food nutrients (sugars), and possibly also drugs. The matrix is well hydrated and crossed by channels conveying oxygen, nutrients and metabolites [65].

Maturation of mixed biofilms depends on oxygen availability and metabolic interactions. End-chain products may be nutrients of different fungal and bacterial species, which may be either partners or competitors [68]. *Streptococcus oralis* combined with *C. albicans* synergistically increases both biofilm formation and virulence factor expression [18; 20; 57; 69]. In early-childhood caries, the presence of *C. albicans* and sucrose (but not glucose) synergistically increases *S. mutans* virulence, resulting in rapid onset of extensive caries lesions. *C. albicans* can produce and tolerate acids and thus contribute to the development of dental caries [18; 19; 70].

Bacteria are able to perceive various environmental parameters, either abiotic, such as physical-chemical signals (pH, osmolality, temperature), or biotic, such as signalling proteins (known as auto-inducers) [71]. When signalling molecules reach a sufficient concentration, bacteria can communicate and coordinate the formation of biofilm or the synthesis of virulence factors. This phenomenon, called quorum sensing, can regulate inter-species and even inter-kingdom growth [72]. *C. albicans* produces at least two signalling molecules, called farnesol and tyrosol [57]. Farnesol is continuously excreted in the environment and plays a role in regulating the yeast-hyphal transition. *In vivo*, quorum sensing coordinates the evolution from colonisation stage to acute infection stage. *In vitro*, farnesol promotes the
formation of *Staphylococcus aureus* biofilm at low levels (0.5-5 nM) and inhibits *S. aureus* growth at higher concentration (180 M) [58]. In addition, some microbial species can produce antimicrobial peptides, although their direct microbe-killing effect is prevented in physiological conditions where they probably act as immunomodulators [73].

*In vitro*, some studies aim to inhibit biofilm formation or to assay pre-formed biofilms, leading to models of early [20; 32; 65] and mature biofilms [74], with corresponding culture duration, sugar pulses in caries models [75], sequential addition of strains, and progressive anaerobic conditions in peri-implantitis models [50].

**Last stages**

The invasion and destruction of soft tissues is mediated by microbial diffusible enzymes, such as lipases, proteases, nucleases and ureases. The destruction and invasion of hard dental tissues that results in tooth cavities starts with localised acid decalcification of the hydroxyapatite crystals constituting enamel and dentin, followed by an enzymatic lysis of organic structures [52].

*In vivo*, the healthy adult’s biofilm is bathed by a saliva flow of approximately 0.35 mL/min [76], which provides nutrients, moistens the mucus membranes, and eliminates part of the bacteria. The biofilm’s thickness is mechanically controlled by salivary flow, tongue and jaw movements, and by chewing solid food. The turnover of epithelial cells and the immune system (in saliva and epithelia) protect soft mucosal surfaces. In contrast, there is no cell turnover on the hard surfaces of teeth, calculus, dentures and dental biomaterials [17; 25].

The last stage of biofilm development is the detachment of microbial cells or aggregates, which then colonise saliva and other supports. Detachment depends on support, microbial community, nutrient availability, hydrodynamics and physical-chemical conditions of the
environment. Microbial aggregate detachment is facilitated by the lysis of extracellular polymers, such as by the production of dextranases by S. mutans and glucanases by C. albicans [66; 77]. In vitro, sophisticated flux systems enable continuous renewal of the culture medium [17; 31; 52; 78].

CHOICE OF MICROBIAL STRAINS

Consortium models

Current consortium models contain cultivable species representative of ecosystems colonising oral mucosa, teeth, dentures and peri-implantitis pockets. They can combine fungal and bacterial reference strains, wild-type strains or mutant strains. The number of species ranges from two to twelve strains. To our knowledge, neither archaea, viruses nor parasites have been used in multispecies bacterial biofilm models. Examples of consortium models combining fungal and bacterial strains are listed in Tables 1 and 2.

Choice of bacterial strains

The choice of bacterial strains commonly selected for caries and periodontitis models warrants update. Peterson et al. (2014) used microarrays and high-throughput sequencing to investigate biofilm physiology and microbial interactions in dental caries [27] and demonstrated that taxonomic profile was not predictive of dental caries. In particular, S. mutans was not always prominent or present in caries ecosystems. Conversely, functional analysis based on RNA expression was more informative. Different bacterial species were shown to display similarities in gene expression patterns, and functional redundancy was common. Extensive listings of cultivable and non-cultivable oral bacteria have recently been published. New prominent species have been identified in samples of healthy saliva [52; 79; 80], dental plaque [23; 27; 79; 80], healthy gingiva [27], periodontitis [27; 79; 81] and oral
candidiasis [2]. The saliva flora is more similar to the tongue flora than to dental plaque [27].

The choice of strains must also take into account co-occurrence and co-exclusion patterns in oral communities [9]. For a single species, biofilm formation can be also strain-dependant [82].

**Choice of fungal strains**

Knowledge on oral fungi is more limited, but several prominent genera have been identified in oral saliva (Table 3). To date, *C. albicans* is the most common species used in oral consortium studies as it is the most amenable to isolation, identification and culture [5]. Bertolini et al. (2015) also used three strains of mutant *C. albicans* [33; 83]. Pereira-Cenci et al. (2008) described a co-culture model with *C. albicans* and *Candida glabrata* [84], while Chew et al. (2015) recently developed a model of vulvovaginal candidiasis containing *C. glabrata* combined with two probiotic lactobacilli, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* [21]. Furthermore, in dental plaque, *Pichia* species could be antagonist to *Candida, Aspergillus* and *Fusarium* species [2].

**In vitro antagonisms between fungal and bacterial strains**

According to the data currently available, bacteria usually decrease *C. albicans* biofilm formation and viability within biofilms, whereas *C. albicans* increases both bacterial growth and biofilm formation. For instance, *C. albicans* growth is inhibited by *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Prevotella nigrescens*, *Porphyromonas gingivalis* and *Streptococcus salivarius*. Motile gram-negative species display a greater inhibitory effect than gram-positive species [20]. The presence of *Streptococcus intermedius* seemed to have no effect [85]. In contrast, both *S. mutans* [19; 84] and *S. gordonii* [86] enhanced *C. albicans* hyphal development and biofilm formation.
formation, partially via the modulation of its signalling pathways. Conversely, *C. albicans* promoted the growth of *S. aureus* [58] and the growth of *Clostridium perfringens* and *Bacteroides fragilis*, which are two strict anaerobes [1]. Furthermore *C. albicans* synergistically promoted *S. gordonii, S. mutans, S. oralis, S. sanguinis* and biofilm formation [17]. The presence of sucrose greatly increased this synergistic effect of *C. albicans* and oral streptococci on biofilm formation [70].

Similarly, Ramirez Granillo et al. (2015) developed a mixed *Aspergillus fumigatus* and *S. aureus* biofilm. Independently of bacterial concentration, they observed a low abundance of *A. fumigatus* biofilm production and abnormal fungal structures (hyphae and conidia) [87].

**Microcosm models**

In microcosm models, the inoculum is constituted of saliva or dental plaque collected from donors [31; 51; 52]. Rudney et al. (2012) recommended pooling saliva or plaque samples from multiple donors and then freezing aliquots in order to reproduce experiments [55]. The resulting *in vitro* microcosms are much more diverse than consortia, but microcosms are difficult to characterise. *Ex vivo* and *in vitro*, culture-independent methodologies are expensive as they involve metagenomics data combined with sophisticated software analysis. Note too that many bacterial species, mostly fastidious anaerobic bacteria, are lost when the taxonomic profile of the microcosm is compared to the initial sample collected *in vivo* [55].

There are few examples of oral microcosm models (Table 1 and 2). De Moraes et al. (2012) validated a model in 24-well tissue cultures plates [51]. More recently, Koopman et al. (2015) and Sousa et al. (2016) developed microcosm models in a sophisticated flux system apparatus [31; 52]. *In vivo*, Zijinje et al. (2010) observed physical interactions between many
oral bacterial species and oral fungi, and hypothesised that fungi could act as bridging
organisms like *Fusobacterium nucleatum* [24]. Rudney et al. (2015) validated a reproducible
oral microcosm model for experiments using plaque with sucrose pulsing, but taxonomic
profiles were limited to bacteria and did not include fungal investigation [23].

**CULTURE CONDITIONS**

**Biofilm support**

*Solid supports and cell culture supports*

Biofilms need to grow onto a support, which can either be a solid material (abiotic) or a cell
culture (biotic). Oral biofilms are usually developed onto solid supports such as bovine
enamel blocks, human enamel disks, human dentine, hydroxyapatite disks, polystyrene
microtiter plates, methacrylate resin disks, soft denture liners, glass coverslips, and titanium
disks (Table 1 and 2). Diaz et al. (2012) successfully developed fungal-bacterial biofilms onto
reconstructed non-keratinised epithelium, using oral cells (OKF6/Tert-2) and oesophageal
cells (EPC2) seeded on collagen type I-embedded fibroblasts (3T3 cells) (Table 2) [17]. Non-
oral cell cultures have also been used, with either bacterial or fungal inoculum. For instance,
Krishnamurthy & Kyd (2014) demonstrated that contact with lung epithelial cells (A549/CCL-
185) increased the formation of a two-bacterial species biofilm compared to a no-contact
model [88]. Kavanaugh et al. (2014) worked with mucus-secreting cells derived from a
human colorectal adenocarcinoma (HT29-MTX) and showed that mucins downregulated
fungal genes involved in surface attachment (*Alsl, Als3*) and suppressed surface adhesion of
*C. albicans* [7]. Recently, Townsend et al. (2016) developed a polymicrobial inter-kingdom *in
vitro* wound biofilm model on complex hydrogel-based cellulose substrata to test commonly
used topical treatments [89].
Pre-treatment of the support

Solid supports and epithelial cell cultures are frequently pre-incubated with human saliva to reproduce the salivary pellicle and provide receptor binding sites for bacteria [78; 84; 90]. Diaz et al. (2012) used the culture medium of the biofilm (Defined Mucin Medium) supplemented with saliva [17]. The main limitation of using saliva is that its composition varies both intra- and inter-individually. To achieve an acceptably reproducible biofilm model, the saliva collection conditions must be standardised (no antibiotics during the previous weeks, hour of sampling, stimulated- or non-stimulated saliva, pooling various donors’ samples). The saliva is then filtered, aliquoted and frozen, ready to perform all the experiments with the same saliva pool [63]. De Moraes et al. (2012) used artificial saliva, which allows other authors to reproduce their model [51].

Culture medium

The culture medium used to grow the final multispecies mix is a key factor that influences the growth, stability, thickness and composition of the biofilm [91].

Consortium models: pre-culture of separate fungal and bacterial strains

*C. albicans* can be pre-grown aerobically at 25°C, 35°C or 37°C, with or without shaking. In different models, the starter culture broth is either a semi-defined medium with 18 mM glucose [84], Sabouraud Dextrose broth [90], Yeast Nitrogen Base broth (YNB) with 100 mM glucose [32], or Yeast Peptone Dextrose medium (YPD) [92]. The final culture medium can contain the mix of every bacterial and fungal pre-culture broth, saliva or fluid universal medium [93].

In parallel, single bacterial species are grown separately in the most appropriate culture broth. There is no consensual medium. Pre-culture broth can be Brain-Heart Infusion (BHI)
BHI broth supplemented with 5% sucrose [32], Ultrafiltered Tryptone-Yeast Extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract, pH 7.0) with 1% glucose [19], Todd-Hewitt Broth with 0.02% Yeast Extract media (THB + 0.02% YE) [92], Universal Medium broth (thioglycolate medium supplemented with 67 mM/L of Sorensen’s solution (UM) [90], or modified Fluid Universal Medium (mFUM) [94] with 0.3% glucose [75]. Bacterial strains are pre-grown for 4 h to 18 h at 37°C, in aerobic, microaerophilic (5% CO₂) or anaerobic (5% CO₂, 95% N at 200 bar) conditions.

Pre-cultures are grown to the exponential growth phase, and an aliquot is diluted in the final culture mix. The optimal inoculum concentration for bacteria and Candida strains generally ranges from 10⁶ to 10⁷ Colony Forming Units (CFU)/mL. The proportion of each species in the final mix must be carefully determined according to strains, support and culture conditions, to prevent any one species from overgrowing [36].

Microcosm models

In microcosm models, the culture medium must be directly adapted to fungal and bacterial strains. In a model of peri-implantitis, Sousa et al. (2016) developed a medium adapted to epithelial cell, fungal and bacterial growth. This unique medium mimicking the peri-implant sulcular fluid contained a tissue culture medium supplemented with horse serum, menadione and haemin [31].

Culture duration

Protocols of current models are summarised in Table 1 and 2. Every protocol has its own unique design, with rinses and steps to promote Candida adhesion to the support, Candida filamentation, or biofilm formation, for instance [22]. Culture duration ranges from 4 hours [17] to 16 weeks [95]. In studies designed to evaluate the efficacy of methods or compounds
to eliminate bacteria in biofilm, the incubation time generally ranges from two to four days [90; 96].

**Culture apparatus**

*Batch cultures*

Batch culture models are grown in polystyrene plates, such as 96-well microtiter plates and 24-well tissue culture plates [97] or more rarely in bottles (Figure 1A). They are inexpensive and allow to test in parallel a large number of conditions [20; 51]. The biofilm is grown in the bottom of wells or onto solid samples placed into the wells (hydroxyapatite, resin or titanium disks, for instance). Extracted teeth specimens can be processed in Eppendorf tubes [98]. In batch cultures, the culture is stopped at given times and submitted to rinses, new medium and/or sugar pulses or addition of antiseptics. Plates are well suited to the screening of several molecules at various concentrations or contact times. Mixed models contain facultative and/or obligate anaerobic bacteria as well as *Candida* sp. Some are grown in a microaerophilic atmosphere [19; 32; 33; 92] or an anaerobic atmosphere [63; 74]. *In vitro*, agitation is important [99]. Shaker speed usually ranges from 75 to 180 rpm. [20; 32; 58].

*Flux systems*

Flux systems have been designed to continuously renew the culture medium, such as in a flow cell ([17; 78] (Figure 1B), an artificial mouth system [52], and a constant depth film fermentor [31]. The model described by Diaz et al. was grown under aerobic conditions [4]. Sousa et al. (2016) managed to create a peri-implantitis model in rabbits [31]. The inoculum was a saliva microcosm, grown onto titanium discs. It was pumped by a peristaltic pump at the rate of 1 mL/min for 8 h. In order to simulate a subgingival environment, an anaerobic
environment was progressively created as follows: day 0, aerobic atmosphere; days 1 to 9, microaerophilic environment (2% O₂, 3% CO₂, 95% N); days 10 to 30, anaerobic environment (5% CO₂, 95% N at 200 bar). After a 30-day incubation period, the discs were surgically positioned onto rabbit tibial bone.

**Qualitative and quantitative methods to assess *in vitro* biofilm models**

The benefits and limitations of each method are presented in Table 4. A flow chart is also available (Table 5) to choose the suitable method.

**Routine laboratory methods**

Routine laboratory methods are a first step in observing consortium and microcosm models. *C. albicans* yeast-hyphal transition and fungal-bacterial interactions are observable directly in biofilms formed on glass coverslips and in the wells of polystyrene plates, with bright-field and phase-contrast microscopes [22]. Light microscopy with x400 magnification is adapted to hyphae/yeast count [32]. In dental plaque and caries models, pH evolution [19; 32; 52; 70; 78] or metabolite releases [52] are monitored in the culture medium bathing the biofilms. In-biofilm cell viability is commonly determined by scraping the microbial deposits formed onto solid supports followed by serial dilutions on appropriate agar plates for CFU count [31; 32; 51; 63; 65; 70; 74; 84; 90; 98], but Falsetta et al. (2014) highlighted the limitations of CFU count data for *C. albicans*, as most hyphae are multicellular with a large biomass compared to yeasts yet like yeasts they form a single CFU [19]. Alternatively, in-well biomass formation can be quantified with colorimetric methods needing a plate spectrophotometer [92]. The XTT assay is based on the reduction of the tetrazolium salt of XTT in formazan by the succinate dehydrogenase system of the mitochondrial respiratory chain in fungal cells, but not bacterial cells [20; 65]. The crystal violet assay dies in violet the total biomass, including fungal cells, bacterial cells and exopolysaccharides [32; 92], but it quantifies both live and
dead cells in the biofilm [74]. Finally, routine histology are suitably adapted to analysing biofilms grown onto epithelial cell cultures [17; 33; 100].

*Imaging*

The LIVE/DEAD® Biofilm viability kit (BacLight, Invitrogen, Paisley, UK) method utilizes mixtures of green-fluorescent (SYTO9) and red-fluorescent (propidium iodide) nucleic acid stains for bacteria. The difference in stain penetration of bacterial and fungal cells allows making difference between healthy cells (green) and bacteria with damaged membranes (red). BacLight® can be used as a quantitative method or as a qualitative method [95; 101]. Figure 2A is an example of LIVE/DEAD® method, applied to a single species C. *albicans* biofilm.

Scanning electron microscopy (SEM) is suitably adapted to observing fungal-bacterial organisation in a biofilm. It is also useful to observe the surface of the biofilm support before and after microbial colonisation, such as enamel add attack in caries models [20; 32; 63; 65; 70; 74; 78; 84; 92; 98]. Figure 2B is an example of a C. *albicans* biofilm observed with SEM. Confocal laser scanning microscopy (CLSM) is suitably adapted to 2D and 3D analysis of the biofilm, combined with fluorescent staining of specific microbial cells and matrix components [17; 19; 22; 33; 65; 70; 74; 78; 92; 100]. Figure 2C is an example of CSLM method, applied to a single species C. *albicans* biofilm. After image reconstruction on appropriate software, it is possible to measure average thickness of the biofilm and to characterise its microbial species and their respective percentages in the biomass [50]. Some fluorophore combinations are also adapted to identify and quantify live and dead cells in the biofilm [74].

*Genetic assays*
Quantitative reverse transcription PCR (RT-qPCR) is suitably adapted to characterising the taxonomic and functional profile of microcosm models based on selected genes [19; 20; 50; 52; 63; 74; 78], including cell viability [74] and hyphal morphology [17], but RT-qPCR remains too expensive for wider use [55].

Fluorescence in situ hybridization (FISH) is based on oligonucleotide probes labelled with fluorescent dyes. FISH can be used to determine the bacterial and fungal composition of a biofilm [102], to visualize spatial distribution in combination with confocal laser scanning microscopy (CLSM) [103], or the colonization of gingival epithelia by subgingival biofilm [94].

A microarray is a miniaturized solid support displaying a very large set of oligonucleotide probes, allowing the screening of > 30,000 genes during a two-day protocol. For instance, microarrays allow the detection of variations in a gene sequence expression, the comparison of a bacterial genome expression at different times of growth or different culture conditions, or the comparison of two bacterial consortia grown in similar culture conditions [104]. In a fungal biofilm model, Cao et al. [105] used microarrays to show the influence of farnesol on C. albicans biofilm: some hyphal-formation-associated genes (including TUP1) were differentially expressed in farnesol-treated biofilms.

CONCLUSION

There is increasing interest in in vitro models of mixed fungal-bacterial biofilms designed to mimic various oral ecosystems. Protocol designs, culture broths and culture conditions are highly diverse, but the supports used to develop biofilms are relatively consensual, as is the choice of C. albicans and bacterial species in consortium models. S. mutans and oral
streptococci are near-standard bacterial species in caries, periodontitis and candidiasis models, but new bacterial and fungal combinations could be explored. Species selection could take into account genomic and proteomic results obtained \textit{in vivo}, as recent studies have revealed new taxonomy profiles, unexpected quantitative compositions and functional expressions in oral microcosms. This review also revealed a broad difference between culture apparatuses and assessment methods, ranging from microtiter plates to custom-made flux systems, and from CFU counts to CLSM and RT-qPCR. These sophisticated and expensive technologies may ultimately lead to therapies designed to clean up oral microcosms or improve oral health at molecular level. However, microtiter plate models still warrant attention, as they are well adapted to the development of new therapeutic agents. Many populations of patients still need basic oral hygiene education, first-line oral care, healthy diet, and medical help to reduce polymedication and combat addictions to alcohol, tobacco and illicit drugs. \textit{In vivo}, these conditions influence oral ecosystems, particularly in children and teenagers, in chronically ill, poly-mediated or malnourished patients, and in frail elderly people. \textit{In vitro} assays with appropriate models could help improve oral care products, drug formulations, or the composition of foods, beverages, and oral nutritional supplements.
<table>
<thead>
<tr>
<th>References</th>
<th>Authors</th>
<th>Model</th>
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<th>Supports</th>
<th>Culture apparatus</th>
<th>Main objective</th>
<th>Assessment methods</th>
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</thead>
<tbody>
<tr>
<td>[17]</td>
<td>Diaz 2012</td>
<td>Oral candidiasis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Streptococcus gordonii</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Streptococcus sanguinis</em>&lt;br&gt;<em>Candida glabrata</em>&lt;br&gt;<em>Escherichia coli</em>&lt;br&gt;<em>Proteus vulgaris</em>&lt;br&gt;<em>Pseudomonas aeruginosa</em>&lt;br&gt;<em>Staphylococcus aureus</em>&lt;br&gt;<em>Streptococcus pyogenes</em>&lt;br&gt;<em>Streptococcus salivarius</em></td>
<td>Reconstructed oral and pharyngeal epithelium</td>
<td>Flux system</td>
<td>To demonstrate a synergistic interaction between commensal oral streptococci and <em>Candida albicans</em></td>
<td>Histology (FISH)&lt;br&gt;CLSM&lt;br&gt;RT-qPCR</td>
</tr>
<tr>
<td>[20]</td>
<td>Park 2014</td>
<td>Oral candidiasis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Streptococcus salivarius</em>&lt;br&gt;<em>Pseudomonas aeruginosa</em></td>
<td>Polystyrene plate</td>
<td>96-well plate</td>
<td>To examine the influence of bacterial presence on biofilm formation of <em>Candida albicans</em></td>
<td>XTT cell viability assay&lt;br&gt;SEM&lt;br&gt;RT-qPCR</td>
</tr>
<tr>
<td>[22]</td>
<td>Schlecht 2015</td>
<td>Oral candidiasis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Staphylococcus aureus</em>&lt;br&gt;<em>Lactobacillus acidophilus</em>&lt;br&gt;<em>Lactobacillus casei</em>&lt;br&gt;<em>Lactobacillus rhamnosus</em></td>
<td>Plastic Petri dish</td>
<td>6-well plate</td>
<td>To analyse how <em>Staphylococcus aureus</em> infection is mediated by <em>Candida albicans</em> hyphal invasion of mucosal tissue</td>
<td>Phase-contrast microscopy&lt;br&gt;Fluorescence microscopy&lt;br&gt;CLSM</td>
</tr>
<tr>
<td>[65]</td>
<td>Matsubara 2016</td>
<td>Oral candidiasis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Lactobacillus acidophilus</em>&lt;br&gt;<em>Lactobacillus casei</em>&lt;br&gt;<em>Lactobacillus rhamnosus</em></td>
<td>Polystyrene plate</td>
<td>96-well plate</td>
<td>To evaluate the inhibitory effects of the probiotic <em>Lactobacillus</em> species on different stages of <em>Candida albicans</em> biofilm development</td>
<td>CFU count&lt;br&gt;XTT cell viability assay&lt;br&gt;SEM&lt;br&gt;CLSM</td>
</tr>
<tr>
<td>[33]</td>
<td>Bertolini 2015</td>
<td>Xerostomia</td>
<td><em>Candida albicans</em> (and mutant strains)&lt;br&gt;<em>Streptococcus oralis</em></td>
<td>Reconstructed oral epithelium</td>
<td>Tissue-culture plate (not detailed)</td>
<td>To analyse the structural and virulence characteristics of <em>Candida</em>-streptococcal mixed-biofilm models on reconstructed oral epithelium, in different conditions of moisture and nutrient availability</td>
<td>CLSM&lt;br&gt;Histology (FISH)</td>
</tr>
<tr>
<td>[51]</td>
<td>de Moraes 2012</td>
<td>Denture stomatitis</td>
<td><em>Saliva microcosm</em></td>
<td>Acrylic resin, soft denture liner</td>
<td>24-well plate</td>
<td>To assess the antimicrobial activity of a triazine derivative</td>
<td>CFU count</td>
</tr>
<tr>
<td>Reference</td>
<td>Year</td>
<td>Model</td>
<td>Bacteria</td>
<td>Polymers</td>
<td>Plate Type</td>
<td>Methodology/Techniques</td>
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<td>[32] Barbosa 2016</td>
<td>Denture stomatitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Streptococcus mutans</em></td>
<td>Polystyrene plate</td>
<td>24-well plate</td>
<td>To study the effects of <em>Streptococcus mutans</em> on biofilm formation and morphology of <em>Candida albicans</em></td>
<td>pH measure&lt;br&gt;CFU count&lt;br&gt;Bright-field microscopy: crystal violet and hyphae quantification&lt;br&gt;SEM</td>
<td></td>
</tr>
<tr>
<td>[74] Sherry 2016</td>
<td>Denture stomatitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Aggregatibacter actinomycetemcomitans</em>&lt;br&gt;<em>Actinomyces naeslundii</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Porphyromonas gingivalis</em>&lt;br&gt;<em>Prevotella intermedia</em>&lt;br&gt;<em>Streptococcus intermedius</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Streptococcus mitis</em>&lt;br&gt;<em>Veillonella dispar</em></td>
<td>Acrylic resin</td>
<td>24-well plate</td>
<td>To assess the activity of three protocols for cleaning removable dentures</td>
<td>CFU count&lt;br&gt;SEM&lt;br&gt;CLSM&lt;br&gt;Live-Dead PCR&lt;br&gt;RT-qPCR</td>
<td></td>
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<tr>
<td>[100] Cavalcanti 2015</td>
<td>Denture stomatitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Streptococcus mutans</em>&lt;br&gt;<em>Streptococcus sanguinis</em>&lt;br&gt;<em>Actinomyces viscosus</em>&lt;br&gt;<em>Actinomyces odontolyticus</em></td>
<td>Acrylic coupons&lt;br&gt;Reconstructed human oral epithelium</td>
<td>24-well plate</td>
<td>To examine the effect of a bacterial component on the virulence and pathogenicity of Candida biofilms and to assess the cellular responses to different biofilm composition</td>
<td>Histology (FISH)&lt;br&gt;CLSM&lt;br&gt;RT-qPCR</td>
<td></td>
</tr>
<tr>
<td>[78] Yassin 2016</td>
<td>Denture stomatitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Lactobacillus casei</em>&lt;br&gt;<em>Streptococcus mutans</em></td>
<td>Acrylic resin</td>
<td>Flux system</td>
<td>To assess the antifungal properties of a fluoridizing releasing denture resin</td>
<td>pH measure&lt;br&gt;SEM&lt;br&gt;CLSM&lt;br&gt;RT-qPCR</td>
<td></td>
</tr>
</tbody>
</table>

FISH: fluorescence in situ hybridisation; CLSM: confocal laser scanning electron microscopy; (RT)-qPCR: quantitative (reverse transcription) polymerase chain reaction; XTT: (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide); SEM: scanning electron microscopy; CFU: colony forming unit

Table 2. Caries and periodontitis models
<table>
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<tr>
<th>References</th>
<th>Authors</th>
<th>Model</th>
<th>Microbial strains</th>
<th>Supports</th>
<th>Culture apparatus</th>
<th>Main objective</th>
<th>Assessment methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>[75]</td>
<td>Reese 2007</td>
<td>Dental plaque</td>
<td><em>Candida albicans</em>&lt;br&gt; <em>Actinomyces naeslundii</em>&lt;br&gt; <em>Fusobacterium nucleatum</em>&lt;br&gt; <em>Streptococcus oralis</em>&lt;br&gt; <em>Streptococcus sobrinus</em>&lt;br&gt; <em>Veillonella parvula</em></td>
<td>Bovine enamel</td>
<td>24-well plate</td>
<td>To observe the biofilm matrix in the dental plaque, before and after glucose-sucrose feeding</td>
<td>TEM</td>
</tr>
<tr>
<td>[84]</td>
<td>Pereira-Cenci 2008</td>
<td>Dental plaque</td>
<td><em>Candida albicans</em>&lt;br&gt; <em>Candida glabrata</em>&lt;br&gt; <em>Streptococcus mitis</em>&lt;br&gt; <em>Candida albicans</em>&lt;br&gt; <em>Lactobacillus casei</em>&lt;br&gt; <em>Streptococcus mutans</em>&lt;br&gt; <em>Streptococcus oralis</em>&lt;br&gt; <em>Streptococcus salivarius</em></td>
<td>Hydroxyapatite&lt;br&gt; Acrylic resin&lt;br&gt; Soft denture liner</td>
<td>24-well plate</td>
<td>To investigate how oral bacteria modulate the development and characteristics of <em>Candida</em> biofilms</td>
<td>CFU count&lt;br&gt; SEM&lt;br&gt; CLSM</td>
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<tr>
<td>[90]</td>
<td>Sampaio 2009</td>
<td>Dental plaque</td>
<td><em>Candida albicans</em>&lt;br&gt; <em>Streptococcus gordonii</em></td>
<td>Bovine enamel&lt;br&gt; Propylene tubes</td>
<td></td>
<td>To assess the antimicrobial activity of a plant extract</td>
<td>CFU count</td>
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<tr>
<td>[92]</td>
<td>Montelongo-Jauregui 2016</td>
<td>Dental plaque</td>
<td><em>Candida albicans</em>&lt;br&gt; <em>Streptococcus gordonii</em></td>
<td>Polystyrene plate</td>
<td>96-well plate</td>
<td>To assess the activity of antifungal and antibacterial drugs</td>
<td>Bright-field microscopy: crystal violet&lt;br&gt; Fluorescent plate reader: Presto Blue&lt;sup&gt;™&lt;/sup&gt; SEM&lt;br&gt; CLSM&lt;br&gt; pH measure&lt;br&gt; CFU count&lt;br&gt; CLSM&lt;br&gt; RT-qPCR</td>
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<tr>
<td>[19]</td>
<td>Falsetta 2014</td>
<td>Dental caries</td>
<td><em>Candida albicans</em>&lt;br&gt; <em>Streptococcus mutans</em>&lt;br&gt; (and mutant strains)</td>
<td>Hydroxyapatite</td>
<td>24-well plate</td>
<td>To study synergistic virulence of <em>Streptococcus mutans</em> and <em>Candida albicans</em> in dental caries biofilm</td>
<td>CFU count&lt;br&gt; CLSM&lt;br&gt; RT-qPCR</td>
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<td>[52]</td>
<td>Koopman 2015</td>
<td>Dental caries</td>
<td><em>Saliva microcosm</em></td>
<td>Glass coverslips</td>
<td>Flux system</td>
<td>To assay the pH-raising effect of arginine</td>
<td>pH measure&lt;br&gt; RT-qPCR</td>
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<tr>
<th>Reference</th>
<th>Year</th>
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<th>Organism(s)</th>
<th>Material</th>
<th>Method</th>
<th>Objective</th>
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<tr>
<td>[98]</td>
<td>2015</td>
<td>Endodontic infection</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Enterococcus faecalis</em>&lt;br&gt;<em>Staphylococcus aureus</em>&lt;br&gt;<em>Streptococcus mutans</em></td>
<td>Human dentin</td>
<td>Eppendorf vials</td>
<td>To assess the antibacterial activity of two plant extracts</td>
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<td>[50]</td>
<td>2016</td>
<td>Periodontitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Actinomyces oris</em>&lt;br&gt;<em>Campylobacter rectus</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Porphyromonas gingivalis</em>&lt;br&gt;<em>Prevotella intermedia</em>&lt;br&gt;<em>Streptococcus anginosus</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Streptococcus mutans</em>&lt;br&gt;<em>Tannerella forsythia</em>&lt;br&gt;<em>Treponema denticola</em>&lt;br&gt;<em>Veillonella dispar</em>&lt;br&gt;<em>Candida albicans</em>&lt;br&gt;<em>Actinomyces naeslundii</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Streptococcus mutans</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Veillonella dispar</em></td>
<td>Hydroxyapatite</td>
<td>24-well plate</td>
<td>To investigate changes in biofilm composition and structure during the shift from a ‘supragingival’ aerobic profile to a ‘subgingival’ anaerobic profile</td>
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<tr>
<td>[63]</td>
<td>2014</td>
<td>Peri-implantitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Actinomyces naeslundii</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Streptococcus mutans</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Veillonella dispar</em></td>
<td>Titanium</td>
<td>24-well plate</td>
<td>To assess surface properties of titanium disks nitrided by cold plasma</td>
</tr>
<tr>
<td>[31]</td>
<td>2016</td>
<td>Peri-implantitis</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Actinomyces naeslundii</em>&lt;br&gt;<em>Fusobacterium nucleatum</em>&lt;br&gt;<em>Streptococcus mutans</em>&lt;br&gt;<em>Streptococcus oralis</em>&lt;br&gt;<em>Veillonella dispar</em></td>
<td>Titanium</td>
<td>Flux system in vitro and secondly rabbit in vivo</td>
<td>To assess three infection protocols with an experimental model for contamination on titanium surfaces</td>
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<td>[70]</td>
<td>2015</td>
<td>Bone destruction</td>
<td><em>Candida albicans</em>&lt;br&gt;<em>Streptococcus mutans</em></td>
<td>Hydroxyapatite</td>
<td>24-well plate</td>
<td>To assess bone hydroxyapatite destruction</td>
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</tbody>
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TEM: transmission electron microscopy; CFU: colony forming unit; SEM: scanning electron microscopy; CLSM: confocal laser scanning electron microscopy; RT-qPCR: quantitative reverse transcription polymerase chain reaction.
Table 3. Consensus members in the oral mycobiome

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<tr>
<th>References</th>
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<th>Main genera</th>
<th>Prominent Candida species</th>
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<tr>
<td>[28]</td>
<td>Ghannoum 2010</td>
<td>20 oral rinse samples</td>
<td>Frequency in the 20 subjects: Candida (75%), Cladosporium (65%), Aureobasidium (50%), Saccharomyces (50%), Non-cultivable categories (36%), Aspergillus (35%), Fusarium (18%), Cryptococcus (4%),</td>
<td>Frequency of Candida species in the 20 subjects: Candida albicans (40%), Candida parapsilosis (15%), Candida tropicalis (15%), Candida kiferensis (5%), Candida metapsilosis (5%),</td>
</tr>
<tr>
<td>[29]</td>
<td>Dupuy 2014</td>
<td>6 saliva samples</td>
<td>Frequency of species occurring in at least 50% of the six subjects: Malassezia (38%), Epicoccum (33%), Candida/Pichia (10%), Fusarium/Gibberella (4%), Cladosporium/Davidiiella (3%), Alternaria/Lewia (2%), Aspergillus/Emericello/Eurotiun (2%), Cryptococcus/Filobasiella (1%),</td>
<td>Candida albicans, Candida utilis/Pichia jadini</td>
</tr>
<tr>
<td>[2]</td>
<td>Mukherjee 2014</td>
<td>24 oral rinse samples</td>
<td>Frequency in the 12 subjects of each group: 12 HIV-non infected patients: Candida (58%), Pichia (33%), Fusarium (33%), Cladosporium, Penicillium, 12 HIV-infected patients: Candida (92%), Epicoccum (33%), Alternaria (25%), Penicillium, Trichosporon</td>
<td>Species of species identified in the Candida genus: Candida albicans (83%), Candida dublinensis (17%), Candida intermedia, Candida sake</td>
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<td>Table 4. Benefits and limitations of each method presented</td>
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<tr>
<td><strong>Biofilm support</strong></td>
<td><strong>Limitations</strong></td>
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<tr>
<td>Solid support</td>
<td>• Simplicity</td>
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<td></td>
<td>• Reproducibility</td>
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<td></td>
<td>• Moderate cost</td>
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<td></td>
<td>• No specific equipment required</td>
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<td></td>
<td>• Large number of samples at the same time</td>
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<tr>
<td>Cell support culture</td>
<td>• Less close to the reality</td>
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<tr>
<td>How to take into account partially the immune response of the host</td>
<td>• Difficulty of development</td>
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<tr>
<td></td>
<td>• Not adapted for a large number of samples</td>
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<td><strong>Human saliva</strong></td>
<td>• Composition varies both intra- and inter-individually</td>
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<td>• To achieve an acceptable reproducibility of a biofilm model, the saliva collection conditions must be standardized (no antibiotics during the previous weeks, hour of sampling, pooling various donors' samples)</td>
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<td><strong>Pre-treatment of the support</strong></td>
<td>• To reproduce the salivary pellicle and provide receptor binding sites for bacteria</td>
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<td>• Closer to reality</td>
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<td><strong>Artificial saliva</strong></td>
<td>• Composition varies both intra- and inter-individually</td>
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<td>• To achieve an acceptable reproducibility of a biofilm model, the saliva collection conditions must be standardized (no antibiotics during the previous weeks, hour of sampling, pooling various donors' samples)</td>
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<td></td>
<td>• Lack of substances (enzymes, sugars)</td>
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<td><strong>Culture apparatus</strong></td>
<td>• To reproduce the salivary pellicle and provide receptor binding sites for bacteria</td>
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<td>• No renewal or renewal rough of the nutrients and growth</td>
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<td><strong>Batch cultures</strong></td>
<td>• Reproducibility</td>
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<td></td>
<td>• Moderate cost</td>
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<td></td>
<td>• No specific equipment required</td>
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<td>• Large number of samples at the same time</td>
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<td><strong>Flux systems</strong></td>
<td>• Require scientific equipment</td>
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<td>• Reproducibility</td>
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<td>• Control of parameters (pH, flux,</td>
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nutriments...)

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<tr>
<th>Routine laboratory methods</th>
<th>Qualitative and quantitative methods to assess in vitro biofilm models</th>
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| Numeration of viable cells (Colony Forming Unit) | • Simplicity  
• Reproducibility  
• Moderate cost  
• No specific equipment required  
• No consideration of viable but non-cultivable bacteria |
| Crystal violet staining | • Simplicity  
• Moderate cost  
• Poor reproducibility  
• Inability to differentiate live or dead bacteria |
| Colorimetric XTT assay | • Simplicity  
• Reproducibility  
• Moderate cost  
• Metabolic activity can be altered by a restricted access to nutrients and oxygen  
• Limited to fungal cells |
| Fluorescence microscopy (i.e LIVE/DEAD Baclight® method) | • Simplicity  
• Multi-purpose method: quantitative method for counting CFUs and viable bacteria with fluorescence microscope or a qualitative method to visualize biofilm colonization with a CSLM  
• Less toxic than conventional assays  
• Limited applications and essentially for bacteria: (i.e: gram stain kit, LIVE/DEAD viability kit...)  
• Few fluorescent stains can be employed simultaneously |
| Confocal scanning laser microscopy (CSLM) | • Multi-purpose method: to estimate the biomass, to quantify extracellular polysaccharides and microbial cells  
• Do not disrupt the biofilm  
• Only semi-quantitative investigation  
• Few fluorescent stains can be employed simultaneously allowing the visualization of just a subset of components in the same image |
| Scanning electron microscopy (SEM) | • High-resolution,  
• Three-dimensional images provide topographical, morphological and compositional information  
• Samples preparation does not preserve the vitality of the biofilm  
• Requires expensive scientific equipment |
| Genetic assays | RT-qPCR | • Powerful and sensitive gene analysis techniques | • Strict rules of sample preparation (No contaminants or PCR inhibitors; choice of the primers sequence…)
• High costs
• Difficulty of execution requiring expensive scientific equipment and skilled technical staff |
|----------------|---------|-----------------------------------------------|--------------------------------------------------|
| Fluorescence in situ hybridization (FISH) | • Capacity to quantify non-culturable organisms | • Cost
• Inability to differentiate viable and nonviable cells
• Impossibility to work on live material
• Only qualitative method |
| Microarrays | • Allow the investigation of the biofilm physiology and micro-organism interactions
• Powerful and sensitive gene analysis techniques | • Strict rules of sample preparation
• High costs
• Difficulty of execution and expensive scientific equipment often lead to the use of platform of sequencing |
Table 5. Flow chart to choose the suitable method

What is the objective of the study?

To evaluate drug/product efficacy

To study the interactions (with other microorganisms and/or cells)

Which kind of data?

Quantitative data

Descriptive data

Not necessary to visualize biofilm

To visualize biofilm

Routines methods

Molecular methods

To visualize constituents (microorganisms, proteins, extracellular polysaccharides, …)

To visualize the morphology/the surface

CFU

Colorimetric assay

ATP bioluminescence

RT-qPCR

Microarrays

CSLM

Bacterial* method

FISH

SEM

AFM

Routines methods

Molecular methods

To visualize biofilm

CFU

RT-qPCR

Microarrays

CSLM

Bacterial* method
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88. Krishnamurthy A, Kyd J. The roles of epithelial cell contact, respiratory bacterial interactions and phosphorylcholine in promoting biofilm formation by Streptococcus pneumoniae and nontypeable Haemophilus influenzae. Microbes Infect. 2014;


FIGURES LEGENDS

Table 1. Oral candidiasis models

Table 2. Caries and periodontitis models

Table 3. Consensus members in the oral mycobioime

Table 4. Benefits and limitations of each method presented

Table 5. Flow chart to choose the suitable method

Figure 1. (A) Schematic representation of batch culture system: microtiter plate or bottle.
(B) Schematic representation of flow cell system.

Figure 2. Comparison of different methods of imaging applied to a single species C. albicans biofilm. (A) Biofilm was stained using SYTO-9 (BacLight®) to stain live biofilm cells green and examined by fluorescence microscopy (x63); (B) Biofilm observed with SEM (Bar, 10μm). (C) Biofilm 3D visualization after z-stack acquisition with CSLM (Bar, 50μm) (Courtesy Pr. G. Ramage)
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Figure 1
338x190mm (96 x 96 DPI)
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CHAPITRE II- Découverte de l’effet antifongique de Solidago virgaurea : inhibition de la transition levure-hyphe et de la formation de biofilm par C. albicans

Un criblage de différentes plantes a été réalisé au laboratoire avec pour objectif d’identifier une plante commune contenant des molécules présentant des propriétés antifongiques. Ce criblage a permis de sélectionner une plante issue de la famille des Asteracées : Solidago virgaurea.

Nous avons établi qu’un extrait aqueux de cette plante commune possède à la fois une activité contre C. albicans et ne présente pas d’activité sur les bactéries orales. S. virgaurea est connue pour contenir des saponines qui sont des molécules détergentes assez fréquentes chez les végétaux. Bien que leur rôle soit encore mal compris, les saponines servent probablement de substances défensives, et en particulier contre les agressions bactériennes et fongiques. Ce sont des agents tensioactifs traditionnellement utilisés comme détergent (ex : Quillaja saponaria, L.). Les saponines sont capables d’interagir avec l’ergostérol des champignons pour former des pores dans les membranes cellulaires. Une étude parue en 1987 (Bader et al., 1987) avait déjà décrit les propriétés antifongiques des saponines triterpénoides de Solidago virgaurea.

Une thèse réalisée en codirection entre le laboratoire MICORALIS (ex-LSBV) et l’Institut de Chimie de Nice et dont le sujet portait sur l’étude phytochimique de S. virgaurea a permis d’identifier une famille de saponines particulièrement active contre la forme filamentouse de C. albicans. Parmi les onze saponines majoritaires caractérisées, grâce à un fractionnement bioguidé de l’extrait aqueux, cinq n’avaient jamais été décrites. Les tests ont cependant montré qu’elles n’étaient pas toutes actives contre le champignon (Laurençon, 2013).

Une étude préliminaire que nous avons réalisée au laboratoire MICORALIS, validée par le travail de thèse sur les analyses phytochimiques, a permis de déterminer que l’extrait aqueux réalisé avec la partie aérienne de la plante (tige et feuilles) donnait les meilleurs résultats. C’est donc cet extrait qui a été choisi pour la suite de nos travaux.
Le travail présenté dans l'article qui suit avait pour principal objectif d’étudier l’effet des extraits de deux sous-espèces de la plante : *S. virgaurea* L. subsp. *Virgaurea* et *S. virgaurea* L. subsp. *Alpestris* sur *C. albicans* et en particulier sur sa croissance, sa capacité à former des hyphes, sa capacité à former un biofilm et également étudier l’effet de ces extraits sur un biofilm déjà formé par le champignon. Le travail porte sur quatre souches de *C. albicans* : une souche de référence (ATCC 10231) et 3 souches sauvages prélevées chez des patients souffrant de candidose orale. L’extrait a également été évalué sur différentes souches bactériennes. En effet l’objectif à plus long terme étant de proposer un traitement efficace contre les candidoses orales, il était important choisir un composé qui ne soit pas nocif pour les bactéries commensales de la flore orale.

Les résultats ont montré que les extraits de *S. virgaurea* n’ont pas inhibé la croissance des quatre souches de *C. albicans* ainsi que des bactéries suivantes : *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius* et *Enterococcus faecalis*. Par contre, nous avons observé une diminution de la transition de la forme levure à la forme hyphé du champignon après 4 et 24h d’incubation en présence des extraits. Incubés directement avec *C. albicans*, les deux extraits végétaux ont limité la formation du biofilm. Sur un biofilm déjà formé, l’ajout d’extraits a permis de diminuer la quantité de biofilm.

Finalement cette étude démontre que les extraits des deux sous-espèces de la plante *S. virgaurea* agissent à la fois sur le changement de morphologie et sur la capacité à former des biofilms par *C. albicans*.
CHAPITRE II

Inhibition of *Candida albicans* yeast–hyphal transition and biofilm formation by *Solidago virgaurea* water extracts

Marlene Chevalier, Etienne Medioni and Isabelle Prêcheur

Published in

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(I.F. 2.458)

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Inhibition of *Candida albicans* yeast–hyphal transition and biofilm formation by *Solidago virgaurea* water extracts

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1Laboratoire Santé Buccale et Vieillissement LSBV URE 01, Faculty of Dentistry, University of Nice Sophia Antipolis, Nice, France

2Dentistry Department, Nice University Hospital, Nice, France

Xerostomia is a decrease of saliva secretion, which can unbalance the oral microflora, mainly to the benefit of *Candida albicans*. The aim of the present study was to find a plant extract that could create an unfavourable environment for *Candida*, and would, therefore, be appropriate for use in a dry-mouth daily-care mouthwash. Water extract from the herbaceous plant *Solidago virgaurea* (Goldenrod) was selected due to its saponin content (plant detergents). Saponin concentrations reached 0.7 and 0.95 mg ml–1 in *S. virgaurea* subsp. *virgaurea* and *S. virgaurea* subsp. *alpestris* extracts, respectively. *C. albicans* was grown in liquid medium and cells were counted by microscopic examination after 0, 4 and 24 h of incubation. *Solidago* extracts did not inhibit the growth of *C. albicans* (four strains), *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius* or *Enterococcus faecalis*. When inocula were incubated with *Solidago* extract for 4 and 24 h, we observed a decrease in *Candida* yeast–hyphal transition. *Candida* biofilms were then prepared in microtitre plates and treated with plant extracts at 0 h, to estimate biofilm formation, or at 18 h to estimate the effect of the saponin on pre-formed biofilms. Biofilm formation and pre-formed biofilms were both strongly inhibited. In conclusion, the *S. virgaurea* extract was efficient against two key virulence factors of *C. albicans*: the yeast–hyphal transition phase and biofilm formation.

INTRODUCTION

Xerostomia is a medical term for the complaint of dry mouth due to a decrease in saliva secretion. Xerostomia can unbalance the oral microflora, mainly to the benefit of *Candida albicans*. *C. albicans* is a human commensal fungus, frequently associated with the normal oral microbiota, occurring in the mouths of up to 80% of healthy individuals. Changes in the oral environment, including xerostomia, can increase *Candida* virulence (Williams & Lewis, 2011). *C. albicans* is a polymorphic fungus which has the ability to rapidly switch between yeast-like round cells and filamentous hyphal cells. Hyphal forms display increased resistance against phagocytosis, enhanced adherence to host surfaces and the ability to invade epithelial cell layers, resulting in tissue damage (Williams & Lewis, 2011; Masuoka, 2004). Besides this, *C. albicans* SAP genes encode secreted aspartyl proteinases, which hydrolyse the proteinaceous part of mucins and proline-rich proteins in the saliva and the oral mucosa. The visco-elastic properties of the oral mucosa coating rely on these salivary mucins. As a result, *Candida* infection can worsen xerostomia (Masuoka, 2004; Villar et al., 2007).

In patients suffering from xerostomia, a biofilm-containing bacteria and *Candida* can be formed both on the oral mucosa and on the surface of dentures, commonly leading to acute or chronic candidiasis, including denture stomatitis. *C. albicans* biofilm formation is initiated when planktonic yeasts adhere to a surface and begin to aggregate to form microcolonies. This first stage, which is vital for biofilm formation, is immediately followed by a proliferation of yeast cells across the substrate surface and the beginning of hyphal development (Douglas, 2003). By using scanning electron microscopy (SEM), García-Sánchez *et al.* (2004) showed that wild-type biofilms are composed of microcolonies connected by ‘bridges’ of hyphae and contain blastospores, hyphae and pseudohyphae in variable proportions. Various morphologies and architectures of biofilms observed *in vitro* and *in vivo* suggest that morphological differentiation to produce hyphae plays an important role in biofilm maturation (Blankenship & Mitchell, 2006; Coleman *et al.*, 2010).

Human host cells and fungal cells are in competition for iron, which is a catalytic cofactor in oxidation–reduction

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**Abbreviations:** SEM, scanning electron microscopy; SVV, *S. virgaurea* subsp. *virgaurea*; SVA, *S. virgaurea* subsp. *alpestris*; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide.

A supplementary figure is available with the online version of this paper.
reactions. The host produces lactoferrin in several secre-
tions including saliva. This glycoprotein displays both iron
sequestering properties and direct action against Candida
cell membranes. In response, Candida produces iron
chelators (siderophores) and iron-transport proteins to
obtain sufficient iron from the host to allow fungal growth
(Nylasi et al., 2005; Chen et al., 2010). Hyposalivation
provokes a decrease in lactoferrin and other immune saliva
components, causing an increased risk of oral candidiasis.

The treatment of xerostomia often includes treatment for
xerostomia-induced candidiasis. Oral azoles and local
polyenes are recommended to treat oral candidiasis, but
only on a limited-time basis because of the risk of the
Candida developing a resistance. Mouthwashes containing
quaternary ammonium (chlorhexidine, hexetidine), vegetal
apolar compounds (essential oils), sodium bicarbonate,
alcohol and other antiseptic compounds can fight Candida
proliferation, but they alter the bacterial part of the biofilm
too and, therefore, carry a risk of worsening xerostomia
symptoms. Some ranges of products, such as mouthwash,
gel, toothpaste and lozenges, aim to fight the symptoms of
dry mouth and mucosal inflammation by using ingredients
such as lactoferrin and/or ovalbumin, lactoperoxidase,
lysozyme, glucose oxidase and vitamins (Bioxtra, Biotène,
Novasial, Oralene), but as yet there is no consensus regarding
the effectiveness of these local treatments (Chen et al.,
2010). All in all, local treatment using metal chelators, such
as saponins, could help to alleviate the symptoms of oral
candidiasis. In addition, Coleman et al. (2010) demon-
strated that C. albicans hyphae and biofilm formation were
disrupted by saponins.

The general goal of the current project was to develop a
daily care mouthwash specifically for dry mouth. The main
cpecifications were to preserve the components of the
healthy biofilm, including salivary mucins and endogenous
bacteria with external polysaccharide coating, such as oral
streptococci, which normally retain water and protect the
oral mucosa, and to create an oral environment unfavour-
able to Candida proliferation, causing inhibition of the
yeast–hyphal transition, iron chelation and reduction of
lipoprotein exudates from serum. We hope to develop a
daily care product that is not toxic to humans or their
environment; therefore, we investigated a plant extract,
which ensures its biodegradability.

Based on the pioneering work of Bader et al. (1995), we
selected Solidago virgaurea (Lin.) saponins as a potential
active compound. In this present paper we compared the
activity of two S. virgaurea subspecies: S. virgaurea L. subsp.
virgaurea (SVV), a common plant in Europe, which grows at
low altitude, and S. virgaurea subsp. alpestris (Waldst. & Kit.)
Gremli (SVA), an alpine plant, which grows at altitudes of
>1 500 m above sea level.

Solidago saponins are a group of heterosides with a
triterpene genin, present in Solidago species (Goldenrod).
Saponins are iron, zinc and calcium chelators. They also
have detergent, haemolytic and antimicrobial properties
due to their capacity to bind membrane sterols. Saponins
are toxic to humans if administered intravenously (causing
haemolysis), but are not toxic when used orally because of
their low digestive absorption rate (Miligate & Roberts,
1995; Matsuda, 1999). S. virgaurea polar extracts (aqueous,
ethanolic or methanolic) contain noticeable levels of sapo-
nins and flavonoids (Tyszkieiwicz, 2008) but only ethanolic
or methanolic Solidago extracts containing saponins that
are purified and submitted to alkaline lysis have proven
antiungal properties (Bader et al., 1995).

The aim of the present work was to screen the antibacterial
and antifungal properties of this Solidago extract against four
C. albicans strains and six bacterial strains (Escherichia coli,
Pseudomonas aeruginosa, Staphylococcus aureus, Strepto-
coccus mutans, Streptococcus salivarius, Enterococcus faecalis);
to investigate the effect of S. virgaurea extract on C. albicans
growth and yeast–hyphal transition; and to evaluate the
effect of Solidago extract on C. albicans biofilm growth, at
both early and intermediate stages.

METHODS

Plant material. Plant materials of the two taxa were collected during
flowering, S. virgaurea L. subsp. virgaurea was collected in the Tinée
valley, Col Saint Martin, la Colmiane, France (44.067°N 7.217°E),
on 23 August 2011 at an altitude of 1 500 m. S. virgaurea subsp.
alpestris (Waldst. & Kit.) Gremli was collected in the Tinée valley,
Piete Roubine, Isola 2000, France (44.18663°N 7.157936°E), on 11
August 2011 at an altitude of 2 130 m. Voucher specimens have been
deposited in the Botanic Garden of the City of Nice (France) (voucher
numbers C-3173 and C-3174, respectively).

Plant water extract was obtained from 6 g dried aerial plant parts by
placing them in 100 ml of distilled sterile water, warming for 2 h at
30 °C and subjecting them to ultrasound twice for 15 min. The
decoction was then filtered. Large particles were precipitated by
addition of 30 mg edible milk calcium caseinate (Protigilip IP4,
Armor Protéines) and 25 µl 1 M citrate buffer (pH 4) ml−1 of
decoction. After 60 min of incubation at 20 °C and filtration, the
decoction was concentrated at 60 °C in a rotary evaporator.

In assays using blood agar plates, isotonic Solidago extracts were
obtained by addition of sodium chloride 0.9%. Powder of escin
(Sigma–Aldrich) in solution, a mixture of saponins obtained from
Aesculus hippocastanum (Horse-chestnut), was used as control.

Saponins cannot be quantified with a spectrophotometer (Oleszek &
Bialy, 2006). In order to estimate the saponins content in Solidago
extracts, we designed a simple screening protocol using the radial
diffusion of haemolysis on 5% sheep’s blood agar plates
(bioMérieux). A dilution of escin was used as control in the 0.25–
5.0 mg ml−1 range. Briefly, 40 µl of saponin solutions were deposited
in 5 mm-diameter pits and the plates were incubated at 37 °C.
Haemolysis radius was measured 24 h later and plotted as a function
of saponin concentration (Fig. S1, available in JMM Online).

Microbial strains and culture conditions. We used three reference
strains recommended by the Association Française de Normalization
(AFNOR) to test antibacterial compounds: E. coli ATCC 25922,
Staph. aureus ATCC 25923 and P. aeruginosa ATCC 27853. We also
tested an oral wild-type strain of E. faecalis obtained from the
Bacteriology Laboratory at Nice University Hospital (courtesy Dr F.
Girard-Pipau). These strains were grown aerobically at 37 °C
overnight, on Mueller–Hinton agar (bioMérieux). Two other oral strains were also tested: Streptococcus ATCC 7073 and a wild-type strain of Streptococcus mutans (courtesy of Dr F. Girard-Pipau). Streptococci were grown on 5% sheep’s blood agar for 5 days at 37 °C, both in an anaerobic chamber and under microaerophilic conditions in a 5% CO2 chamber.

Four C. albicans strains were used: C. albicans ATCC 10231 (AFNOR) and three wild-type oral strains named IM001, IM003 and IM007, isolated at Nice University Hospital Laboratory (Courtesy Dr Martine Gari-Tousaint). In order to increase the yeast–hyphal transition, C. albicans ATCC 10231 was pre-cultivated anaerobically on 5% sheep’s blood agar (bioMérieux) for 48 h at 37 °C. Then all C. albicans strains were cultivated aerobically on Sabouraud Chloramphenicol agar (bioMérieux) for 48 h at 37 °C.

**Microbial growth.** Microbial growth inhibition was investigated by the diffusion method with 100 μl of bacterial (106 c.f.u.) or fungal (105 c.f.u.) inocula smeared onto agar plates, and 40 μl of Solidago extract deposited into pits of 5 mm diameter. Diameter of growth inhibition was measured after 24 h and after 3 days of incubation.

**Effect of plant extracts on Candida yeast–hyphal transition.** To estimate the effect of S. virgareua water extracts on yeast–hyphal transition, C. albicans was cultivated in YEP liquid medium, containing yeast extract (11 mg ml−1, Sigma–Aldrich), peptone (22 mg ml−1, Oxoid) and 2% w/v N-acetyl-D-glucosamine (GlcNAc) (Calbiochem), under aerobic conditions at 37 °C for 24 h. A C. albicans inoculum of 105 cells ml−1 was incubated with S. virgareua water extract at a final saponin concentration of 0.25 mg ml−1. Yeast and hyphae were counted using a KOVA Glastic slide by observation under an optical microscope after 0, 4 and 24 h of incubation. The control consisted of C. albicans incubated with YEP GlcNAc using water acidified with citrate buffer pH 4 instead of plant water extract. The numbers of hyphae and germ tubes were determined and the percentage inhibition of hyphae and germ tube formation was calculated by comparison with control. All assays were performed in triplicate and three independent experiments were performed.

**Effect of plant extracts on the formation of C. albicans biofilms.** C. albicans biofilms were grown on commercially available pre-sterilized, polystyrene, conical bottomed 96-well microtitre plates (Corning). Biofilms were formed by pipetting standardized cell suspensions into wells; 100 μl of a suspension containing 106 cells ml−1 in RPMI 1640 buffered with MOPS (Nett et al., 2011; Ramage et al., 2001). In order to determine whether Solidago water extracts had an effect on biofilm formation, 50 μl of SVV or SVA extracts were added immediately into the microplate wells after the Candida suspensions to a final concentration of 0.25 mg ml−1. C. albicans incubated with acidified water instead of Solidago extract and biofilm-free wells were included to serve as positive and negative controls, respectively. Plates were incubated for 18 h at 37 °C on an orbital shaker at 200 r.p.m. After biofilm formation, the medium was aspirated. Non-adherent cells were removed by thoroughly washing the biofilms twice with PBS (pH 7.2). A semi-quantitative measure of biofilm formation was calculated using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay (Hawser et al., 1998; Nett et al., 2011). Briefly, 100 μl volumes of water were added to each of the prewashed biofilms and into the control well. Then 50 μl volumes of the XTT reaction mixture (activation reagent and XTT reagent) prepared according to manufacturer’s recommendations (Cell Proliferation kit XTT, AppliChem) were added. Plates were incubated in the dark for 2 h at 37 °C. A colorimetric change resulting from XTT reduction and representing a direct correlation of metabolic activity of the biofilm was measured in a microtitre plate reader (ELx800, Biotek Instruments) at 490 nm. An inhibitory percentage was calculated by the following formula: [(control—treatment)/control]× 100. Assays were performed in triplicate and three independent experiments were performed.

**Effect of plant extracts on pre-formed C. albicans biofilms.** Candida biofilms were obtained as described before, but 100 μl SVV or SVA extracts (final concentration 0.75 mg ml−1) or water acidified (control) were added into wells after 18 h of incubation. Microtitre plates where incubated for 2 hours on an orbital shaker at 200 r.p.m. Biofilms were then washed and measured as described before.

**Scanning electron microscopy.** For SEM, C. albicans biofilms were grown on polymethylmethacrylate (PMMA) discs (8 mm diameter) (Pesci-Bardon et al., 2006). Biofilms were formed by dispensing standardized cell suspensions (1 ml of a suspension containing 106 cells ml−1 in RPMI 1640) onto resin discs within six-well cell culture plates (Corning) and incubating at 37 °C. Discs were removed 18 h later and washed with 0.1 M phosphate buffer (2 × 3 min). Biofilms were treated for 2 h with SVV, SVA or acidified water (control) as described before. Discs were then washed in 0.1 M phosphate buffer (2 × 3 min). Discs were placed in fixative (4%, v/v, formaldehyde in PBS) overnight. Samples were dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, 100% for 20 min) and, finally, air-dried in a desiccator. Samples were observed at × 100, × 500 and × 1500 magnification in a scanning electron microscope (JSM-5310LV, JEOL) in low vacuum mode at 25 and 30 kV. Images were processed for display using SemAforE software (JEOL AB).

**Statistical analysis.** Statistical analyses were performed using StatView software version 5.0 (1998, SAS Institute). For multigroup comparisons, the Kruskal–Wallis test was used to determine if any group exhibited statistically significant different percentages of inhibition of biofilm formation or of yeast–hyphal transition. If the Kruskal–Wallis test demonstrated a post hoc analysis using the Mann–Whitney U test correction was used to adjust the significance value (P) for the number of comparisons.

**RESULTS**

The saponin concentration was estimated by an indirect method of quantification. It reached 0.7 ±0.15 mg ml−1 in the SVV extract and 0.95 ±0.15 mg ml−1 in the SVA extract.

**Microbial growth**

The SVV and SVA plant extracts did not inhibit bacterial (E. coli, Staph. aureus, P. aeruginosa, Strept. mutans, Strept. salivarius, E. faecalis) or fungal (C. albicans, four strains) growth by the diffusion method on agar plates (data not shown).

**Effect of plant extracts on C. albicans yeast–hyphal transition**

C. albicans growth in liquid medium was not inhibited by the addition of plant extracts (data not shown). However, these extracts did inhibit yeast–hyphal transition (Table 1). Shorter forms of germ-tubes were also observed.
Table 1. Impact of *S. virgaurea* extracts on *C. albicans* hyphal formation

At 4 and 24 h, the number of hyphae and germ tubes was determined and the percentage was calculated by comparison with the total. Values are means ± SD. *P*-values represent significant difference between *Solidago* treatments and controls (no treatment) using Kruskal–Wallis and Mann–Whitney tests.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>SVV</th>
<th>SVA</th>
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<tbody>
<tr>
<td>ATCC 10231</td>
<td>51.5 ± 13.0</td>
<td>1.6 ± 4.6*</td>
<td>7.7 ± 6.6†</td>
</tr>
<tr>
<td>IM001</td>
<td>25.2 ± 8.6</td>
<td>14.3 ± 5.7†</td>
<td>10.7 ± 6.3§</td>
</tr>
<tr>
<td>IM003</td>
<td>18.7 ± 8.0</td>
<td>1.1 ± 1.1*</td>
<td>0.1 ± 1.6*</td>
</tr>
<tr>
<td>IM007</td>
<td>16.9 ± 6.7</td>
<td>2.7 ± 2.0*</td>
<td>4.3 ± 2.9*</td>
</tr>
</tbody>
</table>

*P<0.0001.
†P<0.05.
‡P<0.005.
§P<0.0005.

Effect of plant extracts on the formation of *C. albicans* biofilms

*Solidago* extracts strongly inhibited *C. albicans* biofilm formation.

All *C. albicans* strains produced a significant biofilm (OD<sub>600</sub>: ATCC 10231, 2.06 ± 0.47; IM001, 3.09 ± 0.55; IM003, 2.22 ± 0.62 and IM007, 3.38 ± 0.17). Compared to control cultures without *Solidago* extracts, SVV and SVA extracts significantly reduced biofilm formation for all the strains tested (*P<0.0005). The percentages of reduction in biofilm formation observed were: ATCC 10231, 98.41 ± 1.97%; IM001, 99.18 ± 2.45%; IM003, 97.32 ± 5.94%; and IM007, 96.49 ± 3.73%, for SVV, and ATCC 10231, 95.86 ± 4.88%; IM001, 96.00 ± 2.22%; IM003, 99.46 ± 5.94%; and IM007, 95.14 ± 4.11%, for SVA (Fig. 1). Using the non-parametric test of Mann and Whitney, there were no statistically significant differences between SVA and SVV treatments.

Effect of plant extracts on pre-formed *C. albicans* biofilms

*Solidago* extracts significantly decreased *C. albicans* pre-formed biofilms. Exposure of pre-formed 18 h biofilms to SVV and SVA extracts for 2 h resulted in significant reduction in viability compared to control biofilms for all the strains tested (*P<0.0005). The percentages of reduction observed were: ATCC 10231, 77.85 ± 13.35%; IM001, 91.16 ± 4.82%; IM003, 79.21 ± 8.28%; and IM007, 90.85 ± 7.19%, for SVV, and ATCC 10231, 92.37 ± 7.79%; IM001, 82.17 ± 5.93%; IM003, 76.26 ± 10.71%; and IM007: 91.91 ± 4.84%, for SVA (Fig. 2). Using the non-parametric test of Mann and Whitney, there were statistically significant differences between SVA and SVV treatments. SVA was more efficient than SVV against *C. albicans* ATCC 10231 (*P=0.0243) and SVA was more efficient than SVA against *C. albicans* IM001 group (*P=0.038). However, there were no statistically significant differences between SVV and SVA against *C. albicans* IM003 and IM007 (*P>0.05). SEM examination showed a decreased number of both yeast and hyphal forms when *Candida* biofilms were grown on resin discs (Fig. 3).

DISCUSSION

In the present study, we investigated a new strategy to relieve dry mouth symptoms and focused on the creation of an unfavourable environment for *C. albicans*. For this purpose we investigated *Solidago* water extracts containing saponins on *C. albicans* biofilm formation. Cells were incubated for 18 h, in the presence or absence of *S. virgaurea* water extracts (final concentration 0.25 mg ml<sup>-1</sup>), and assayed for XTT reduction activity. A reference strain, *C. albicans* ATCC 10231, and three wild-type strains, IM001, IM003 and IM007, were tested. Kruskal–Wallis and Mann–Whitney tests were used to compare *Solidago* treatments and controls (no treatment), *P<0.005.

![Fig. 1. Effect of *Solidago virgaurea* water extracts containing saponins on *C. albicans* biofilm formation. Cells were incubated for 18 h, in the presence or absence of *S. virgaurea* water extracts (final concentration 0.25 mg ml<sup>-1</sup>), and assayed for XTT reduction activity. A reference strain, *C. albicans* ATCC 10231, and three wild-type strains, IM001, IM003 and IM007, were tested.](http://jmm.sgmjournals.org)
Saponins, which are plant detergents with iron-chelator properties. Saponins could deprive \textit{Candida} from the iron available in serum exudates and saliva. However, \textit{in vivo}, the precise role of plant iron chelators in mediating conditions affecting the oral mucosa and microflora may be controversial. Hameed \textit{et al.} (2008) performed iron deprivation experiments by using a synthetic chelator (bathophenanthroline disulfonic acid), which promoted hyphal development without affecting the growth of \textit{Candida} cells, or their ability to form biofilms on catheters.

The present plant extraction process allowed the preservation of a natural edible aroma (saponins/liquorice), dye (flavonoids/yellow) and maybe other compounds of interest such as anti-inflammatory acid phenols (salicylic acid), or chalcones (flavonoids) with intrinsic yeast–hyphal transition and biofilm inhibitory properties (Messier \textit{et al.}, 2011).

In order to develop a dry mouth-specific mouthwash and to preserve oral mucosa hydration, we first aimed to preserve oral bacteria in the healthy microflora, which are natural competitors of \textit{C. albicans}. For this reason, we first verified that \textit{Solidago} extracts did not inhibit bacterial growth. This absence of antibacterial activity could be explained because saponins can interfere with sterols, namely phytosterols (plants), cholesterol (mammalian cells and human viruses) and ergosterol (fungi), while most of bacteria are lacking in membrane sterols (Alvarez \textit{et al.}, 2007).

\textbf{Anti-	extit{Candida} properties are required in a dry mouth-specific mouthwash (ten Cate \textit{et al.}, 2009; Humphrey & Williamson, 2001); however, in clinical practice, a full eradication of \textit{C. albicans} from oral ecosystems is not a therapeutic objective (ten Cate \textit{et al.}, 2009). In response to the host environment, \textit{C. albicans} yeast–hyphal transition is important for virulence, and Saville \textit{et al.} (2003) demonstrated that mutants that are locked in yeast or hyphal forms were not as virulent. It has also been shown that filamentation is not a compulsory prerequisite during the formation of a biofilm (Blankenship & Mitchell, 2006; Douglas, 2003). Thus, the second aim of this work was to inhibit the \textit{C. albicans} yeast–hyphal transition using \textit{Solidago} extracts, and in experiments, such an effect was

![Figure 2](image)

**Fig. 2.** Effect of \textit{Solidago virgaurea} water extracts containing saponins on pre-formed \textit{C. albicans} biofilms. Cells were incubated for 18 h, 100 \textmu{l} of \textit{S. virgaurea} water extract (final concentration 0.75 mg ml\textsuperscript{-1}) was added and incubation was prolonged for 2 h. Biofilms were assayed for XTT reduction activity. A reference strain, \textit{C. albicans} ATCC 10231, and three wild-type strains, IM001, IM003 and IM007, were tested. Kruskal–Wallis and Mann–Whitney tests were used to compare \textit{Solidago} treatments and controls (no treatment), *P<0.005.

![Figure 3](image)

**Fig. 3.** SEM images of \textit{C. albicans} ATCC 10231 biofilm formation on resin discs. (a, d) Controls, \textit{C. albicans} morphology displayed yeast and hyphal cells. (b, e) \textit{C. albicans} treated with \textit{Solidago virgaurea} subsp. \textit{virgaurea} water extract exhibited a decreased number of yeast and hyphal cells. (c, f) Similar results were seen with \textit{Solidago virgaurea} subsp. \textit{alpestris} water extract. Bars, 100 \textmu{m} (a–c) and 50 \textmu{m} (d–f).
observed. This could be explained by the difference in total lipid content of hyphal forms, which is higher than that of the yeast and rather immature forms (C16:0 versus C18:0 fatty acids) due to an increased biosynthesis of lipids in hyphal forms (Bahn et al., 2007). Additional studies will now be necessary in order to investigate whether Solidago saponins can reduce Candida pathogenesis linked to hyphal forms, focusing on membrane polarization and production of extracellular proteolytic enzymes (SAP), as well as the synthesis of adhesins promoting adherence to epithelial cells, denture resin, Candida cells and other oral bacteria (Hwp1, Als, Ala1) (Hope et al., 2008; Alvarez et al., 2007; Martin & Konopka, 2004). Potential interactions between saponins and Candida membrane-associated lipo- mannans should also be investigated, as well as resistance-inducing factors (Masuoka, 2004).

Candida hyphae can co-aggregate to form a threedimensional scaffolding colonized by oral bacteria, leading to macroscopic deposits on oral surfaces (Villar et al., 2007). Although in vitro mutant cells fixed in either hyphal or yeast form could develop into biofilms (Douglas, 2003), these biofilms were rudimentary and were not as stable as wild-type biofilms (Blankenship & Mitchell, 2006). The third aim of this work was to investigate whether Solidago extracts could prevent C. albicans biofilm growth, at both early and intermediate stages of development. This was confirmed by growth onto two different supports, microtitre plates and PMMA resin discs mimicking prosthetic dentures. SEM results suggested that saponin extracts displayed anti-adherent activity but the XTT method is only able to demonstrate that there is less metabolic activity after both treatments (Nett et al., 2011). However, the anti-biofilm effects of saponins observed in the present study are in line with the results of Coleman et al. (2010) who showed that, similarly, plant derived saponins disrupted C. albicans biofilms. Several authors demonstrated that, unlike brand antiseptic mouthwashes, fluconazole and polyenes (nystatin and amphotericin B) were inefficient against pre-formed Candida biofilms in vitro (Ramage et al., 2011; Lamfon et al., 2004). The S. virgareus extracts used in this study displayed anti-biofilm properties similar to those of antiseptic mouthwashes (used at the exposure time recommended by the manufacturers) containing quaternary ammonium compounds [chlorhexidine gluconate (Cordosyl), GlaxoSmitKline; hexetidine-triclosan (Oraldene), Warner-Lambert] or essential oils in combination (Listerine, Pfizer). However, unlike antiseptic mouthwashes, these Solidago extracts did not exhibit any fungicidal or bactericidal properties. In vivo, inhibition of hyphal forms by a saponin-containing mouthwash should also reduce macroscopic biofilms too. This property could be reinforced by the intrinsic detergent properties of saponins against lipid molecules, particularly serum lipoprotein exudates (Enjalbert & Whiteway, 2005). Currently, serum is commonly added to Candida culture medium to promote the yeast–hyphal transition. Besides, saponins have both haemolytic and iron chelator properties, and iron is necessary for Candida growth (Matsuda, 1999). Thus, saponins could interfere with blood exudates from inflamed oral mucosa and indirectly reduce Candida proliferation by sequestering iron.

In conclusion, we showed that S. virgareus water extracts had no antibacterial or antifungal activity sensu stricto. However they displayed inhibitory effect on the C. albicans yeast–hyphal transition, strongly inhibited biofilm formation and decreased pre-formed biofilms. Some Candida strains were more inhibited by SVV and others by SVA, so it could be beneficial to mix these two plant extracts. Chemical analysis is in progress, in order to better understand how saponins can inhibit the Candida yeast–hyphal transition.

ACKNOWLEDGEMENTS

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1021


CHAPITRE III - Aspects moléculaires : S. virgaurea bloque l’expression de gènes impliqués dans l’adhésion et la morphologie au niveau transcriptionnel

Nous avons montré que l’extrait de S. virgaurea inhibait la conversion levure-filament de C. albicans. Cette propriété limite sa virulence à la fois sous forme planctonique et sous forme de biofilm. L’extrait inhibe aussi l’aptitude de C. albicans à former des biofilms et diminue la quantité de biofilm déjà formé.

L’objectif de cette seconde étude était d’approfondir les résultats obtenus en travaillant sur l’extrait S. virgaurea L. subsp. Alpestris. Etant donné que l’extrait contient des composés susceptibles de détériorer la membrane de C. albicans, nous avons tout d’abord testé l’intégrité de celle-ci. Puis, d’un point de vue moléculaire, nous avons mesuré l’expression des principaux gènes impliqués dans le changement de morphologie (HWP1, ECE1, HGC1), l’adhésion (ALS) et la formation de biofilm (ALS, HWP1, ECE1, HGC1). De même nous avons mesuré l’expression d’un gène codant pour une protéine exportable (SAP6).

Jusqu’alors nous avions mis en évidence la diminution de l’adhésion sur des surfaces inertes. Il nous a donc semblé intéressant de vérifier que la diminution de l’adhésion était également effective sur des cellules épithéliales. Enfin, nous avons analysé l’action synergie de l’extrait végétal combiné à des antifongiques prescrits en première intention dans les candidoses orales (amphotéricine B, nystatine, miconazole) et à un bain de bouche contenant de la chlorexhidine, grâce à la méthode de l’échiquier (checkerboard).

Une partie des travaux présentés dans cet article a été initiée lors d’une mobilité de formation d’une durée de deux semaines effectuée dans l’équipe « Infection & Immunity Research Group Lead ; University of Glasgow Dental School » sous la direction du Pr. Gordon Ramage. Ce laboratoire a publié de nombreux travaux sur C. albicans au niveau de la cavité orale, dont une partie sur le mode d’action de composés connus et de nouveaux composés, incluant des composés naturels (Sherry et al., 2012).

Les résultats ont confirmé que l’extrait de S. virgaurea diminue fortement l’adhésion de C. albicans aux surfaces inertes mais également aux cellules épithéliales. Nous avons également démontré qu’il inhibe l’expression de plusieurs gènes impliqués dans l’adhésion, le changement...
de morphologie et la formation de biofilm. Et enfin nous avons montré que, la combinaison entre l’extrait végétal et du miconazole, de la nystatine ou un bain de bouche contenant de la chlorexhidine agit de manière synergique sur l’élimination du biofilm.
Prevention of *Candida albicans* biofilm formation by *Solidago virgaurea* extract involves transcriptional blockade of adhesion-specific genes

Marlène Chevalier, Alain Doglio, Ranjith Rajendran, Gordon Ramage, Isabelle Prêcheur and Stéphane Ranque

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**Journal of Medical Microbiology**

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| Corresponding Author: | Marlène CHEVALIER  
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                        NICE, FRANCE |
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                   Alain DOGLIO  
                   Ranjith RAJENDRAN  
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                   Isabelle PRÈCHEUR  
                   Stéphane RANQUE |
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                   Results/Key findings: The SV-extract was shown to strongly inhibit yeast adherence and its hyphal transition on inert substrates and epithelial cells, but it did not affect viability and growth of yeasts in liquid culture. Moreover, we demonstrated that the plant extract acutely inhibited expression of several cell-wall-associated proteins encoding genes involved in yeast adhesion and hyphal morphology. In addition, when used in combination with miconazole and nystatin, or a commonly antiseptic mouthwash, the SV extract promoted a synergistic antifungal action.  
                   Conclusions: C. albicans species cause frequent infections of the oral cavity owing to their ability to form biofilm-surface-associated microbial communities. By preventing expression of adhesion and hyphae-associated genes, the SV-extract may represent an innovative and very promising new antifungal product that could be of value for oral use. |
Prevention of *Candida albicans* biofilm formation by *Solidago virgaurea* extract involves transcriptional blockade of adhesion-specific genes

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Key words:

*Candida albicans, Solidago virgaurea*, biofilm, genetic control, antifungal therapy

Subject category: Prevention and Therapy

Word count: 4886
**Abstract**

**Purpose:**
We previously discovered that a crude aqueous extract from the medicinal plant *Solidago virgaurea* (SV) was able to inhibit formation of *Candida albicans* biofilm. In the present study we investigated the inhibitory mechanism.

**Methodology:**
Analysis of how SV-extract effected adhesion, biofilm formation and liquid culture growth of different *C. albicans* laboratory and clinical isolates were performed *in vitro*. Analysis of *C. albicans* gene expression was carried out using quantitative PCR-based approaches. The synergistic effects of SV-extract combined with the antifungals amphotericin B, nystatin, miconazole, and a commonly used antiseptic mouthwash, were analyzed with checkerboard microdilution assay.

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The SV-extract was shown to strongly inhibit yeast adherence and its hyphal transition on inert substrates and epithelial cells, but it did not affect viability and growth of yeasts in liquid culture. Moreover, we demonstrated that the plant extract acutely inhibited expression of several cell-wall-associated proteins encoding genes involved in yeast adhesion and hyphal morphology. In addition, when used in combination with miconazole and nystatin, or a commonly antiseptic mouthwash, the SV extract promoted a synergistic antifungal action.

**Conclusions:**
*C. albicans* species cause frequent infections of the oral cavity owing to their ability to form biofilm-surface-associated microbial communities. By preventing expression of adhesion and hyphae-associated genes, the SV-extract may represent an innovative and very promising new antifungal product that could be of value for oral use.
INTRODUCTION

The polymorphic fungus *C. albicans* is the most frequent commensal and opportunistic pathogenic yeast in the oral cavity [1, 2]. While usually benign in healthy individuals, recalcitrant *C. albicans* infections of the oral cavity are commonly observed in mildly immunocompromised individuals or in the most fragile patients at the extreme age of the life [3]. *C. albicans* develops within a complex microbial biofilm that protects it from the environment and immune system, forming co-operative synergistic and antagonistic interactions with the oral microbiome [4, 5]. *C. albicans* adhesion represents the first step of infection (0 to 11h), immediately followed by proliferation of adherent yeasts and hyphae formation (12 to 24h) that represents a critical step before *C. albicans* biofilm formation. Finally, the maturation phase (≈24 to 72 h) is characterized by a dense network of filamentous forms (pseudohyphae and hyphae), and become encased in the exopolymeric matrix [6–8]. This is governed by a gene network of transcription regulators and their target genes [9, 10]. In particular Efg1, Cph1 and/or Bcr1 transcription factors involved in biofilm formation [11]. The zinc finger transcription factor Bcr1 is a master gene required for expression of members of the agglutinin-like sequence (ALS) gene family notably the cell-wall glycoproteins Als3p and Als1p. Bcr1p also tightly regulates [12, 13] expression of hyphae-specific genes like hyphal wall protein (Hwp1) and extent of cell elongation 1 protein (Ece1) [12]. Als1p, Als3p, and Hwp1p proteins have been described to maintain cell wall integrity, to promote hyphae initiation and to increase binding of *C. albicans* to oral epithelial cells [12, 14, 15]. Moreover, Als3p has also been proposed to act as a major *C. albicans* invasin [16]. While *ECE1* is usually considered as a hyphal-induced gene it seems to be not essential for *C. albicans* growth or hyphal formation, although adhesion is reduced in its absence [17, 18]. *C. albicans* also expresses a family of secreted aspartyl proteinases (SAPs) genes that have been described as a
major virulence factor and which are mainly regulated by the cell morphotype and the 
environmental factors. While SAP1 to SAP3 are expressed predominantly in yeast cells, hyphal 
cells mainly express SAP4 to SAP6 [19, 20]. Lastly, hyphal G cyclin 1 (HGC1) is activated by hyphae-
inducing signals and necessary for hyphal morphogenesis and regulation [17, 21]. These 6 genes 
are involved in morphology, pathogenicity (SAPs) or both (HGC1, HWP1, ECE1, ALS) [17].

Biofilm formation is considered as a key virulence factor of C. albicans that can provide the 
community protection against antimicrobial agents as compared with those in a nomadic state 
(e.g. planktonic cells) [22]. C. albicans in biofilm can be 100-fold more resistant to antifungal 
fluconazole and 20- to 30-fold more resistant to antifungal amphotericin B than planktonic cells 
[23]. Although, C. albicans can cause infectious lesions in either yeast and filamentous forms, 
previous studies have shown that its ability to form biofilm is a key factor to host invasion and 
tissue destruction [24–26]. Moreover, the most commonly used antifungal drugs have been 
mostly developed to target exponentially growing fungal cells and are limited, or not effective, 
against biofilm structures. So, the challenge to search novel strategies and new 
chemotherapeutic options to control fungal biofilm is to develop new antifungal oral therapies 
able to prevent biofilm formation and/or to alter mature biofilm architecture. By preventing 
biofilm formation and favoring the release of planktonic yeasts, such a class of inhibitors are 
expected to reduce the resistance of pathogenic oral biofilm and moreover to synergistically 
increase the action of others antifungal drugs. Interestingly, we previously established that a 
crude water extract of the medicinal plant, the Solidago virgaurea (SV), was able to strongly 
inhibit C. albicans biofilm formation and to destroy pre-formed biofilms [27]. The aim of the 
present study was thus to further investigate the mechanism of action of the SV—extract on C.
*albicans* biofilm formation and to evaluate its potential as a new potential antifungal compounds.

**MATERIAL AND METHODS**

*Preparation and use of plant extract*

The SV-extract was obtained as previously described [27], with minor modifications. Briefly, 11.5 g of dried aerial parts of SV was incubated in 100 ml of distilled sterile water for 4 h at 45°C and treated twice by ultrasound. After filtration, large particles were precipitated by addition of 30 mg edible milk calcium caseinate (Protilight IP4, Armor Protéines) and 25 µl of 1 M citrate buffer (pH 4). The decoction was then filtered and autoclaved before any experiments. Control experiments were carried out using acidified-water with citrate buffer (pH4) instead of SV-extract. As specified, the crude aqueous SV-extract or acidified-water (control) were used in any experiments of this study diluted to one third (33.3% final).

*Candida strains, growth and culture conditions*

The *C. albicans* ATCC 10231 and three oral clinical isolates of *C. albicans* isolated from patients followed at the dentistry department of the Nice University Hospital were used in this study. Clinical isolates were identified as *C. albicans* using routine biochemical identification performed at the laboratory of bacteriology of the Nice University Hospital. Working stocks of yeast cells were maintained at 4°C on Sabouraud agar plates (SAB; Oxoid, Cambridge, UK). Yeasts were propagated in liquid culture in yeast peptone dextrose (YPD) medium (Oxoid, Cambridge, UK) containing 2% w/v N-acetyl-α-d-glucosamine (Calbiochem, Darmstadt, Germany) as described previously [27]. Numeration of planktonic yeasts and hyphae were
achieved using KOVA Glasstic slide. Before adhesion-experiments on solid support yeasts were suspended in RPMI-1640 medium (Sigma Aldrich) before seeding. Yeast adhesion on Thermanox™ coverslips (Nunc Inc, Thermo Fisher Scientific) was performed in a well of a 24-well flat bottom plate containing 1 ml of *C. albicans* ATCC 10231 suspension in RPMI (1 × 10⁶ cells/mL). After incubation (24h at 37°C), coverslips were gently washed in PBS and transferred to a glass slide for microscopic analysis with a scanning electron microscope (JEOL 6700F).

**Propidium and chitin assays**

To assess the effect of the SV-extract on yeast membrane integrity a propidium iodide (PI) uptake assay was performed. *C. albicans* (ATCC 10231) were standardized to 5 × 10⁷ cells/mL in RPMI-1640 and treated for different times with the SV-extract (60 min in total). Every 10 minutes, 100 μl of yeasts were removed, washed in PBS, suspended in PI-solution (20 μM in PBS) and incubated for 15 min at 37°C in dark. PI-uptake was then measured in black 96-well microtiter plate with a fluorescent plate reader (Ex 535/Em 617; FluoStar Omega, BMG Labtech). Microscopic observations were performed at 10x and 63x magnification (Zeiss, Model Axiovert A1, Germany). Permeabilized-yeasts used as control were obtained by incubating yeasts for 60 minutes in 70% ethanol solution.

The contribution of chitin was investigated using nikkomycin Z, a specific inhibitor of the chitin synthesis pathway as previously described [28]. Briefly, planktonic yeasts (5 × 10⁷ cells/mL) were treated with the SV-extract in presence or absence of nikkomycin Z (0.4 μg/ml; Sigma-Aldrich) for 24 h at 37°C. For experiments with adherent *C. albicans*, cells (5 × 10⁷ cells/mL) were incubated 24 h at 37°C in RPMI to allow yeast adhesion and biofilm formation, then biofilm was
treated with SV-extract ± nikkomycin Z for another 24 h. Following incubation of planktonic cells and biofilms the metabolic activity was quantified using the XTT reduction assay.

**Gene expression analysis**

The effect of SV-extract on *C. albicans* ATCC 10231 adhesion and filamentation was analyzed by qRT-PCR-based quantitative transcriptional analysis. Standardized cells (1 × 10⁸ cells/mL) diluted in RPMI were placed in a 6 wells plate at 37°C in presence of SV-extract (33.3%) or acidified-water for 4h and 24 h, in triplicate. At each time point, yeasts were collected and RNAs extracted with the MasterPure Yeast RNA Purification Kit (Tebu-bio, Le Perray En Yvelines, France). cDNA was then synthesized using Power SYBR® Green RNA-to-CR™ 1-Step Kit (Life Technologies, Paisley, UK), following manufacturer’s instructions. Primers used for qRT-PCR analysis are presented in Table 1. Targeted putative virulence genes of *C. albicans* were *ALS1* and *ALS3* (agglutinin-like sequence), *HWP1* (hyphal wall protein), *ECE1* (Extent of cell elongation 1), *HGC1* (Hyphal G cyclin 1) and *SAP6* (secreted aspartyl proteinases). The *ACT1* housekeeping gene served as an endogenous reference control for *C. albicans* taking into account its stability in sessile and planktonic yeasts [29].

Cycling conditions consisted of 30 min at 48°C for reverse transcription, 10 min at 95°C and forty cycles of 15 s at 95°C and 60 s at 60°C. Each parameter was analyzed in duplicate using StepOnePlus Real-Time PCR System (v. 2.3, Applied Biosystems, USA). Gene expression was normalized to the housekeeping gene (*ACT1*) and presented in the form of expression levels relative to *ACT1*.
Yeast adhesion to oral epithelial cells

Yeast adhesion to human epithelial cells (hECs) was performed using TR146, an oral epithelial cell model commonly recognized as a representative for the human gingival mucosa [30]. TR146 cells were cultured in flasks (75 cm²) (Nunc Inc, Thermo Fisher Scientific) at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose 4.5 g/L supplemented with 10% Fetal bovine serum (FBS). SV-extract pre-treated C. albicans (24 h at 37°C) were washed and suspended in 0.9% NaCl, approximately 10⁵ TR146 and 10⁶ fungi were mixed and incubated for 45 min at 37°C with gentle orbital shaking. Then the cells-fungi mixture 1:1000 were stained with Calcofluor White (CW [Invitrogen, Paisley, UK]) and FUN® 1 (Invitrogen, Paisley, UK) for 30 min. Cell-wall chitin of fungi was specifically labeled with the CW (blue-fluorescence), while the FUN® 1, that passively diffused into cytoplasm of most eukaryotic cells, was used to stain epithelial cells with green fluorescence. Then mixture was transferred to a glass slide for observation under fluorescent microscope (Zeiss, Model Axiovert A1, Germany) at 10x and 63x magnification. Frequency of C. albicans adhesion to TR146 was measured by counting any TR146 showing at least one adherent fungus over the total number of TR146.

Checkerboard microdilution assay

The C. albicans MICs (minimal inhibitory concentration) of each compound were determined by the micro-broth dilution method according to the Clinical and Laboratory Standards Institute guidelines [31]. Each well of the 96-well microtiter plate contained a clinical strain of C. albicans in RPMI 1640 medium (final concentration of 2.5x10³ cells/mL) and serially diluted test agents in combinations.
The concentrations for miconazole were 0.156–40 mg/L, for nystatin 0.025–125 mg/L and for amphotericin B 0.156–40 mg/L. In order to calculate a fractional inhibitory concentration index (FIC index) for SV extract and for commercial mouthwash (Eludril) a value of 250 was attributed for the initial solution (without dilution). For these compounds values were comprised between 250 and 3.75. 96-well microtiter plates were incubated at 37°C for 24 h, and optical density measured at 630 nm. MIC\textsubscript{80} were determined as the lowest concentration of the drugs (alone or in combination) that inhibited viability by 80% compared with that of drug-free wells. The FIC index is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. It was calculated using the formula: FIC index = MIC\textsubscript{(combination)}/ \( \text{MIC (drug A alone)} + \text{MIC (combination)}/ \text{MIC (drug B alone)} \). FIC index < 0.5 indicates synergy; 0.5–4.0, indifference; and >4, antagonism [32].

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical significance between treated and control groups were analyzed by Student’s t-test. A p-value < 0.05 was considered statistically significant.

RESULTS

The SV-extract blocks C. albicans hyphae-transition phase and yeast adhesion to oral epithelial cells

In vitro C. albicans growth usually leads to fully mature biofilms with a network of planktonic yeasts, hyphae, and pseudohyphae [33]. As illustrated in Fig. 1 a-b, our culture conditions allowed for an efficient C. albicans biofilm formation on inert Thermonox support with presence
of numerous hyphae. In contrast, SV-extract addition almost completely inhibited the yeast adhesion to the support, and subsequent biofilm formation (Fig. 1c). Moreover, direct visual observation performed at higher magnification showed that the rare adherent SV-treated yeasts did not exhibit gross morphological abnormalities, but were mainly detected under a small oval form lacking their ability to form hyphae shape (Fig. 1d). In addition, we also investigated the possibility that SV-extract may prevent yeast adhesion to hECs. Indeed, C. albicans virulence is mainly associated with its ability to adhere to mucosal cells promoting severe deleterious cellular effects and epithelium disruption [34]. In our assay conditions, C. albicans adhesion to hECs was rapid and very efficient allowing most hECs (63.8 ±13%) to bind with at least one C. albicans (Fig. 2). hECS-bound fungi were mostly under hyphae shape and form heap with cells. Of note, hEC morphology was strongly affected in the presence of high concentration of adhering C. albicans in particular many apoptotic cells, showing characteristic cell-ballooning, were observed suggesting that yeast binding to hECs severely affected hEC cell viability. In contrast, after 24h of yeast pre-treatment with the SV-extract, yeast adhesion to hECs was drastically reduced (19.5 ±13.7%) leaving most hECs free of any yeast binding (Fig. 2).

Inhibition of biofilm formation by the SV-extract was dose dependent (not shown) with an EC₈₀ readily achieved when the crude aqueous SV-extract was diluted 3-fold. However, this working-dose was previously described to contain around 0.25 mg ml⁻¹ of saponin-like products [27]. We excluded a possible saponin-mediated detergent action by showing first that the growth of planktonic forms of the C. albicans 10231 strain and of the 3 different clinical isolates was not affected by the SV-extract (Fig. 3). On the contrary, planktonic growth of the 10231 strain and of 2 clinical isolates was rather stimulated in the presence of the plant extract indicating that the cell viability of the planktonic yeasts was not affected. Moreover, lack of SV-associated toxic
effects on yeast integrity was also confirmed using a PI-uptake assay (Fig. 4) that established
that PI uptake in yeast was not increased with time in the presence of the SV-extract, suggesting
that this saponin-containing product did not destabilize cell wall through a detergent action.
Taken together, adhesion experiments carried out on solid support and live hECs clearly
indicated that the SV-extract was very efficient to prevent yeast adhesion and likely hyphal
transition. These results may suggest that the SV-extract exhibited an anti-fungal action, acting
rather like a fungistatic antifungal agents, able to efficiently prevent biofilm formation to favor
yeast growth under planktonic form.

SV-extract inhibits expression of hyphae-specific genes

To gain further insight into the mechanism of SV mediated inhibition of *C. albicans* hyphal
growth and biofilm formation, we analyzed the expression profile of critical adhesion and
hyphal growth-associated genes (Fig. 5). *C. albicans* cells were incubated in the presence of the
SV-extract and the level of expression of each gene was quantified by qRT-PCR and normalized
with housekeeping gene (*ACT1*). The expression of *HWP1, ALS3, ECE1* and *SAP6* was strongly
inhibited in the presence of extract, this inhibition was observed early after SV-extract addition
(4 h) and remained very significant even after 24 h. *HGC1* expression was also strongly reduced
early after SV-extract addition while it became meaningless after 24 h of incubation (Fig. 5). In
contrast, the expression of *ALS1* did not appear to be modified in the presence of SV-extract at
4 h (1.2 fold) and was even stimulated at 24 h establishing that gene inhibition by the SV-extract
was not general. Taking together, this genetic analysis strongly suggested that SV-extract can
block yeast adhesion and biofilm formation through a mechanism that appears to take place at
the transcriptional level by preventing the expression of key yeast adhesins. How this
transcriptional blockade take and are some other master genes affected in the presence of the
SV-extract remains an interesting question that should be solved with further works.

**Antifungal activity of commonly used drugs is enhanced in the presence of SV-extracts**

**Checkerboard microdilution assay**

We then reasoned that the SV-extract may act synergically with many other antifungal agents
that are commonly used for treatment of oral candidosis and that display different antifungal
actions. The synergistic effect of the SV-extract with three oral antifungal drugs (AmB,
miconazole and nystatin) and a commonly used antiseptic mouthwash containing chlorhexidine
was analyzed by checkerboard assay on *C. albicans* biofilm formation. After the FIC
determination, the FICI was calculated for each combination to determine whether the
interaction of SV with AmB, miconazole, nystatin or mouthwash was positive, negative or
neutral. The FICI of SV in combination of AmB, miconazole, nystatin and mouthwash were
2.006, 0.5, 0.032 and 0.18, respectively, revealing a significant synergistic interaction between
of the SV-extract and miconazole, nystatin and the mouthwash to inhibit biofilm formation. For
AmB, we quantified a value comprised between 0.5 and 4 which indicate that the SV-extract
did not seem to interact synergistically.

**DISCUSSION**

Medicinal plants have been used for decades to improve health and treat some specific
disorders. SV (also known as European goldenrod or woundwort) is a widespread herbaceous
perennial plant of the family Asteraceae, known for a long time for its astringent, diuretic, antiseptic and other medicinal properties [35]. We previously discovered that the crude water extract from SV was very efficient to prevent formation of candida biofilms and that it was also capable to eradicate pre-formed biofilms [27]. This study thus revealed the potential value of this plant extract as a new candidate against oral Candida infection.

*Candida* biofilm formation begins with adherence of yeast cells to a substrate (the adherence step). Results from our previous study were confirmed and extended in the present study by showing that the adherence step to inert support and to hECs was strongly affected in the presence of the plant extract. In addition, the few number of yeasts which were yet able to adhere to coverslips or to human cells mostly displayed a small oval yeast-form (blastospores) and never reached the hyphal transition step characterized by appearance of elongated projections to generate yeast filamentous forms, including hyphae and pseudohyphae. These observations suggested that the plant extract was able to prevent yeast adhesion to support leading to the lack of biofilm formation.

We first hypothesized that the SV extract may exert some toxic effect on yeast viability taking into account the presence several secondary metabolites such as flavonoids, phenolic compounds, monoterpenes, sesquiterpenes, triterpenes, polyacetylenes and saponins [36]. Some of these compounds, notably the saponins, are known to bind membrane sterols causing cell membrane damage through detergent action [37]. However, we excluded some toxic effects since cell growth in liquid culture was not affected and moreover we even observed a stimulating effect with some Candida isolates. In addition, PI-uptake was not increased in the presence of the SV-extract indicating lack of membrane permeabilization through a possible
detergent action. Concurrently, we also demonstrated that chitin, that is an essential structural
polysaccharide component of cell walls and septa in fungi, was not targeted by the plant-extract
(not illustrated).

We then further investigated the molecular basis of the SV-extract-associated inhibitory action
on biofilm formation. Recent progress in expression profiling and genetic manipulation has
increased our understanding of the regulatory pathways and mechanisms that govern C.
*albicans* biofilm development and biofilm-based drug resistance [18]. In particular, some
*Candida* genes have been described to encode for several cell-wall related proteins which play
a direct role in cell–substrate or cell–cell adherence or hyphal development [18, 22].
Surprisingly, we observed that expression of several genes encoding for such wall-associated
proteins (i.e., Eap1p, Hwp1p, Ece1p, Als1p) were strongly inhibited in presence of the SV-
extract. Obviously, by lacking Ece1p, Eap1p, Hwp1p and Als1p, the SV-treated yeasts become
unable to bind substrate or to initiate hyphal development. These observations enable us to
propose a robust mechanism that may be sufficient to explain *C. albicans* biofilm formation by
the SV-extract. Interestingly, this transcriptional inhibition of cell-wall related proteins by the
SV-extract appeared to be rather specific considering that, at same time, cell viability and cell
growth in liquid culture was not affected at all. Molecular mechanisms relying a specific
transcriptional inhibition of SV-extract upon adherence specifically interferes with expression
of cell wall related proteins cannot be understood at this stage of our work and will require
further works to be solved.

Development of new antifungal products able to prevent biofilm formation are of critical
interest taking since biofilm lifestyle confers numerous advantages to the pathogens, including
high tolerance to environmental stresses such as antimicrobials and host immune responses. Interestingly, we demonstrated in the present study that when used in combination with the SV-extract, the common antifungal molecules miconazole, and nystatin, or a current antiseptic mouthwash, showed a very significant increase of their antifungal activity. Synergistic action of the SV-extract with these antifungal products could be first explained by the fact that by preventing biofilm inhibition the SV-extract may favor the action of inhibitors known to be more efficient against growing planktonic yeasts [8]. Second, miconazole, nystatin and the SV-extract are likely to inhibit *candida* development by acting against different targets or stage block synthesis of ergosterol, since we showed that the SV-extract act at transcriptional level it that combination of inhibitors acting at different stages of biofilm development may greatly favor an antifungal synergism. Concurrently, taking into account the fact that chitin, which constitutes a specific element of the fungus cell wall, may favor *C. albicans* biofilm susceptibility to azole-derivatives products [38], we performed here additional studies (not illustrated) to investigate a possible involvement of chitin to mediate the SV-extract action. Using nikkomycin (NKV), a competitive analogue of chitin synthase substrate UDP-N-acetylglucosamine that prevent chitin presence in cell wall [39], we produced chitin-deprived *Candida* to demonstrate that both planktonic and sessile cells were not more or less susceptible to the SV-extract when cultivated with NKZ strongly suggesting that the cell wall chitin was not targeted by the SV-extract.

During the past decades a dramatic increase in invasive fungal infections have been documented, especially caused by different species belonging to the *Candida* genus. The development of new inhibitor of *Candida* biofilm is thus urgently needed. The currently used antifungal drugs have mostly been developed to target exponentially growing fungal cells and are poorly or not effective against biofilm structures. So, development of new inhibitors able to
inhibit biofilm formation and/or to disarticulate mature biofilm architecture represent
stimulating new orientations for the proposal of new antifungal therapies. In this context, the
SV-extract represents a very innovative and promising new plant-derivative antifungal product.
In particular, it is the first natural product, easy to produce at low price, able to prevent
adhesion, hyphal transition and subsequent pathogenic biofilm formation with very high
efficiency. Moreover, due to its mechanism of action that involves specific inhibition of
adhesion and hyphal-related genes, this antifungal plant extract may open the way to the
development new antifungal strategies.

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Conflict of interest: Patent. Composition with saponins. Madinier (Prêcheur) Isabelle, Géribaldi
(Starita) Mireille. Applicant Univ Nice Sophia Antipolis ETA (FR); CT Hospitalier Univ D (FR);
REFERENCES


FIGURES LEGENDS

**Table 1:** Forward and reverse primers used in real-time PCR for the reference genes

**Table 2:** *MIC* (minimal inhibitory concentration) was defined as the lowest concentration of the drugs (alone or in combination) that inhibited viability by 80% compared with that of drug-free wells. The values represent the majority of three independent tests. The FIC was calculated as the MIC of the combination divided by the MIC of each individual component extract.

†: The FIC index was then calculated as the sum of each component FIC in a combination and interpreted as either synergistic (≤0.5), indifferent (0.5–4.0) or antagonistic (≥4.0).

**Fig. 1:** SEM images of *C. albicans* ATCC 10231 biofilm formation on Thermanox™ Coverslips. (a, c) Controls, *C. albicans* morphology displayed yeast and hyphal cells. (b, d) SV-extract treatment, (B) lack of *C. albicans* adhesion, (d) morphology of SV-treated *C. albicans* at high magnification Bars, 100 μm (a-b) and 1 μm (c-d).

**Fig. 2:** Effect of SV extract on *C. albicans* adhesion to cells. *C. albicans* were pre-treated 24 h with 33.3% of SV or water acidified (control). For the assay, approximately 10^5 cells TR146 and 10^8 fungi were mixed and incubated on an orbital shaker at 37°C for 45 min. The mixture was stained with Calcofluor White and FUN® 1 for 30 min before observation under fluorescent microscope with 10x (a, b) and 63x (c, d) magnification. The percentage of cells with adherent *C. albicans* was determined by dividing the number of cells showing at least one adhering-fungi by the total number of adherent cells [e]. Results represent the average of three independent experiments ± SD. **p < 0.005 when compared with the SV untreated controls.
Fig. 3: Growth of *C. albicans* ATCC 10231 and 3 clinical strains in a YEP liquid medium after 4 and 24 h of SV-extract treatment compared to untreated control. Results were obtained by counting of total cells (yeast+hyphae) and represent the average of three independent experiments ± SD. *p < 0.05 or ‡p < 0.005 when compared with the SV untreated controls.

Fig. 4: Propidium iodide uptake assay. SV-extract do not alter cell wall permeability. *C. albicans* (ATCC 10231) planktonic cells (5×10^7 cells/ml) were treated with SV (33.3%) for 10, 20, 30, 40, 50, and 60 min. [a]: A panel of images illustrating the proportion of *C. albicans* permeabilized (in red) by SV extract after 60 min of incubation compared with the untreated control and positive control (Ethanol 70%). Magnification x10 and x63 in insert.

[b]: After treatment, the cells were washed by centrifugation, resuspended in PI (20 µM in PBS), and incubated for 15 min at 37°C. These were then transferred to a black 96-well plate for PI quantification in a fluorescent plate reader (Ex 535/Em 617).

Fig. 5: Effect of SV extract on expression of *C. albicans* genes after 4 h or 24 h of treatment. *C. albicans* cells were incubated in the absence (control) or presence (33.3%) of SV-extract in RPMI-1640 medium at 37°C for 4 or 24 h. Following incubation, expression of the indicated genes were determined by qRT-PCR. Expression level of each gene is displayed after normalization with internal control housekeeping gene *ACT1*. The histogram shows the relative expression fold change of genes by SV treatment with respect to the control. Results represent the average of three independent experiments ± SD. *p < 0.05 or ‡p < 0.005 when compared with the SV untreated controls.
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<tr>
<td></td>
<td>Reverse: TGCGAGAGATTGAGAAGAAGTTT</td>
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<tr>
<td>ALS1</td>
<td>Forward: TTCTCATGAATCAGCACTCCACAA</td>
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<tr>
<td></td>
<td>Reverse: CAGAATTTTCAACCATACTTGGTTTC</td>
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<tr>
<td>ALS3</td>
<td>Forward: CAACCTGGTTATTGAAACAAAAAC</td>
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<td></td>
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<td>Compound</td>
<td>Clinical strain Biofilm formation</td>
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DISCUSSION
La cavité orale est un écosystème complexe qui associe des éléments anatomiques et physiologiques variés : des structures fonctionnellement spécialisées (dents, langue, lèvres...), plusieurs types de revêtements épithélial-muqueux, un important réseau lymphatique et sanguin, un système glandulaire développé qui permet la production de salive. Un aspect remarquable de l’écosystème oral est la coexistence de tissus mous qui desquament et se renouvelent continuellement, et de tissus durs dont la destruction est irréversible. Cet écosystème subit des modifications et des contraintes permanentes dues notamment aux changements d’oxygénation, de température, aux apports en nutriments réguliers, au pH et aux flux salivaire et sulcalaire. La grande diversité de niches et ces fréquents changements conduisent à une très grande diversité de microorganismes, comprenant au sens large virus, bactéries, champignons et parasites, ainsi qu’à des interactions importantes entre les microorganismes et avec les cellules de l’hôte. De par sa position à l’entrée des voies digestives et respiratoires, la bouche a également un rôle important dans la défense de l’organisme contre les infections. Il est désormais reconnu qu’il existe un lien fort entre microbiome oral et santé générale. Toute altération de la flore orale a donc des conséquences qui peuvent aller du simple inconfort à des pathologies plus graves comme les candidoses, les caries, les parodontites, la dénutrition et les pneumonies d’inhalation. Il existe aussi un lien de causalité présumptif ou établi entre les parodontites et le diabète, les cancers des voies aérodigestives supérieures, des maladies auto-immunes comme la polyarthrite rhumatoïde, des pathologies cardiovasculaires (Aida et al., 2011; Gil-Montoya et al., 2015).

Les microorganismes s’organisent et interagissent entre eux au travers de la formation de structures complexes de type biofilms. Le développement de nouvelles techniques permettant de créer et de modéliser in vitro des biofilms représente donc une étape essentielle qui permet de simplifier les études visant à analyser leur structure, leur genèse, l’effet de substances actives d’origines variées, des méthodes de désinfection ou les interactions inter-espèces ou avec les cellules de l’hôte. Notre revue de la littérature (chapitre I) illustre pleinement que ce sujet est en pleine expansion et suscite un intérêt marqué tant au niveau académique que pour la validation de procédés industriels. Si l’on considère uniquement les biofilms mixtes bactéries-champignons, des modèles de biofilms contenant plus de 10 souches différentes (Thurnheer et al., 2016) ont été développés. Les supports utilisés ne sont plus seulement des supports inertes (plastique, résine, dents...) mais également des cellules ou
tissus généralement sous forme d’épithéliums reconstruits. Les techniques et méthodes d’analyse se sont également diversifiées permettant maintenant de visualiser et/ou quantifier le biofilm de manière précise et reproductible. Toutefois le développement de modèles de biofilm *in vitro* reste long, difficile et coûteux. De plus il ne permet d’intégrer que des microorganismes cultivables et élimine ainsi de nombreux microorganismes potentiellement responsables de diverses pathologies. L’essor de la modélisation mathématique de la formation de biofilm permet maintenant d’effectuer de nombreuses expériences difficilement réalisables en laboratoire pour des raisons de coût ou de faisabilité. Les constants développements effectués permettent de prendre en compte certaines complexités du biofilm et permettent déjà de simuler la résistance des biofilms à des agents antibotiques.


Les extraits bruts aqueux de ces deux sous-espèces ont été testés sur 4 souches de *C. albicans* : une souche de référence et 3 souches sauvages prélevées chez des patients souffrant de candidose orale. Il était important de vérifier l’activité sur des souches sauvages qui ont potentiellement acquis des résistances et auraient ainsi un profil différent de la souche de référence. Les extraits ont tous les deux montré une activité inhibitrice de la conversion levure-hyphe de ce champignon sans inhiber sa croissance ni celle des bactéries endogènes de la muqueuse buccale. Ce type d’activité est particulièrement adapté pour un traitement spécifique qui ciblerait uniquement la forme virulente de *C. albicans* sans déséquilibrer la flore commensale. Bloquer les facteurs de virulence du champignon sans altérer sa viabilité est
d’ailleurs considéré comme un nouveau paradigme dans le domaine des antifongiques (Gauwerky et al., 2009).

Un autre facteur de virulence du champignon est sa capacité à adhérer aux surfaces puis à former des biofilms. Afin de déterminer si la présence d’extrait limitait la capacité d’adhésion de *C. albicans*, celui-ci a été incubé en présence d’extrait végétal pendant 18h dans des conditions lui permettant de former des biofilms. Des études préliminaires réalisées au laboratoire ont montré que la quantité de biofilm formé par ces souches était maximale à 18h avant de diminuer progressivement. Les résultats ont démontré que les extraits limitaient l’adhésion du champignon à plus 95% en comparaison avec un contrôle non traité. Cette propriété est donc intéressante à titre préventif comme par exemple pour éviter la prolifération du champignon sur les prothèses dentaires. Cependant, il était très intéressant d’étudier également l’effet de *S. virgaurea* sur des biofilms déjà constitués, qui reflètent davantage la situation réelle de l’action d’un antifongique oral. Nous avons ainsi testé les extraits sur des biofilms déjà formés et « âgés » de 18h. En comparaison avec des biofilms non traités, les pourcentages de réduction de biofilm, après traitement avec les extraits, étaient compris entre 76.26% et 91.91%. Nos travaux établissent donc pour la première fois que l’extrait aqueux de *S. virgaurea* présente une activité antifongique puissante, inhibitrice des premières étapes de la formation du biofilm à *C. albicans* (adhésion) et également capable de détruire un biofilm mature déjà formé. Ces observations laissent envisager une action complète, à la fois préventive et curative.

Suite à cette première étude, nous avons souhaité mieux comprendre sur quelles bases moléculaires reposait le mécanisme d’action de l’extrait végétal sur *C. albicans*. L’activité des deux extraits étant similaire, nous n’avons considéré dans ces études que la sous-espèce alpestris. Ce travail a été réalisé en collaboration avec l’équipe « Infection & Immunity Research Group Lead » dirigée par le Pr. Gordon Ramage à Glasgow (Ecosse) qui a publié plusieurs articles sur l’effet de composés naturels sur *C. albicans*. L’étude de composés naturels, et particulièrement d’extraits végétaux, présente des particularités et notamment en raison du fait que la composition chimique n’est pas parfaitement définie. J’ai ainsi eu l’opportunité de réaliser une mobilité de formation d’une durée de deux semaines dans cette équipe afin d’acquérir de nouvelles techniques mais également de découvrir la recherche à l’international. Les travaux initiés au cours de cette mobilité, et poursuivis en France, ont permis notamment
d’exclure que la présence de détergents (saponines) dans l’extrait n’exerce pas un effet lytique sur les levures en perméabilisant leur membrane. Les détergents sont en effet utilisés en laboratoire pour la lyse des membranes cellulaires. Nous avons également pu exclure que la chitine qui est un constituant essentiel des parois de champignons, soit la cible de l’action de l’extrait. En effet, la chitine est une des cibles des antifongiques (azolés) disponibles sur le marché, et nous avons vérifiés que lorsque C. albicans est dépourvu de chitine il reste sensible à l’extrait végétal.

Nous avons ensuite étudié par PCR quantitatives en temps réel, l’expression des principaux gènes impliqués dans le changement de morphologie (HWP1, ECE1, HGC1), l’adhésion (ALS) et la formation de biofilm (ALS, HWP1, ECE1, HGC1)(Fan et al., 2013; Finkel & Mitchell, 2010). Nous avons également mesuré l’expression d’un gène codant pour une protéine exportable (SAP6). Les résultats démontrent que l’extrait induit une répression significative de l’expression de tous ces gènes sauf d’ALS1, qui code pour une glycoprotéine de la paroi. La plupart des gènes étudiés ici est étroitement liée à la forme hyphé du champignon, or étant donné que l’extrait de S. virgaurea inhibe la conversion de levure en hyphé, les résultats obtenus en PCR quantitative semblent cohérents. Ils démontrent de manière claire que l’extrait agit à un niveau transcriptionnel sur l’expression de gènes clés pour l’évolution morphologique du champignon sans pour autant agir d’une manière lytique sur la viabilité de C. albicans. Bien qu’ils ne permettent pas de conclure sur le mode d’action de l’extrait, ces résultats sont nouveaux car ils permettent de proposer un nouveau modèle d’action antifongique d’un composé naturel sur un biofilm pathogène.

Dans ce contexte, il nous a semblé pertinent d’étudier l’effet de l’extrait végétal combiné à des antifongiques locaux prescrits en première intention dans les candidoses orales (amphotéricine B, nystatine, miconazole) et à un bain de bouche contenant de la chlohexidine sur la formation d’un biofilm de C. albicans. La chlohexidine est un antiseptique à large spectre qui possède une action fongicide sur les Candida (Fathilah et al., 2012). Compte tenu du mécanisme d’action de ces antifongiques, clairement différent de celui, proposé ici, pour l’extrait de S. virgaurea, une action synergique entre ces agents semblait probable. Les tests ont été réalisés sur une souche de référence ainsi que sur une souche sauvage (résultats non publiés). Pour ces deux souches, les résultats ont montré une action synergique significative entre l’extrait et le miconazole, la nystatine et le bain de bouche. Par contre, l’extrait combiné

La toxicité de l’extrait végétal constitue un paramètre important à étudier si l’on souhaite pouvoir utiliser ce produit naturel dans une formulation thérapeutique. Des expériences ont été réalisées sur des cellules humaines en culture primaire de cellules orales non kératinisées. Pour cela, nous avons utilisé le rouge neutre qui est une sonde vitale qui rentre dans les cellules par un mécanisme de diffusion passive et se concentre dans les lysosomes. L’accumulation intracellulaire du rouge neutre dépend de la capacité de la cellule à maintenir des gradients de pH grâce à la production d’ATP. La concentration utilisée dans ce manuscrit (33%) est toxique pour les cellules. Par contre à une concentration inférieure (1%) aucune toxicité n’a été observée. Cette concentration est plus en adéquation avec les concentrations en principes actifs observées dans les solutions commerciales.

Tous les travaux et essais entrepris au cours de ce travail de doctorat n’ont pas encore fait l’objet d’articles scientifiques. Ainsi il nous semblait intéressant de vérifier l’effet de l’extrait végétal sur un biofilm plus complexe composé de bactéries orales et du champignon *C. albicans*. Pour cela, un travail entrepris parallèlement à l’étude de *S. virgaurea* avait pour objectif de créer un biofilm non pathogène composé de bactéries représentant la flore naturellement présente au niveau du palais ou de l’intérieur des joues. De plus les problématiques de bouche sèche et de candidoses étant aggravées par l’âge, il nous a semblé important de tenir compte des bactéries présentes dans la flore orale des personnes âgées. Les recherches bibliographiques (Preza et al. 2009; Aas et al. 2005; Simon-Soro et al. 2013) ont permis de mettre en évidence l’implication de nombreuses souches bactériennes. Une sélection a été faite en éliminant les bactéries qui ont des conditions de culture trop exigeantes ou qui ne forment
pas de biofilm. Ainsi les bactéries suivantes ont été choisies : *Streptococcus salivarius*, *Fusobacterium nucleatum*, *Rothia mucilaginosa* et *Gemella haemolysans*. Parmi ces microorganismes certains sont anaérobies stricts (*F. nucleatum*), anaérobies facultatifs (*S. salivarius* et *G. haemolysans*) ou aérobies (*R. mucilaginosa* et *C. albicans*). Une mise au point a donc été nécessaire afin de réussir à faire pousser ces cinq microorganismes ensemble. Comme certaines espèces comme *S. salivarius* ont tendance à prendre le dessus sur les autres espèces, différentes mises au point concernant la proportion de bactéries à introduire, et à quel moment, ont été réalisées afin d’obtenir un biofilm où toutes les espèces introduites au départ étaient encore présentes à l’arrêt de la culture. Il est à noter que, dans ce modèle de biofilm *in vitro* *C. albicans* est dominant à la fois sous forme d’hyphes et levures. A ce jour, uniquement des observations morphologiques ont été réalisées. Elles ont confirmé l’effet de l’extrait de *S. virgaurea* sur l’inhibition de conversion de levure à hyphes du champignon, y compris lorsqu’il est inclus dans un biofilm mixte.

De nombreuses molécules sont capables de provoquer un changement de morphologie chez *C. albicans* comme le farnesol, des molécules sécrétées par les bactéries ou encore des molécules lipidiques (Shareck & Belhumeur, 2011). Mais actuellement seulement quelques études ont montré des effets similaires avec des composés naturels (Tsang et al., 2012; Vediyappan et al., 2013). Ainsi, l’étude de Vediyappan et al. (2013) a prouvé l’effet de saponines triterpénoides issues de feuilles de *Gymnema sylvestre*, une plante médicinale, sur la morphologie du champignon. Tandis que Tsang et al. (2012) ont montré que la purpurine, un pigment rouge issue des racines de la plante *Rubia tinctorum L.*, possède une activité à la fois sur la morphologie et la formation de biofilms par *C. albicans*. Cependant, dans leur étude, la purpurine n’a pas permis de diminuer efficacement la viabilité d’un biofilm déjà formé, contrairement à notre extrait.

Actuellement *C. albicans* est encore l’espèce majoritairement isolée au niveau de la cavité orale. Cependant des espèces telles que *C. parapsilosis*, *C. tropicalis* et *C. glabrata* sont de plus en plus fréquemment retrouvées dans les prélèvements. Parmi elles, seule *C. tropicalis* est capable de former de vrais hyphes. Néanmoins elles ont toutes la capacité de former des biofilms. Une étude parue récemment (Araújo et al., 2017) vient de montrer que les gènes
impliqués dans la formation de biofilms par ces quatre souches n’étaient pas exactement les mêmes. Ainsi par exemple la famille des *Agglutinin-Like Sequences* n’interviendrait pas dans le processus d’adhésion de *C. glabrata*. Il serait donc intéressant de confirmer les propriétés anti-biofilm de l’extrait de *S. virgaurea* sur d’autres espèces de *Candida*. 
CONCLUSION

A travers ces différentes études, nous avons montré que l’extrait végétal aqueux de S. virgaurea agissait sur les quatre principaux facteurs de virulence du champignon C. albicans qui sont : le changement de morphologie de levure à hyphé, ses capacités d’adhésion à différents substrats et tissus, la formation de biofilms et la production d’enzymes hydrolytiques et antioxydantes. Toutefois, S. virgaurea n’a pas d’effet sur la croissance et la viabilité du champignon.

Au niveau moléculaire, l’extrait induit une répression significative de l’expression de plusieurs gènes impliqués dans le changement de morphologie (HWP1, ECE1, HGC1), l’adhésion (ALS) et la formation de biofilm (ALS, HWP1, ECE1, HGC1). Au niveau structural, l’extrait n’altère ni la paroi ni la membrane du champignon.

D’autre part, l’extrait est actif uniquement contre les champignons mais n’affecte pas la flore bactérienne et ainsi ne risque pas de déséquilibrer le biofilm endogène. De plus, une action synergique contre la formation du biofilm a été observée lorsque l’extrait était combiné à certains antifongiques topiques.

Ces différentes propriétés font de l’extrait de S. virgaurea un candidat intéressant pour le développement de nouveaux traitements antifongiques locaux contre les candidoses cutanéomuqueuses.
ANNEXES
ANNEXE I- Les bains de bouche antiseptiques pourraient augmenter la xérostomie chez les patients polymédiqués

Plus de 2 % de la population souffre de sécheresse buccale ou xérostomie, qui atteint surtout les femmes et les personnes âgées. La sécheresse buccale survient typiquement dans le cadre des maladies auto-immunes, avec un syndrome sec et une destruction irréversibles des cellules qui sécrètent la salive, les larmes et les sécrétions muqueuses. Mais actuellement, la majorité des sécheresses buccales est due à la prise de certains médicaments qui inhibent la sécrétion salivaire, sans détruire les cellules qui sécrètent la salive. De plus, un risque de sécheresse buccale existe dès qu'un malade prend 4 ou 5 médicaments différents par jour, quels que soient ces médicaments. Or il est reconnu que la sécheresse buccale est un facteur favorisant les candidoses orales. Afin d’éliminer ces candidoses des antifongiques sont prescrits et bien souvent l’utilisation de bains de bouche antiseptiques est aussi recommandée. Les bains de bouche usuellement prescrits peuvent contenir divers principes actifs et excipients, notamment des antiseptiques à large spectre, des détergents de synthèse ou des principes actifs agissant contre les symptômes des infections ou inflammations bucco-dentaires. Ainsi, les ammoniums quaternaires, l’héxétidine, la chlorhexidine, l’éthanol, et de nombreuses huiles essentielles inhibent le développement ou détruisent les bactéries du biofilm oral en même temps que les Candida.

Afin de mieux cibler les facteurs de risque conduisant à une sécheresse buccale, une étude clinique observationnelle a été menée au CHU de Nice. Bien que l’objectif principal fût de connaître l’impact des médicaments sur la sécheresse buccale, d’autres critères ont été intégrés aux questionnaires comme par exemple les habitudes dans le domaine alimentaire et les produits d’hygiène bucco-dentaire utilisés. Ainsi, l’étude suivante avait pour objectif de voir si l’utilisation régulière, et pendant plus de deux semaines, de bains de bouche antiseptiques augmentait la sécheresse buccale chez les patients polymédiqués. Cette étude a été réalisée sur 120 patients suivis à l’hôpital et prenant plus de 4 médicaments par jour, répartis en deux groupes : 60 patients d’âge moyen adressés par divers services (âge moyen 44,0 ± 8,7 ans) et 60 patients plus âgés hospitalisés dans un service de gérontologie (âge moyen 84,5 ± 8,0 ans). Les résultats de cette étude ont montré que l’utilisation régulière, et pendant plus de deux semaines, de bains de bouche antiseptiques augmentait la sécheresse buccale chez les patients
polymédiqués. Il est donc nécessaire de trouver des alternatives aux bains de bouche antiseptiques classiquement utilisés avec pour objectif de limiter les formes pathogènes et réduire la biomasse sans pour autant éradiquer la flore orale, qui est nécessaire à la santé bucco-dentaire.
ANNEXE I

Antiseptic mouthwashes could worsen xerostomia in patients taking polypharmacy

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ORIGINAL ARTICLE

Antiseptic mouthwashes could worsen xerostomia in patients taking polypharmacy

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Abstract

Objective. Polypharmacy is a common cause of xerostomia. This study aimed to investigate whether xerostomia could be an adverse drug event of mouthwashes, when they are used for longer than 2 weeks by patients taking polypharmacy. Materials and methods. This cross-sectional observational study included 120 hospitalized patients (60 middle-aged and 60 elderly patients), taking polypharmacy (24 drugs daily) and at risk of drug-induced xerostomia. Xerostomia was assessed by questioning participants. Results. A total of 62.5% of patients complained of xerostomia. In the middle-aged group (mean age = 44.0 (8.7) years; 35.0% women) xerostomia seemed independently associated to mouthwashes, at the limit of significance (OR = 5.00, 95% CI = 0.99–25.3, p = 0.052). Active principles in mouthwashes were mainly quaternary ammonium compounds (91.9%). Mouthwashes may disturb the healthy balance of the biofilm moisturizing the oral mucosa. The biofilm contains mucus, salivary glycoproteins with oligosaccharides side chains able to sequester water and endogenous bacteria surrounded by a glyocalyx. Oral bacteria are fully susceptible to quaternary ammonium (chlorhexidine, hexetidine, cetylpyridinium chloride) and to other antiseptics used in mouthwashes, such as betain, resorcin, triclosan, essential oils and alcohol. However, caregivers currently recommend such dental plaque control products to patients suffering from xerostomia in order to reduce the risk of caries and periodontitis. Conclusion. This study is the first report that use of antiseptic mouthwashes for more than 2 weeks could worsen xerostomia in patients taking polypharmacy. Oral care protocols should avoid this iatrogenic practice, particularly when xerostomia alters the quality-of-life and worsens malnutrition.

Key Words: adverse drug event, biofilm, iatrogenic disease, xerostomia

Introduction

Alterations of saliva physiology include xerostomia, hyposalivation and altered saliva composition. Xerostomia is a subjective feeling of oral dryness. Mouth dryness is a term regarding dryness in the oral cavity, objectively diagnosed by for instance a dental mouth mirror sticking to the buccal mucosa of the cheek due to dryness. Xerostomia varies substantially between individuals [1,2]. According to Glorie et al. [3], dry mouth is not necessarily related to decreased salivary flow. Some patients experience a feeling of oral dryness, despite seemingly normal, objectively measured levels of saliva secretion [4], whereas others do not complain about dry mouth, despite objectively diagnosed hyposalivation [5]. However, most individuals experience a sensation of oral dryness when their salivary output is less than about half of the normal output in health, but with great variation [2].

The prevalence of xerostomia reaches 10–20% in the general population, primarily in women, and 50% in the elderly [1,6]. Symptoms of mouth dryness include a sensation of thirst, soreness and dryness of the lips and oral mucosa [7,8]. It is associated with an increased risk of caries, oral candidiasis, removable denture intolerance, taste disturbance and pneumonia, with a subsequent risk of eating difficulties.
choking, loss of appetite and malnutrition [6,8–10]. Treatment protocols may include: general and local hydration, saliva substitutes and lubricants, central (pilocarpine, cevimeline) and local (sugar-free chewing-gums and candies) secretagogues, antifungal treatment, topical analgesics before meals, suppression or replacement of xerogenic drugs, dietary modification and/or dietary supplements and oral hygiene reinforced with antiseptic oral care products. No treatment or combination treatment is fully satisfactory in combating xerostomia [1,2,11–13].

Common causes of xerostomia include dehydration, autoimmune (Sjögren’s syndrome) and endocrine (diabetes) diseases, hepatitis C virus (HCV) infection and radiation therapy of head and neck tumors [12,14,15]. Many commonly prescribed medications are associated with the feeling of mouth dryness, despite normal saliva production [1,2]. In the elderly, the main cause of xerostomia is medication and, in particular, the use of ≥4–5 drugs per day [2]. More than 500 medications are associated with xerostomia, with special emphasis placed on psychotropic drugs (anticholinergic drugs/atropinics, neuroleptics, tricyclic antidepressants, antipsychotics, benzodiazepines), followed by anti-hypertensives, diuretics, anti-neoepastics, opiates, bronchodilators, proton pump inhibitors, antihistamines and others [6–9,16,17]. However, few medications except for true anticholinergic drugs have been demonstrated to affect salivary function and polypharmacy remains the most prevalent cause of mouth dryness [4,14,16]. Actually, xerostomia is not listed either among indications of antiseptic mouthwashes or among their side-effects. However, we observed that, in all the previous series investigating polypharmacy and xerostomia, no attention had ever been paid to topical medications such as antiseptic mouthwashes [2,16,17].

Antiseptic mouthwashes are efficient against bacterial species colonizing the oral biofilm and their use must not exceed 2 weeks [18]. However, misuse of antiseptic mouthwashes for longer than a 2-week period is frequently reported by patients, with the risk to unbalance the oral bacterial biofilm coating oral mucosa. Besides, the biofilm contains salivary and bacterial glycoproteins, the primary function of which are to retain water [19].

Due to the absence of consensual treatment for xerostomia [2,6], it might be necessary to combat iatrogenic factors. We hypothesized that, in addition to low saliva secretion induced by systemic drugs, mouth dryness could be worsened by biofilm alterations induced by local antimicrobial medications.

The objective of the present work was to investigate the link between xerostomia and the use of antiseptic mouthwashes for a duration of time longer than 2 weeks, in patients taking polypharmacy.

Materials and methods

Study design and patients

This cross-sectional observational study included 120 patients from Nice University Hospital: 60 middle-aged patients (Mean age = 44 (8.7)) from the Department of Infectious Diseases and 60 elderly patients (Mean age = 84.5 (8.0)) from the Department of Geriatrics. Patients recruited in the Infectious Diseases Department suffered mainly from human immunodeficiency virus (HIV) infection or HCV chronic hepatitis. Patients recruited in the Geriatrics Department suffered from various cardiovascular, endocrine, psychiatric and other chronic disorders. Both of these populations had a high probability to be given polypharmacy. Such patients frequently complained of xerostomia and exhibited mucosal dryness.

We enrolled consecutive patients seen at the Department of Dentistry for routine dental examination. All participants taking four drugs or more daily were eligible for the study and there was no exclusion criterion. All participants gave written informed consent. The study was approved by the Clinical Research Department of Nice University Hospital and by the local Ethics Committee (May 6, 2013; registration number 20100108).

Data collection

The main variable was subjective xerostomia. According to the protocol described by Thomson et al. [20], participants were asked the question ‘How often does your mouth feel dry?’ with four possible answers: ‘Always’, ‘Frequently’, ‘Occasionally’ or ‘Never’. Patients who answered ‘Always’ or ‘Frequently’ were considered as suffering from xerostomia.

Other data were obtained from patient interviews, routine dental examinations and hospital medical files. Collected data includes gender, age and common known associations with xerostomia: Sjögren’s disease, dehydration, head and neck radiation therapy, tobacco use, previous or current illicit drug addiction, HIV or HCV infection, depressive disorders, diabetes mellitus, Parkinson’s disease, number of drugs taken per day and loss of appetite. Recent non-voluntary weight loss and body mass index (BMI: [mass in kg]/[height in m]²) were also noted. Xerostomia and use of antiseptic mouthwashes for longer than 2 weeks duration were recorded. Routine oral parameters were charted: oral candidiasis (denture stomatitis, acute stomatitis, erythematous stomatitis), oral pain, oral ulcerations, active dental caries, edentulousness, removable denture(s) and masticatory ability [21].

Patients’ medications were also recorded. Each psychotropic agent was categorized as follows: muscarinic antagonists (true anticholinergic/atropinic
drugs), adrenergic alpha-antagonists, opioid agonists, serotonin 5-HT2 blockers, histamine H1 antagonists, dopamine D2 receptors blockers or GABA-A receptor agonists. Some were classiﬁed in more than one category; for instance, risperidone is a selective blocker of dopamine D2 receptors and serotonin 5-HT2 receptors and it was attributed to both categories. Other drugs were charted as follows: paracetamol, glucocorticoids, antibacterial agents, antifungal agents, anti-HIV agents, diuretics, adrenergic beta-blockers, angiotensin-converting enzyme inhibitors, sodium potassium pump inhibitors, iron supplements, calcium channel blockers, platelet aggregation inhibitors, coumarin anticoagulants, heparin, proton pump inhibitors, anti-diabetic agents, etc.

Data analysis

Analysis was performed separately for the middle-aged patients group and for the elderly patients group, using SAS statistical package, version 9.1.3 (SAS Institute, Inc., Cary, NC). In univariate analysis, the association between xerostomia and quantitative parameters were assessed using Student’s t-test or Wilcoxon test if Student’s t-test hypothesis was not veriﬁed. Association between xerostomia and qualitative variables were assessed using the chi-square test or Fisher’s exact test in case of small expected frequencies.

Multivariate analysis was performed using logistic regression. The analysis was adjusted on risk factors known to be associated with xerostomia (‘woman’, ‘number of drugs taken per day’ and ‘use of psychotropic drugs’) [1,2]. In addition, the variables associated with p < 0.1 in the univariate analysis were included in the multivariate model. Statistical signiﬁcance was accepted at 5% (p ≤ 0.05).

Results

Patients of both groups were heavily medicated with up to 18 drugs per day. Seventy-ﬁve out of the 120 patients (62.5%) suffered from subjective xerostomia and 37 patients (30.8%) reported regular use of antiseptic mouthwashes. They used them once or more daily at home for more than 2 months and they continued this habit during their hospitalization. Most of the antiseptic mouthwashes contained quaternary ammonium compounds (34/37: 91.9%): chlorhexidine gluconate (n = 19, combined with chlorobutanol, alcohol and levomenthol), hexetidine (n = 12, combined with alcohol and menthol), cetylpovidinium chloride (n = 3, combined with chlorobutanol, eugenol, menthol and castor oil). Other mouthwashes contained sodium bicarbonate (n = 1), alcohol and anethole combined with other essential oils (mint, cinnamon, clove and benzoin) (n = 1) and salicylic acid combined with alcohol, levomenthol, resorcinol and veratrol (n = 1). No patient reported the use of antimicrobial oral care products speciﬁcally designed for daily oral hygiene. For the inclusive patients, no element in favour of dehydration at the clinical or biological level was noted in the medical record. In this study, we observed only denture stomatitis and no acute or erythematous stomatitis. However, we did not make an oral sample in search of Candida.

The two groups of patients are described in Tables I and II. In the group of middle-aged patients, risks factors for xerostomia were as follows: younger age (42.5 (9.4) years vs 46.5 (6.9) years; p = 0.08), woman (46.0% vs 17.4%; p = 0.024), use of antiseptic mouthwashes for a duration of time longer than 2 weeks (43.2% vs 17.4%; p = 0.039), tobacco use (83.8% vs 60.9%; p = 0.046) and use of GABA treatment (43.2% vs 17.4%; p = 0.039). In the group of elderly patients, risks factors for xerostomia were as follows: number of drugs taken per day (9.0 (2.9) vs 6.6 (3.2); p = 0.005), use of sodium potassium pump inhibitors (36.4% vs 13.6%; p = 0.055) and use of psychotropic drugs (57.9% vs 22.7%; p = 0.008).

In the group of middle-aged patients, multivariate analysis showed an association between xerostomia and the variable ‘use of antiseptic mouthwashes’, at the limit of signiﬁcance (adjusted odds ratio (OR) = 5.00, 95% CI = 0.99–25.3; p = 0.052) (Table III). However, in the group of elderly patients, the association between xerostomia and the variable ‘use of antiseptic mouthwashes’ was not statistically significant (OR = 1.70, 95% CI = 0.44–6.62; p = 0.44).

In the younger population, the multivariate model included, in addition to the forced variables cited above, the variable ‘age’ (year), ‘tobacco use’ (yes/no) and ‘use of GABA treatment’ (yes/no). The variable ‘use of GABA treatment’ was no longer associated to xerostomia after adjustment (p = 0.53) and did not modify the association between ‘use of antiseptic mouthwashes’ and xerostomia, therefore the variable was removed from the ﬁnal model. As previously described, we observed in younger patients an association between xerostomia and female gender or tobacco. In this series, a younger age was also associated to xerostomia.

In the group of elderly patients, multivariate analysis conﬁrmed a signiﬁcant association between xerostomia and the number of drugs taken per day. The multivariate model included, in addition to the forced variables, the variable ‘use of sodium potassium pump inhibitor’ treatment. This variable was no longer associated to xerostomia after adjustment (p = 0.28) and did not modify the association between ‘use of antiseptic mouthwashes’ and xerostomia, therefore it was removed from the ﬁnal model (Table III). In elderly patients we only observed a tendency of association between xerostomia and psychotropic drugs consumption (Table III).
Table I. Description of the population included in the study.

<table>
<thead>
<tr>
<th></th>
<th>Middle-aged group (n = 60)</th>
<th>Elderly group (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years</td>
<td>44.0 (8.7)</td>
<td>84.5 (8.0)</td>
</tr>
<tr>
<td>Women</td>
<td>21 (35.0)</td>
<td>43 (71.7)</td>
</tr>
<tr>
<td>Tobacco use</td>
<td>45 (75.0)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Previous or current illicit drug addiction</td>
<td>26 (43.3)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>HIV infection</td>
<td>41 (68.3)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>HCV infection</td>
<td>41 (68.3)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Depressive disorders</td>
<td>29 (48.3)</td>
<td>13 (21.7)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (3.3)</td>
<td>10 (16.7)</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>0</td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>0</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
<td>0</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Dehydration</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Head and neck radiation therapy</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mean number of drugs taken per day</td>
<td>5.2 (1.2)</td>
<td>8.1 (3.2)</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>22 (36.7)</td>
<td>28 (46.7)</td>
</tr>
<tr>
<td>Recent non-voluntary weight loss</td>
<td>28 (46.7)</td>
<td>37 (61.7)</td>
</tr>
<tr>
<td>Mean Body Mass Index (BMI), kg/m²</td>
<td>22.8 (4.1)</td>
<td>23.6 (4.5)</td>
</tr>
<tr>
<td>Subjective xerostomia</td>
<td>37 (61.7)</td>
<td>38 (63.3)</td>
</tr>
<tr>
<td>Use of antiseptic mouthwashes &gt;2 weeks</td>
<td>20 (33.3)</td>
<td>17 (18.3)</td>
</tr>
<tr>
<td>Oral candidiasis</td>
<td>8 (13.3)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>Oral pain</td>
<td>13 (21.7)</td>
<td>12 (20.0)</td>
</tr>
<tr>
<td>Oral ulcerations</td>
<td>4 (6.7)</td>
<td>4 (6.7)</td>
</tr>
<tr>
<td>Active dental cariesa</td>
<td>25 (49.0)</td>
<td>1 (2.7)</td>
</tr>
<tr>
<td>Edentulousness (no residual tooth)</td>
<td>9 (15.0)</td>
<td>23 (38.3)</td>
</tr>
<tr>
<td>Removable denture(s)</td>
<td>27 (45.0)</td>
<td>25 (41.7)</td>
</tr>
<tr>
<td>Mean masticatory ability, b %</td>
<td>50.9 (31.5)</td>
<td>22.8 (30.6)</td>
</tr>
</tbody>
</table>

Results are expressed as mean (standard deviation) or number (%).

- The percentage of active dental caries was calculated in dentate patients only (51 younger and 37 elderly patients).
- Masticatory ability, expressed as a percentage, was recorded without removable dentures: an index to quantify the couples of antagonistic teeth (100%: 32 healthy teeth; 0%: no couple of antagonistic teeth).

Discussion

This study showed that, in a population of hospitalized adults taking polypharmacy (mean age = 44), the regular use of antiseptic mouthwashes was independently associated to xerostomia. Despite a high prevalence of xerostomia in patients who are administered polypharmacy (62.5% in the present series of 120 subjects), antiseptic mouthwashes had never been included in the list of the drugs associated with xerostomia. Apart from antiseptic mouthwashes, in the group of middle-aged patients we could not attribute xerostomia to any specific medication or pharmacodynamic pathway. Only 14 patients were given true anti-cholinergic drug (muscarinic antagonists), which was insufficient to correlate these drugs to xerostomia. These results are in line with those of previous authors with larger series, who did not evidence any association between xerostomia and xerogenic medications, other than true anti-muscarinic medications [3,16].

Many risk factors may be involved in xerostomia and the present study faced several difficulties. First, it is difficult to validly and reliably assess the degree of xerostomia [20,22]. Second, drugs classification is complex and we proposed a coding system based on pharmacodynamic rather than therapeutic classes. Finally, the present study was a cross-sectional study and causality between the use of mouthwashes and secondary mouth dryness or conversely the feeling of mouth dryness and secondary use of mouthwashes can be debated. However, a microbiological approach would favor the first hypothesis. Actually, antiseptic mouthwashes efficiently fight bacterial proliferation. The impact of antiseptic mouthwashes on mouth dryness could be explained by an unbalance of the endogenous microbial biofilm coating the oral mucosa. Eliasson et al. [23] showed that a feeling of xerostomia was related to a deficiency in minor salivary gland secretions. Mucin-rich saliva moistens the oral mucosal surfaces more efficiently than the salivary flows produced during meals by the parotid, submandibular and sublingual glands. Salivary mucins are glycoproteins with large oligosaccharides side chains able to sequester water and lubricate the oral mucosa [11]. They contribute to the extracellular matrix of the oral biofilm [24]. However, the healthy biofilm is also composed of bacteria, such as Streptococcus salivarius, Streptococcus mitis, Rothia mucilaginosa, Gemella haemolysans and Fusobacterium nucleatum, themselves enveloped by glycoprotein capsules or glycocalyx able to retain water [25,26]. These bacterial species are fully susceptible in vitro to antiseptics commonly used in oral care products, including quaternary ammonium, betain, resorc, triclosan, essential oils and alcohol [6]. Fluorides also display antimicrobial properties against cariogenic and other viridans streptococci [27,28]. The unbalanced bacterial biofilm can in turn be colonized by Candida albicans [29], which is able to produce secretory aspartyl proteinases (Sap2), specifically known to disrupt mucins [30]. Use of antiseptic mouthwashes for a duration of time of more than 2 weeks could, thus, initiate or worsen mouth dryness by a direct action on the oral biofilm. Considering these preliminary results, microbial biofilm analysis would help to understand whether use of
Table II. Drug treatment of the population included in the study.

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Middle-aged group (n = 60)</th>
<th>Elderly group (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscarinic antagonists</td>
<td>7 (11.7)</td>
<td>7 (11.7)</td>
</tr>
<tr>
<td>Adrenergic alpha-antagonists</td>
<td>11 (18.3)</td>
<td>14 (23.3)</td>
</tr>
<tr>
<td>Opioid agonists</td>
<td>21 (35.0)</td>
<td>15 (25.0)</td>
</tr>
<tr>
<td>Serotonin 2 (5-hydroxytryptamine) 2, 5-HT2) blockers</td>
<td>5 (8.3)</td>
<td>9 (15)</td>
</tr>
<tr>
<td>Histamine 1 (H1) inhibitors</td>
<td>11 (18.3)</td>
<td>10 (16.7)</td>
</tr>
<tr>
<td>Dopamin 2 (D2) receptors blockers</td>
<td>7 (11.7)</td>
<td>7 (11.7)</td>
</tr>
<tr>
<td>Gamma-aminobutyric acid -A (GABA-A) receptor agonists</td>
<td>20 (33.3)</td>
<td>29 (48.3)</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>2 (3.3)</td>
<td>37 (61.7)</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>3 (5.0)</td>
<td>4 (6.7)</td>
</tr>
<tr>
<td>Antibacterial agents</td>
<td>8 (13.3)</td>
<td>4 (6.7)</td>
</tr>
<tr>
<td>Antifungal agents</td>
<td>4 (6.7)</td>
<td>3 (5.0)</td>
</tr>
<tr>
<td>Anti-HIV agents (non-nucleoside reverse transcriptase inhibitors, NNRTI)</td>
<td>13 (21.7)</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HIV agents (nucleotide reverse transcriptase inhibitors, NRTI)</td>
<td>37 (61.7)</td>
<td>0</td>
</tr>
<tr>
<td>Anti-HIV agents (protease inhibitors, PI)</td>
<td>21 (35.0)</td>
<td>0</td>
</tr>
<tr>
<td>Diuretics</td>
<td>2 (3.3)</td>
<td>21 (35.0)</td>
</tr>
<tr>
<td>Adrenergic 1 beta-blockers</td>
<td>5 (8.3)</td>
<td>15 (25.0)</td>
</tr>
<tr>
<td>Angiotensin converting enzyme (ACE) inhibitors</td>
<td>0</td>
<td>25 (41.7)</td>
</tr>
<tr>
<td>Sodium potassium pump inhibitors (SPPI)</td>
<td>0</td>
<td>17 (28.3)</td>
</tr>
<tr>
<td>Iron supplements</td>
<td>1 (1.7)</td>
<td>9 (15.0)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>0</td>
<td>13 (21.7)</td>
</tr>
<tr>
<td>Platelet aggregation inhibitors</td>
<td>2 (3.3)</td>
<td>13 (21.7)</td>
</tr>
<tr>
<td>Coumarin anticoagulants</td>
<td>2 (3.3)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>Heparin</td>
<td>0</td>
<td>9 (15.0)</td>
</tr>
<tr>
<td>Proton pump inhibitors (PPI)</td>
<td>1 (1.7)</td>
<td>18 (30.0)</td>
</tr>
<tr>
<td>Antidiabetic agents</td>
<td>2 (3.3)</td>
<td>10 (16.7)</td>
</tr>
<tr>
<td>Psychotropic drugs*</td>
<td>17 (28.3)</td>
<td>27 (45.0)</td>
</tr>
</tbody>
</table>

Results are expressed as number (%).
*Psychotropic drugs: patients receiving muscarinic antagonists, adrenergic alpha-antagonists, opioid agonists, 5-HT2 blockers, H1 inhibitors, D2 receptor blockers and/or GABA-A receptor agonists.

mouthwashes exacerbates xerostomia among persons taking polypharmacy.

This study showed that hospital stay did not prevent tobacco smoking and confirmed that it was a risk factor of xerostomia [31]. In parallel with the present results, smoking could have systemic and topical effects on xerostomia. It is possible that the hospitalization contributes to reduce the tobacco consumption but we have no quantified data allowing us to compare before and after. The results are given according to the answers of patients and maybe under-estimated in particular for the sick of the Department of Geriatrics.

Among elderly people (mean age = 85), we did not find a significant link between complaints of mouth dryness and the regular use of antiseptic mouthwashes. The first explanation was that elderly patients were more heavily medicated than younger patients (average = 8.2 drugs/day vs 5.2 drugs/day) and the risk of xerostomia increases with the number of drugs taken daily [2]. However, other causes of mouth dryness among elderly could have been taken in account, such as age-related saliva alterations and mouth breathing [1,2]. Besides, elderly people in their 80s frequently suffer from swallowing problems. In order to avoid choking, they are given crushed medicines or opened capsules mixed with food [32].

A topical antimicrobial action of active ingredients on the oral biofilm cannot be excluded to explain the high prevalence of xerostomia among elderly patients taking polypharmacy. This would be in line with literature data, confirmed by the present study, assessing that the risk of dry mouth increases when patients are prescribed four or more drugs per day, whatever drugs are prescribed, except for true atrophic drugs which have a clear pharmacodynamic action on salivary secretory cells [3,16]. In other words, topical factors directly in contact with the oral mucosa, such as tobacco smoking, alcohol (in drinks or in mouthwashes), antiseptic mouthwashes or crushed medicines, could be inducers of xerostomia by disrupting the endogenous microbial biofilm [31–33].

According to recommended regimens, the duration of use of antiseptic mouthwashes should not exceed 2 weeks. However, in this study, many patients used them as if they were common hygiene products. They reported the ‘expectation of improving dry mouth symptoms’ or ‘slowing down the progression of caries or periodontal diseases’. Antiseptic mouthwashes are also commonly recommended as daily oral care products to fight mouth dryness, dental caries and gingival inflammation in hospitals or at home [6,12,27,28]. As far as xerostomia may severely alter the quality-of-life of chronically ill or elderly patients [34–36], the use of antiseptic mouthwashes should be taken into account in patients taking polypharmacy.

In conclusion, patients and caregivers should be aware that long-term, routine use of the most common mouthwashes might be harmful and increase the risk of xerostomia, especially in patients taking polypharmacy. These antimicrobial products should be left aside and replaced by conventional oral hygiene procedures whenever xerostomia worsens quality-of-life or nutritional status, particularly with frail chronically ill patients.
Additional studies would be necessary on the biofilm in the case of xerostomia. Research and quantification of bacterial species of the healthy oral biofilm capable of maintaining hydration due to their glycoalyx such as *Rothia mucilaginosa*, *Prevotella intermedia* or *Micrococcus luteus* would be particularly interesting. Usually these bacterial markers are not isolated and quantified in the studies on the oral mucosal flora.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Antiseptic mouthwashes and xerostomia

ANNEXE II- Un exemple de l’utilisation des modèles de biofilms in vitro

Thérapie photodynamique versus irrigation ultrasonique : Interaction avec un biofilm microbien endodontique

En parallèle à ce doctorat nous avons collaboré à d’autres travaux sur les biofilms oraux. L'objectif de cet article était de mettre au point un biofilm composé de plusieurs bactéries représentant la flore du canal radiculaire de la dent. Le traitement du canal radiculaire (ou traitement endodontique) est nécessaire lorsque la pulpe de la dent est infectée suite à une carie ou une nécrose liée à un traumatisme. L'infection peut alors se répandre par les canaux radiculaires jusqu'à l'apex de la dent et provoquer un granulôme ou un abcès.

La désinfection du canal est le facteur clé de la réussite de tout traitement endodontique. La pulpe dentaire est physiologiquement stérile. Lorsqu'elle est contaminée, le biofilm endocanalaire est composé de plusieurs centaines d’espèces bactériennes différentes, typiquement issues de la flore cariogène. Sa reproduction en laboratoire est donc impossible. Cependant, il est indispensable de tester tout nouveau protocole de désinfection in vitro avant son application clinique. Il était donc nécessaire de mettre au point un modèle reproductible. C'est pourquoi nous avons conçu et réalisé un modèle de biofilm multi-espèces, à partir d'espèces cultivables et représentatives de l’écosystème de l'endodonte infecté. La recherche bibliographique a permis de sélectionner Streptococcus salivarius, Enterococcus faecalis, Prevotella intermedia et Porphyromonas gingivalis. Ce sont des représentants de différents groupes colonisateurs, qui coexistent dans l’espace endodontique. Pour cette étude, nous avons recueilli et décontaminé des dents extraites chez des patients du pôle Odontologie du CHU de Nice. Puis, un biofilm a été formé directement dans les dents par inoculation au niveau de l’endocanal. Le biofilm a été maintenu en culture durant 7 jours, afin de lui permettre de coloniser et de s’organiser à la surface de la dentine (multiplication des bactéries et production d’exopolymères). Nous avons ainsi préparé et inoculé 30 dents avec le biofilm expérimental, qui ont été réparties en 3 groupes afin de tester 3 techniques différentes de décontamination endodontique :

- Irrigation ultrasonore en synergie avec EDTA 17% et NaOCl 2.6%
- Laser LED de longueur d’onde 635 nm
- Laser diode de longueur d’onde 650 nm

L’efficacité du traitement qui correspondait à l’absence de biofilm résiduel a été examinée grâce à des cultures bactériennes en aérobiose et anaérobiose.

Dans un premier temps, les cultures bactériennes ainsi que les images réalisées en microscopie électronique à balayage ont montré que le biofilm s’était correctement développé au niveau de la dentine. Dans un second temps les résultats ont montrés que la technique d’irrigation ultrasonore était la technique de décontamination endodontique la plus efficace parmi les 3 techniques testées.
ANNEXE II

Photodynamic therapy versus ultrasonic irrigation: Interaction with endodontic microbial biofilm, an ex vivo study

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Photodynamic therapy versus ultrasonic irrigation: Interaction with endodontic microbial biofilm, an ex vivo study

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KEYWORDS
Root canal disinfection;
Biofilm;
Photodynamic therapy;
Photosensitizer;
Passive ultrasonic irrigation

Summary
Introduction: Photodynamic therapy was introduced as an adjuvant to conventional chemical debridement during endodontic treatment to overcome the persistence of biofilms. The aim of this study was to evaluate the ability of photodynamic therapy (PDT) to disrupt an experimental microbial biofilm inside the root canal in a clinically applicable working time.

Materials and methods: Thirty extracted teeth were prepared and then divided in three groups. All samples were infected with an artificially formed biofilm made of Enterococcus faecalis, Streptococcus salivarius, Porphyromonas gingivalis and Prevotella intermedia bacteria. First group was treated with Aseptim Plus® photo-activated (LED) disinfection system, second group by a 650 nm Diode Laser and Toluidine blue as photosensitizer, and the third group, as control group, by ultrasonic irrigation (PUI) using EDTA 17% and NaOCl 2.6% solutions. The working time for all three groups was fixed at 3 min. Presence or absence of biofilm was assessed by aerobic and anaerobic cultures.

Results: There was no statistically significant difference between results obtained from groups treated by Aseptim Plus® and Diode Laser (P < 0.6267). In cultures of both groups there was a maximal bacterial growth. The group that was treated by ultrasonic irrigation and NaOCl and EDTA solutions had the best results (P < 0.0001): there was a statistically significant reduction of bacterial load and destruction of microbial biofilm.

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Conclusion: Under the condition of this study, Photodynamic therapy could not disrupt endodontic artificial microbial biofilm and could not inhibit bacterial growth in a clinically favorable working time.

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Introduction and background

Clinical management of microorganisms and their elimination from root canal space is the main goal of endodontic treatment [1,2]. Most failures occur when treatment procedures have not reached an acceptable measure for control and elimination of infection [3–5]. The success rate of endodontic treatments is higher (94%) when the canal is bacteria-free (verified by bacteriological methodologies used just before filling [10]). On the presence of bacteria, this rate is diminished by about 68% [4]. It is apparent that the mechanical debridement combined with chemical irrigation removes the bulk of the infecting microorganisms. But because the infection of root canal system is 3-dimensional, the residual bacteria are still detectable in an important area of the teeth just before filling the root canal [6–8], particularly in small accessory and lateral canals [9]. The antimicrobial susceptibility or resistance to the polymorphous micro flora, which includes anaerobic, facultative anaerobic and aerobic bacteria, may determine the outcome [9]. The important role of Enterococcus faecalis in root canal treatment failures and persistent periapical infection is well established in literature [7,10,11,12]. Although E. faecalis possesses several virulence factors, its ability to cause periapical disease originates from its ability to persist as a pathogen in the root canals and dentinal tubules of infected teeth [13–15]. Against the traditional views that suggest that the most robust group of organisms are the survivors of root canal treatment, the application of ecological parameters indicates the most important factor is the ability of bacterium to adapt itself to new limiting factors in its corresponding niches. Furthermore, as in every natural microenvironment, the adaptive capabilities of individual organisms are exponentially augmented when growing in biofilm communities. These microorganisms are enclosed in an extracellular polysaccharide matrix. The base of this ecological approach to root canal infections is founded on this concept that the most dangerous pathogen is not an individual species, in a planktonic form, but a polymicrobial entity that undergoes different physiological and genetic changes initiated by changes in root canal environments [16,17]. As an essential part of debridement of root canal space, irrigation makes it possible to achieve a cleaner root canal space more than of that which can be obtained only with mechanical instrumentation [18,19].

Hence, the need for an efficient root canal disinfection method drives researchers toward looking for other more effective technologies in Endodontics. There is no one unique irrigant that can possess all required criteria for the best root canal disinfection, so dual irrigant protocols using NaOCl and EDTA solutions are mainly used in Endodontics [20–22]. The use of ultrasonic devices during irrigation has been proposed to confront the problems observed during cleaning and disinfection of the root canal system [22] and the results are significant in reducing survival bacteria [23,24]. Passive ultrasonic irrigation (PUI) seems to enhance theses results [19], due to an acoustic streaming into the root canal [23].

Since the first laser device was developed by Maiman in 1960 [24], this machine has been used in various fields of dentistry [25–32]. Antimicrobial effects of lasers have driven researchers to use this technology for the purpose of root canal disinfection [33–37]. Er:YAG laser (λ = 2940 nm) [40], Nd:YAG laser (λ = 1064 nm) [41], KTP (λ = 532 nm) [42] and Diode Lasers (λ = 630 nm, 810 nm [38] to 980 nm [39]), were tested and showed an effective and significant elimination of the bacterial contamination, but most of the studies were performed on mono-bacterial (E. faecalis) infected root canals in planktonic form [43,44]. On the other hand, the use of these wavelengths at high power, have considerable disadvantages that make them hazardous for antibacterial purposes [45]. Melted dentin, cracking on the surface, and slight debris formation are other disadvantages of some lasers with high levels of energy [45–49] and, in addition, the complexity and high cost of these devices [50]. The antimicrobial effect of Photodynamic therapy (PDT) has been shown in several publications [51–54] and applied in different fields of dentistry [53,54]. Photodynamic therapy (PDT), sometimes called photo-chemotherapy, is a form of phototherapy using nontoxic light-sensitive compounds (photosensitive agents) that are exposed selectively to a defined wavelength light, whereupon they become toxic to target malignant and other diseased cells. PDT has proven ability to kill microbial cells, including bacteria, fungi and viruses. PDT is popularly used in treating acne. It is used clinically to treat a wide range of medical conditions, including wet age-related macular degeneration and malignant cancers [53], and is recognized as a treatment strategy, which is both minimally invasive and minimally toxic. The physical phenomenon is based on the role of complementary colors [54]. Oskar Raab and Hermann Von Tappeiner used for the first time, in 1900–1904, the term ‘‘Photodynamic action’’ to describe the toxic effect of Acridine orange (a dye) on Paramaecia, when exposed to the sunlight. These researchers told about the importance of the combination of light and atmospheric oxygen. Many years later, in 1975, the first medical treatment using PDT was proposed by Kelly et al. [55] to treat bladder cancer without adverse effects on sound cells. Then PDT, since 90s, was really developed as a an antimicrobial therapy for infectious diseases and the terms of Photo-Activated Antimicrobial Chemo-Therapy and Photo-Activated Disinfection were used [56,57]. This therapy was describe as minimally invasive, to be used several times in the same place, and in combination with surgery, radiotherapy and chemotherapy particularly to treat cancers. In dentistry, Wilson [58], published the first application of PDT in this field, to remove dental plaque. Then, in 2000s, antimicrobial effects on oral biofilms of PDT and its use
to diagnose (PDD) and treat oral cancers were significantly
developed [59].

Bacteria could not develop resistance to oxygen derived
cytotoxic reactive species such as free radicals and singlet oxygen [60]. Bacteria that grow in biofilms, explored in
diseases like cystic fibrosis or periodontitis, are also
sensitive to PDT [61,62]. In addition to direct effect on extracellular molecules, singlet oxygen has a photo-damage
effect on polysaccharides of the extracellular matrix of poly-
mers within the bacterial biofilm [50]. Light wavelength,
intensity and energy, the amount of absorbance of pho-
tosensitizer by cells, and exposure time are also other
important factors which may influence the results [63].
The upper layers (1–5 mm) of most tissues are penetrated
by light at wavelengths of ~630 nm; deeper penetration is
achieved at 700–800 nm. Diode Laser systems are used
predominantly. Recently, non-laser light sources, such as
light-emitting diodes (LED), have also been applied in PDT
[64–67]. Different methods, different light sources and
many photosensitizers have been examined, all in vitro, ex
vivo and in vivo [9,63,68–72].
The aim of this study was to evaluate the capability of
two different PDT protocols in disrupting microbial biofilm
growth in root canal space, compare their efficiency to PUI
using EDTA 17% and NaOCl 2.6% solutions.

Materials and methods

Selection and preparation of teeth

Thirty-four roots obtained from 50 extracted human single
and multi-rooted teeth were selected. The presence of just
one canal was confirmed by digital radiography. The teeth
were stored in saline solution until starting the experiments.
All roots were reduced from original height to 14 mm. After-
ward, all the samples were placed in an Ultrasonic heated
bath (Fisher Scientific Inc., Schwerte, Germany) in order to
clean off the dust and dirt accumulated during cutting. All
apices were closed with Photac™ Fil Quick Applicap™ Glass-
tonomer cement (3M ESPE AG, Seefeld, Germany), to avoid
leak loss of irrigants during the root canal preparation.

Root canal shaping

The same operator performed all of the preparation steps.
A # 10 K-File (MicroMega, Besançon, France) was intro-
duced into the canal to determine the working length, and con-
firm the absence of any obstacles in the canal. Root canal
shaping was implemented using the Protaper® rotary sys-
tem (Dentsply-Maillefer, Ballaigues, Switzerland) according
to the manufacturer instructions. The final file used was the
F2 file (# 25, 09 taper).

Between the use of each instrument, the canals were
flushed with 1 ml of NaOCl 2.6% solution using a syringe and
a 26G needle (PentaForte S.p.A, Campili, Italy). A
# 10 K-File was used to verify the glide path of the root
canal. A final rinsing, aiming to remove smear layer and
debris was performed using 1 ml EDTA 17% solution
(Root canal enlarger Edeta, Produit Dentaire S.A, Vevey,
Switzerland). The solution was advanced into the canals for
1 min using an endodontic ultrasonic smooth file, IRRISAFE®
(ACTEON, Merignac, France) mounted on an ultrasonic unit
(PiezOn® Master 400 EMS Electro Medical Systems SA, Nyon,
Switzerland). Then, 2 ml of NaOCl 2.6% were injected in
the canal and agitated for 1 min with the same ultrasonic file as
mentioned before. To finish the rinsing, 2 ml of NaOCl 2.6%
solution was injected into the canals to clean up any rem-
nants without ultrasonic agitation. All prepared teeth were
kept in saline solution until ex vivo infection procedures
were conducted. All tooth samples were rinsed with water
several times to remove any possible remaining debris and
disinfecting solutions from the root canal walls. The teeth
were then sterilized with an autoclave (130 °C during 1 h).

Biofilm ex vivo creation

The biofilm created in the laboratory for this study was
composed of 4 different bacterial species, Porphyromonas
gingivalis (ATCC 33277), Streptococcus salivarius (ATCC
7073), a wild-type strain of E. faecalis, and a wild-type strain
of Prevotella intermedia that were obtained from the labo-
ratey of bacteriology (Hôpital Archet 2 – Nice – France). E.
faecalis and S. salivarius were grown aerobically overnight
and at 37 °C on Mueller-Hinton agar plates or on 5% sheep
blood agar plates (BioMérieux, Marcy l’Etoile, France),
respectively. P. gingivalis and P. intermedia were cultivated
anaerobically on 5% sheep blood agar plates at 37 °C for 3
and 5 days, respectively. For the four strains a standardized
 suspension containing 10⁶ cells ml⁻¹ in Schaedler broth (Bio-
Rad, Marnes la Coquette, France) was prepared respecting
the following proportion: 5% S. salivarius, 21% E. faecalis,
37% P. intermedia, and 37% P. gingivalis.

The biofilm was applied onto the teeth by dispensing
2.2 ml of standardized cell suspension within 24-well cell
culture plates (Corning Inc., Union city, CA, USA). Cell cul-
ture plates were incubated anaerobically at 37 °C on an
orbital shaker (150 r.p.m). After 24h, 0.5 ml of Schaedler
broth was added into every well. Seven days after the
inoculation, teeth were removed and washed with 0.1M
phosphate buffer saline twice, for 3 min each time. Then,
the teeth were randomly divided into 5 groups: 2 groups of
2 teeth (as negative and positive control group), and 3
groups of ten teeth to be treated with different methods to
 disrupt the biofilm.

Disinfection procedures

Control groups
Cultures were achieved from the 2 teeth of the negative con-
trol group (no inoculation) in order to verify that the canal
is bacteria-free and a SEM examination is performed under
low vacuum to verify the absence of smear layer (Fig. 1).
Cultures were also realized from the 2 teeth of the posi-
tive control group in order to verify that canal is infected by
the biofilm and a SEM examination is performed under
low vacuum to verify the presence of biofilm (Fig. 2A and B).

Group A, LED
The ten teeth from group A were treated with PDT protocol
using an LED with a wavelength of 635 nm as a source of
light (Aseptim Pluss®, Leutkirch im Allgö, Germany). Light
was delivered using a disposable conical soft plastic tip. The
Photosensitizer was a solution of dilute, pharmaceutical grade Toluidine blue, which was supplied in 0.8 ml syringes. The solution was introduced into the root canal by mean a G26 needle.

For an easy performance of the treatment protocol, teeth were mounted on a base made from Aquasil™, a vinyl polysiloxane impression material (Dentsply DeTrey GmbH, Konstanz, Germany). The work desk was cleaned by alcohol 70% to avoid contamination. Then all procedures were carried out under sterile conditions next to the flame. The excess of the product was collected during injection using a sterile pipette tip and a suction device. The solution was rubbed around inside the root canal one minute using a #10 K-file to respect the manufacturer’s instructions continued the procedure. The sterile, specially designed flexible tip attached to the LED device was inserted into the canal space until a tug-back sensation was felt. Activation of the photosensitizer was commenced for 120s according to the manufacturer’s instructions. Once the procedure was completed, the canal was rinsed with 2 ml of sterile water to remove the photosensitizer from the canal. Afterward, the canal was dried with a sterile paper point.

**Group B, Laser**

A so-called DeltaCube™ soft laser with a wavelength of 650 nm (Laser 3 S, Pessac, France) with a maximum energy of 60 mW was used. The light was delivered into the canals using a 300 μm optic fiber in a continuous manner for 120s. The best duration of activation of a photosensitizer (a 15 μg/ml solution of Toluidine blue O; Sigma–Aldrich, Schnelldorf, Germany) by a laser was measured through a pilot study. Before activation, the photosensitizer was agitated for 1 min by means of a #10 K-file. The photosensitizer was exposed to a red laser light, and after 120 s, the photosensitizer lost its original blue color to pink, in which the light absorbance may change. Because the Laser beam could not diffuse in all directions, the fiber was moved all along of the canals repeatedly to activate the photosensitizer in all regions of the canal space (WL minus 2 mm). After treatment, the canal was rinsed with 2 ml of sterile water and dried by sterile paper points.

**Group C, PUI**

The ultrasonic irrigation was performed by injecting 1 ml EDTA 17% solution (Produit Dentaire S.A., Vevey, Switzerland) in the root canals. The solution was agitated in the canals for 1 min by means of an endodontic ultrasonic soft file (Irrisafe®, ACTEON, Mérignac, France) that was mounted on an ultrasonic unit (Pmax®, ACTEON, Mérignac, France) as described for final irrigation before infection protocol. Two ml of NaOCl 2.6% solution was injected in the canal and was agitated for 1 min with the same device. Another 2 ml of NaOCl 2.6% was injected into the canals. Finally, the canal was rinsed with 2 ml sterile water and dried with sterile paper points.

**Bacterial load control: microbiological sampling and culturing**

Once the clinical procedures were accomplished, microbiological samples were taken from the canals. A # 10 K-file was used to rub the canal walls to collect any possible viable bacteria. Then the samples were cultivated in 5% sheep blood agar. The technique of assessment of culturing was inspired by the protocol of Bonsor et al. [71, 72]. A design of 5 parallel lines was created on the agar plate and this was repeated 3 times more, that gave a 5 growing areas to the culture pattern (Fig. 1A). If growth occurred in the well area, a score of one was allocated. If the growth occurred in both the well and the first five streaked lines, this was scored two and so on up to a maximum score of five (Fig. 1B).

Aerobic cultures were performed on all the teeth. Then 3 teeth from each group that were randomly selected underwent anaerobic culturing. For all of the teeth an aerobic culture was performed and for 3 teeth of each group that were randomly selected, an anaerobic culture was done. The plates were incubated at 37°C. The bacterial growth was observed and the scores recorded all 24, 48 and 72 h after culturing.

SEM observation of biofilm inside the root canal before treatment (Fig. 3A and B), and after LED, Diode and PUI treatments (Fig. 4A and B) from all samples were then taken.

**Figure 1** (A) Schematic view of culturing and scoring according to Bonsor et al. [73, 74]. (B) Transfer on an agar plate with 5 scores design streaking. Score of 5 is equivalent to heavy bacterial load, score of 2 equivalent to approximately $1.5 \times 10^8$ bacteria, score of 0 equivalent to no culturable bacteria.
Figure 2  Bacterial load scores per root canals treated: A and B treated by Diode Laser and Toluidine blue, C and D by Aseptim and E and F by PUI. Bar chart shows the numbers of the root canals with aerobic (A, C, E (10 specimens each)) and anaerobic (B, D, F (3 specimens each)) bacterial loads at 24, 48 and 72 h after the treatment.

Figure 3  (A) Biofilm in root canal 72 h after inoculation – SEM ×2000. (B) Another view SEM ×7500.
Statistical analysis

ANOVA and post hoc tests statistical analysis tests were achieved; all groups were compared by multiple two by two sample tests by Fisher’s PLSD and were confirmed by the Student Newman–Keuls test.

Results

Group A, LED

Based on the scoring method previously described, none of the canal cultures had scores of 0 or 1 in the group treated by LED Aseptim® system, meaning that in all canals there was a bacterial load after treatment. Under aerobic conditions, 24 h after treatment, cultures taken from 3 canals showed a score of 2 that was reduced to 2 canals after 48 h and remained unchanged during final observation at 72 h. During the first observation, there were 3 canals with a bacterial load score of 3 that was augmented to 4 canals two days after inoculation and then returned to 3 canals at the end of the experiment. A score of 4 was registered in only 1 culture after 24 and after 48 h of treatment, while after 72 h, 2 cultures showed the same score. Of the 10 root canals evaluated in this group, only three showed maximum bacterial infection with a score of 5 during the observation (from 24 to 72 h after the treatment). The distribution of aerobic bacterial load scores for the culture taken at the different times of bacterial growth is shown in Fig. 2A.

The cultures taken from the root canals did not present anaerobic bacterial load scores of 0, 1 or 2. At the time points of 24 and 48 h after treatment, two root canals had anaerobic bacterial load scores of 3 (Fig. 4A) and one went up to a score of 4 at 72 h. Only one root canal showed a complete anaerobic bacterial infection with a load score of 5 during the entire cultivation experiment. There was no significant difference between the bacterial load scores obtained from anaerobic and aerobic cultivations. Anaerobic bacterial load scores of root canals treated through the Aseptim® system is shown in Fig. 2B. Remnant biofilm inside the dentinal tubules can be seen in Fig. 4A (SEM).

Group B, Diode Laser and Toluidine blue

Teeth presented both aerobic and anaerobic bacterial load. Under aerobic conditions after 24 h, cultures taken from 5, 1, 1 and 3 root canals had bacterial load scores of 2, 3, 4 and 5, respectively. There were no root canals presenting load scores of 0 or 1. Forty-eight hours after treatment, there was a dramatic decrease in the number of root canals with scores of 2 from 5 to 1 and respective increases in number of root canals with scores of 3 from 1 to 4. One root canal with a score of 4 and four root canals with scores of 5 were recorded in this step of observation. The bacterial load scores remained unchanged after 72 h of incubation (Fig. 2C and D).

Interestingly, all 3 root canals evaluated under the anaerobic condition presented a high level of bacterial load score at 5, only after 72 h of incubation (Fig. 2C and D).

Group C, PUI, NaOCl and EDTA

No bacterial load for all samples, under aerobic and anaerobic conditions. PUI using EDTA and NaOCl solutions ultrasonically agitated is effective at disrupt the biofilm and at disinfecting the root canal (Fig. 2E and F). Cleaned surfaces can be seen in Fig. 4B (SEM).

Statistical analysis showed that there is a statistical significant difference between the three groups when culturing under aerobic conditions (P < 0.0001 in all observations at 24, 48 and 72 h).

There was no statistically significant difference between cultures obtained from PDT by diode laser group and from PDT by Aseptim® protocol (P ≤ 0.6267 for the final observation at 72 h), all of which were confirmed by the Student Newman–Keuls test.

Ultrasonic irrigation showed the best results to disrupt the microbial biofilms (P < 0.0001). All statistical plots and tables of cultures taken under aerobic conditions for all observations are shown in Fig. 2.

The same statistical analyses procedures were performed for the cultures incubated under anaerobic conditions. A significant difference was observed in 3 groups and for all observations at 24, 48 and 72 h (P < 0.0001). The ultrasonic irrigation had the best effects on reducing bacterial load in anaerobic conditions (P < 0.0001). However, Aseptim® had
statistically better effects than photodynamic therapy by Diode Laser to reduce bacterial load in anaerobic conditions \((P < 0.0043\) for final observation at 72 h). All statistical plots and tables of statistical analyses of cultures taken under anaerobic conditions for all observations are shown in Fig. 2.

Discussion

Hundreds of protocols have been introduced in literature aiming to obtain a 100% bacterial free root canal space. PDT was introduced as a measure to attain this goal. PDT was shown to have ability to kill the planktonic bacteria in vitro study essays.

In the majority of publications concerning PDT and Endodontics, this protocol has played a great role in the reduction of bacterial colonies. However the effect of PDT on microbial biofilm may be related to that of NaOCl used during instrumentation [72]. Subsequently it was suggested always as an adjuvant to conventional root canal treatment procedures and not as a substitute. PDT was performed at the point of chemo-mechanical debridement completion, when the microbial biofilm present in the canal might be logically stressed and disturbed by root canal instrumentation and irrigation. Because intact biofilm remnants attached to canal walls may still be detectable after chemo-mechanical debridement, we investigated the interaction of an intact biofilm with Photodynamic therapy and ultrasonic irrigation in an ex vivo trial. In this study we used an artificially formed biofilm in which there was *E. faecalis* whose role in treatment failures was previously described. We used two different methods of PDT using a 650 nm Diode Laser and LED and we compared the outcome of both of them with passive ultrasonic irrigation.

Garcez et al. [69,70] used PDT as a supplement to chemo-mechanical debridement and placed the photosensitizer in contact with the microbial biofilm resistant to antibiotherapy. A further 4 min were considered for its activation by light, resulting in a total of a 6 min working time. He suggested even a two-visit treatment protocol to obtain bacteria-free root canals.

Soukos et al. [71] examined the effects of PDT on *E. faecalis* in a planktonic state, but he submerged the specimens in photosensitizer solution for 5 min and spent 5 min for photo activation. The reduction of EF in planktonic form was only 77.5%.

Souza et al. [76] irrigated the specimens with two different solutions, one NaOCl 2.5% and other NaCl 0.85%. Then they incubated the canals for 2 min with Toluidine blue and Methylene blue as the photosensitizing agents and for 4 min the Laser irradiated the canal. Ten minutes for a single canal [71] and two visits, becomes 60 min for a multi-rooted tooth in the case of lack of anatomical variation, a problem that is found in other protocols.

Because in the protocols mentioned above and in many of similar publications [63,75–78] the procedures are quite time consuming and not feasible in everyday dental practice, we tried to find and standardize a suitable amount of time for treatment that would be clinically accepted.

The other factor was the photosensitizer itself. A large variety of concentrations from too low (10 μg/ml) to too high (100 μg/ml) of Toluidine blue were tested [79–81]. Because of our objective of feasibility and safety in the clinic (to prevent any possible toxicity and colorization of hard and soft tissues from the photosensitizing agent) we used Toluidine blue with a concentration of 15 μg/ml. The concentration and exact composition of photosensitizer of Aseptim Plus® system was protected by a patent (Scican, States Patent 5,601,430, United States Patent and Trademark Office, sold by Micro-Mega in France).

According to the manufacturer instructions, a 3-minute total procedure time was established for all procedures. In this protocol 1 min was dedicated to agitating the photosensitizer in the root canal with a hand # 10 K file and 2 min were committed to activating the photosensitizing agent with light. The duration of photo activation was tested also for PDT by diode laser through a pilot study. The color modification observed was quite/very much the same with the Aseptim® solution after 120 s of irradiation by LED.

As the photosensitizing agent must act as a chromophore corresponding to the exact complementary color of the activator light, in this study we observed that the color of the photosensitizer changed totally from blue (which is a complementary color of the 650 nm red light of Diode Laser and 630 nm of LED) to pink after 30s of irradiation. The energy absorbed by the photosensitizer could not be the same during all of the irradiation. During another 90s of activation time, there were different absorption coefficients of the photosensitizer regarding its continuous color changes.

Maximum energy absorption by photosensitizer occurred approximately during the first 30s of the experiment.

However, the period of 180s was respected in the ultrasonic irrigation group too. We used 2 different solutions, EDTA 17% and NaOCl 2.6%. One ml of each was injected using an up and down movement for 30s at WL minus 2 mm and then was agitated for 60s.

As explained above, all solutions used in all three groups were in a passive state during activation and we did not add photosensitizer, NaOCl, or EDTA.

Standardization of the working time in all three groups aims to measure the capability of those methodologies to act on microbial biofilm.

Diode Laser light was delivered via a 300–μm optic fiber but the LED light was transmitted into the canal by a conical plastic tip that allowed for a better distribution of photons in all directions. Diode Laser light has the collimated characteristic of any other Laser: to activate the entire photosensitizer we had to move the fiber tip up and down (Working Length minus 2 mm) in a helicoidally manner. However there was a slight refraction of the Laser light, so the light might miss some regions in the canal. Moderately better results were achieved by Aseptim Plus® device system which may be due to its non-collimated light produced by LED and/or by the composition and concentration of the photosensitizer.

Ultrasonic irrigation promoted best results in our study. It was efficient time-wise and the most economical. Ultrasonic irrigation enhances the effects of NaOCl and EDTA by acoustic streaming. The solution can reach all parts of the canal even in non-instrumented parts of the root canal where we may find a portion of intact biofilm. But, our results may have to be limited by the carry over effect of antibacterial solutions. Carry-over effect means that the medicament, in active form, follows along with the sample.
into the dilution series and even to the culture plate (or liquid culture), where surviving microbes are calculated. A high enough concentration of the disinfectant, in such a situation, can cause a false negative result: the microbes are not killed, but residual medicament in the culture media prevents their growth by a bacteriostatic effect. Thus, carry-over, if undetected, gives a too positive picture of the antibacterial effectiveness of the medicament. Various inactivating agents are used to prevent the effects of carry-over. Citric acid has been used in the root canal to neutralize Ca(OH)₂, sodium thiocarbonate neutralizes NaOCl, and a mixture of Tween 80 and alpha-lecithin inactivates chlorhexidine (CHX). However, in the literature, this point is controversial because in numerous studies, carry over effect of NaOCl solution is considered as negligible at concentration of 3%. In the study of Rossi-Federer et al. [85], the experiment to determine the effect of carry-over of NaOCl resulted in similar colony counts for the samples from teeth irrigated with NaOCl and water indicating that carry over of NaOCl had no noticeable effect in his experiment.

According to Haapasalo et al. in 2007 [86], this effect is observed in vitro with more powerful disinfectant solutions such as the MTAD. On the other hand, most of the studies showing this effect are made on models (tooth discs, extracted teeth) infected by planktonic bacteria or mono-bacterial biofilm. In the case of multiple bacterial biofilms, this effect seems to be minimized. An other effect, described by Haapasalo [84] is the inhibitory effect of dentin on the antibacterial medicaments: Hydroxyapatite, which is the main inorganic component of dentin, showed a similar effect on calcium hydroxide as dentin, preventing the killing of E. faecalis [82,87]. Although the result does not exclude the role of the organic part of the dentin in calcium hydroxide inactivation, it emphasizes the importance of the inorganic dentin components. The strong effect of dentin on the antibacterial action of a saturated calcium hydroxide solution can probably be attributed to the buffering action of dentin against alkali [87]. Studies with dentin powder have shown that dentin has an inhibitory effect on the antibacterial effectiveness of 1% sodium hypochlorite. Dentin powder (18%, w/v) greatly delayed the killing process of E. faecalis, which was used as a test organism [87]. When hypochlorite was preincubated in dentin in a closed test tube for 24 h before adding the bacteria, killing all of the bacteria required 24-hour incubation with hypochlorite, whereas after 1-hour incubation all of the bacterial cells were still viable [85–88].

In a pilot study carried out prior to this study to assess our biofilm growth and concentrations of each strain, we used tooth discs on which our biofilms grew. We treated them with the same techniques to test removal of our biofilm. We did not notice any carry over effect following the NaOCl treatment. The SEM observations of samples clearly confirmed the absence of bacteria or trace of biofilm in NaOCl treated root canals (Fig. 4A), while in those treated with the Diode Laser and the Aseptim system, there were still either bacteria present in the tubuli, or traces of biofilm (Fig. 4B).

Also found in this study was the abundant presence of E. faecalis and some chains of S. salivarius that were observed using the Gram staining test (after the photodynamic therapy of both PDT methods).

This proves the previously described resistance of E. faecalis to treatments and the risks of treatment failures due to the presence of this bacterium [8,14–21]. But the fact that all other bacteria were not cultivable does not mean necessarily that they were killed by Photodynamic therapy or even by Ultrasonic irrigation methodology. This could be related to non-viable bacterial cells already there before treatment. This cannot be assessed precisely in the present experimental conditions. Biomolecular techniques as real time PCR [86] or FISH (rRNA 16s) [83] methods have to be used to determine what species are persistent.

Conclusion

Under the conditions of this study, PUI using EDTA and NaOCl solutions was found to be effective in order to eliminate an in vitro-created seven days-old biofilm. PDT is not effective in this purpose. Photodynamic therapy using a non-collimated light and an optimized concentration of photosensitizing agent may have a role in disrupting the biofilm and decreasing the bacterial load of intact microbial biofilm; these effects are statistically inferior to those obtained by passive ultrasonic irrigation.

The present study suggests further in vitro and in vivo studies as:

- Understanding the real interaction of root canal instrumentation on bacterial biofilm.
- The establishment of protocols using a continuous injection of photosensitizer and simultaneous activation by light to overcome the energy absorbance modification related to the photosensitizer’s color changes during activation.
- The interest of using Er:YAG Laser before Photodynamic therapy to remove the smear layer and increase wettability of root canal walls.

Conflict of interest

The authors declare to do not have any conflict of interest.

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COMMUNICATIONS

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...
La vie mettra des pierres sur ton chemin,
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