Inhibition of *Candida albicans* biofilm formation and modulation of gene expression by probiotic cells and supernatant

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Oral candidiasis is a disease caused by opportunistic species of *Candida* that normally reside on human mucosal surfaces. The transition of *Candida* from budding yeast to filamentous hyphae allows for covalent attachment to oral epithelial cells, followed by biofilm formation, invasion and tissue damage. In this study, combinations of *Lactobacillus plantarum* SD5870, *Lactobacillus helveticus* CBS N116411 and *Streptococcus salivarius* DSM 14685 were assessed for their ability to inhibit the formation of and disrupt *Candida albicans* biofilms. Co-incubation with probiotic supernatants under hyphae-inducing conditions reduced *C. albicans* biofilm formation by 75% in all treatment groups. Likewise, combinations of live probiotics reduced biofilm formation of *C. albicans* by 67%. When live probiotics or their supernatants were overlaid on preformed *C. albicans* biofilms, biofilm size was reduced by 63 and 65% respectively. Quantitative real-time PCR results indicated that the combined supernatants of SD5870 and CBS N116411 significantly reduced the expression of several *C. albicans* genes involved in the yeast–hyphae transition: *ALS3* (adhesin/invasin) by 70% (*P*<0.0001), *EFG1* (hyphae-specific gene activator) by 47% (*P*<0.0061), *SAP5* (secreted protease) by 49% (*P*<0.0001) and *HWP1* (hyphal wall protein critical to biofilm formation) by >99% (*P*<0.0001). These findings suggest the combination of *L. plantarum* SD5870, *L. helveticus* CBS N116411 and *S. salivarius* DSM 14685 is effective at both preventing the formation of and removing preformed *C. albicans* biofilms. Our novel results point to the downregulation of several *Candida* genes critical to the yeast–hyphae transition, biofilm formation, tissue invasion and cellular damage.

INTRODUCTION

Although >150 species of fungi exist within the ubiquitously distributed *Candida* genus, most do not normally colonize the human body due to their inability to grow at 37 °C (Schauer & Hanschke, 1999; Williams & Lewis, 2011). *Candida albicans*, however, innocuously colonizes the oral cavity of ~80% of healthy individuals. In immunocompetent individuals, *C. albicans* is maintained in a harmless state by the host’s innate immune defences. Nevertheless, certain factors can predispose individuals to subsequent *C. albicans* infection (oral candidiasis) by shifting the oral environment to one that favours the replication of *Candida*. These factors commonly involve the weakening of the host’s immune system, and include, amongst others, the use of dentures (Campisi et al., 2008), steroid inhaler use (Fukushima et al., 2005), xerostomia (Radfar et al., 2003), age (Weerasuriya & Snape, 2008), AIDS (Egusa et al., 2008), the use of broad-spectrum antibiotics (Soysa et al., 2008) and poor oral hygiene in general (Ishijima et al., 2012). Most cases of

Abbreviations: qRT, quantitative real-time; SFS, sterile-filtered supernatant.
oral candidiasis are mildly debilitating, causing inflammation and a variety of superficial mucosal lesions. However, in severely immunocompromised individuals such infections can become systemic, carrying with them a 30–50 % mortality rate (Vazquez, 2010).

*C. albicans*, which accounts for ~ 50 % of all oral candidiasis cases (Thompson et al., 2010; Zomorodian et al., 2011), is a polymorphic fungus that can exist commensally as unicellular budding yeast cells or in a pathogenic filamentous hyphal morphology (Gow et al., 2012; Shapiro et al., 2011). During its commensal, budding state, *C. albicans* reversibly adheres to oral epithelial cells through weak hydrophobic and electrostatic interactions ( Cotter & Kavanagh, 2000).

The presence of various environmental triggers causes increased expression of the EFG1 (enhanced filamentous growth protein 1) gene, which encodes a transcription factor essential for the transition to a pathogenic hyphal state (Bailie & Douglas, 1999; Ramage et al., 2002). Efg1p subsequently causes increased expression of several hyphae-specific genes that promote a stronger covalent attachment to receptors on host tissues, weaken the host immune system, damage host cells, and promote tissue invasion ( Sudbery, 2011). Examples of these include: HWp1 (Staab et al., 1999), ALS3 (Drago et al., 2000; Hoyer, 2001; Phan et al., 2007) and various SAP genes (Gropp et al., 2009; Ramage et al., 2006; Reinholdt et al., 1987; Rüchel, 1986; Silverman et al., 2010).

Once hyphae have formed, attachment to oral epithelial cells and various other surfaces, such as teeth, dentures and other prostheses, further propagates oral candidiasis pathogenesis by promoting the formation of *Candida* monomicrobial and polymicrobial biofilms ( Jenkinson et al., 1990; Sardi et al., 2010). The National Institutes of Health has estimated that ~ 80 % of human infections are a direct result of the formation of pathogenic biofilms ( Harriott & Noverr, 2011). Once *Candida* biofilms are established, the expression of virulence factors increases, and fungal susceptibility to antimicrobials and phagocytosis decreases drastically ( Net et al., 2010; Rajendran et al., 2010; Williams & Lewis, 2011). In the case of *Candida*—bacterial polymicrobial biofilms, the disruption of the oral mucosa and suppression of host defences by *C. albicans* can act as a catalyst for commensal oral bacteria that are normally associated with oral health, e.g. viridans streptococci, to cause serious disease, e.g. bacterial endocarditis ( Herzberg et al., 1997). Evidently, the formation of *Candida* biofilms poses a serious health risk, not only by promoting oral candidiasis, but by setting the stage for more serious, invasive *Candida* or bacterial infections in susceptible populations, such as the immunocompromised and the elderly.

Probiotics have long been studied for their ongoing beneficial effects in the human gastrointestinal system. Only a few clinical studies to date have examined the preventative effects of probiotics on oral candidiasis, but they have shown very promising results. Hatakka et al. (2007) performed a 16-week, randomized, double-blinded, placebo-controlled study of 276 elderly participants fed cheese containing a mixture of *Lactobacillus* and *Propionibacterium* probiotics, and results indicated a 32 % reduction in salivary yeast counts and an increase in salivary flow. Similarly, Mendonça et al. (2012) showed that consumption of Yakult LB, which contains *Lactobacillus casei* and *Bifidobacterium breve* probiotics, significantly reduced Candida prevalence and increased anti- *Candida* salivary IgA levels in healthy elderly participants. In addition to modulating clinical parameters, probiotics have been shown to successfully prevent *Candida* biofilm formation *in vitro* and *in vivo*. Ishijima et al. (2012) demonstrated the ability of *Streptococcus salivarius* DSM 13084 to prevent hyphal growth and biofilm formation of *C. albicans* in plastic Petri dishes, and protect mice from oral candidiasis through an unknown mechanism independent of bacteriocin activity ( Ishijima et al., 2012). Their results indicated that probiotics may protect the host from oral candidiasis by binding *C. albicans*, and preventing its adhesion to mucosal and abiotic surfaces.

This study aimed to expand the findings of Ishijima et al. (2012) using a multi-strain probiotic combination previously shown *in vitro* to reduce the risk of other oral indications, such as dental caries. The goal of our study was to determine whether or not the combination of *Lactobacillus plantarum* SD5870 and *Lactobacillus helveticus* CBS N116411 (Paturi et al., 2008), with or without *S. salivarius* DSM 14685 (Burton et al., 2013), was effective at both preventing the formation of and removing preformed *C. albicans* biofilms. Combinations of live bacteria or their sterile-filtered supernatants (SFSs) were utilized. Finally, we sought to elucidate a mechanism of biofilm prevention via our candidate probiotic strains, by examining the modulation of several hyphae-specific *C. albicans* genes.

**METHODS**

* C. albicans, bacterial strains, media and growth conditions. *C. albicans* strain TIMM 1768, isolated from the blood of a candidiasis patient and previously shown to induce oral candidiasis in animal models, was used in this study (Ishijima et al., 2012; Kamagata-Kiyoura et al., 2003). For stock cultures, *C. albicans* TIMM 1768 was grown on Sabouraud dextrose agar (SDA) plates for 18 h at 30 °C. *L. plantarum* SD5870 (Nutracelix) and *L. helveticus* CMS N116411 (Lallemand Health Solutions) were grown on de Mann–Rogosa–Sharpe agar plates for 22 h at 37 °C in anaerobic jars. *S. salivarius* DSM 14685 (BLIS Technologies) was grown under the same conditions on CABK12 agar ( Ishijima et al., 2012).

For biofilm and quantitative real-time (qRT)-PCR assays, individual colonies of all cultures were used to inoculate 10 ml YEPD (1 % yeast extract, 2 % peptone, 2 % glucose). *C. albicans* was grown aerobically at 30 °C for 18 h, *L. plantarum* was grown anaerobically at 37 °C for 24 h, *L. helveticus* was grown anaerobically at 37 °C for 44 h and *S. salivarius* was grown anaerobically at 37 °C for 22 h. Co-cultures of L. plantarum, L. helveticus and S. salivarius were grown by inoculating a single colony of each bacterium into 10 ml YEPD, and incubated anaerobically at 37 °C for 24 h. Supernatant from all strains was collected and filtered using a syringe and 0.2 μm filter to generate SFS.
C. albicans biofilm prevention assays. Biofilm assays were performed as outlined by Abe et al. (1994) with minor variations. A 3 h incubation of C. albicans cells in YEPD, supplemented with 0.5 % FBS (v/v) at 37 °C in 5 % CO₂, was found previously to induce robust biofilm formation (Ishijima et al., 2012; Sudbery 2011). Assays were performed on two surfaces.

Polystyrene plates (96-well)

Three combinations of SFSs were prepared: (i) combined SFS of individual cultures of SD5870 and CBS N116411; (ii) combined SFS from individual cultures of SD5870, CBS N116411 and DSM 14685, (iii) SFS from SD5870, CBS N116411 and DSM 14685 co-culture. For each well, 100 μl SFS was mixed with 5 x 10⁵ live C. albicans in 100 μl YEPD supplemented with 1 % FBS (v/v), and subsequently incubated for 3 h at 37 °C and 5 % CO₂. Negative control samples contained 100 μl YEPD. After the incubation period, biofilm formation was assessed as per Abe et al. (1994). Cells were fixed with 70 % ethanol, stained with 1 % (w/v) crystal violet and OD₆₀₀ readings were taken.

Polyurethane discs

SD5870, CBS N116411 and DSM 14685 were grown individually as described previously. SFSs were prepared in one of two ways: (i) SD5870 and CBS N116411 mixed in equal ratios, and (ii) SD5870, CBS N116411 and DSM 14685 mixed in equal ratios. C. albicans cells (1 x 10⁵) in 50 μl YEPD supplemented with 5 % FBS (v/v) were incubated on polyurethane discs for 3 h at 37 °C and 5 % CO₂, under the following treatment conditions: (i) 5 x 10⁵ cells of each of SD5870 and CBS N116411 in 450 μl YEPD, (ii) 3.3 x 10⁴ cells of each of SD5870, CBS N116411 and DSM 14685 in 450 μl YEPD, (iii) 450 μl SD5870 and CBS N116411 SFS mixed in equal ratios, (iv) 450 μl SD5870, CBS N116411 and DSM 14685 SFS mixed in equal ratios or (v) 450 μl YEPD as a negative control. Biofilm formation was quantified by determining the C. albicans c.f.u. present on each disc. Each disc was washed five times in PBS, suspended in 1 ml PBS and submerged for 15 min in a sonicating water bath. Serial dilutions from each sample were plated on SDA agar and incubated at 37 °C overnight.

C. albicans biofilm disruption assays. Biofilm disruption assays were performed using a modified protocol originating from Abe et al. (1994).

Polystyrene plates (96-well)

C. albicans biofilms were formed by adding 5 x 10⁵ c.f.u. to 200 μl 0.5 % (v/v) FBS-supplemented YEPD media and incubating the plates for 3 h at 37 °C and 5 % CO₂. The supernatant was subsequently removed and replaced with one of the following treatments: (i) 200 μl combined SD5870 and CBS N116411 SFS mixed in equal ratios, (ii) 200 μl 0.5 % FBS harvested from a co-culture of SD5870, CBS N116411 and DSM 14685 probiotics or (iii) 200 μl PBS alone as a negative control. The plates were reincubated at 37 °C and 5 % CO₂ for an additional 3 h. The reduction in size of C. albicans biofilms adhered to the plates was quantified as described earlier.

Polyurethane discs

C. albicans biofilms were formed for 3 h at 37 °C in 5 % CO₂. The discs were washed three times using fresh media and then exposed to one of the following treatments: (i) 500 μl of SD5870 and CBS N116411 SFS mixed in equal ratios, (ii) 500 μl SD5870, CBS N116411 and DSM 14685 SFS mixed in equal ratios, (iii) 5 x 10⁵ cells of each of SD5870 and CBS N116411 in 500 μl YEPD, (iv) 3.3 x 10⁴ cells of each of SD5870, CBS N116411 and DSM 14685 in 500 μl YEPD or (v) 500 μl PBS alone as a negative control. The discs were reincubated at 37 °C and 5 % CO₂ for an additional 1 h. The reduction in size of C. albicans biofilms was quantified by determining the C. albicans c.f.u. adhered to the discs as described earlier.

Regulation of C. albicans gene expression by probiotic SFS. To determine the effects of probiotic SFS on the transcription of C. albicans genes related to adhesion, biofilm formation and virulence, qRT-PCR was performed on C. albicans samples after culturing them under biofilm-inducing conditions. Briefly, 1 x 10⁷ c.f.u. C. albicans in 50 μl YEPD media supplemented with 5 % FBS (v/v) were co-cultured for 3 h in 1.5 ml microcentrifuge tubes at 37 °C and 5 % CO₂ under the following conditions: (i) 1 μg amphotericin B ml⁻¹ solution (Sigma-Aldrich) in 450 μl YEPD (positive control), (ii) 450 μl SD5870 and CBS N116411 SFS mixed in equal ratios, (iii) 450 μl SD5870, CBS N116411 and DSM 14685 SFS mixed in equal ratios or (iv) 450 μl YEPD alone (negative control). Each sample was centrifuged for 5 min at 13 000 r.p.m., supernatants were discarded and RNA was isolated using a Yeast Ribopure RNA Purification kit (Ambion). Concentration, purity and quality of the isolated RNA samples were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific). RNA (1 μg) from each sample was immediately reverse transcribed into cDNA (without RNase inhibitor) using a High Capacity cdNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer’s instructions. The conditions for reverse transcription were 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. The resulting 20 μl of each cDNA sample was diluted in 380 μl Nuclease-Free Water (Ambion) and stored at ~20 °C until further use. qRT-PCRs were carried out on clear 384-well Armadillo PCR Plates (Thermo Scientific). Each 20 μl reaction comprised: 0.25 μl both forward and reverse primers (Table 1) for the C. albicans genes ACT1, ALS3, EAP1, EFG1, NRG1, HWPI or SAP5 (Invitrogen) previously diluted to 100 μM, 15 μl Power SYBR Green PCR Master Mix (Applied Biosystems) and 4.5 μl Nuclease-Free Water (Ambion). Reactions were performed in a 7900 HT Sequence Detection System (Applied Biosystems) using the following thermocycling conditions: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C and then 1 min at 60 °C, for 40 cycles. Each reaction was performed in triplicate on the same plate. The specificity of each primer pair was confirmed by the presence of a single melting temperature peak. The Ct was automatically calculated using the accompanying SDS 2.3 sequencing software (Applied Biosystems). The reference gene used for this experiment was ACT1, as described previously (Theberge et al., 2013). Results were analysed using the 2⁻ΔΔCt relative expression method.

Statistical analysis. For biofilm assays, statistical significance was determined using a one-way ANOVA with Dunnett’s multiple comparison post-test, comparing treatment conditions to the negative controls. For qRT-PCR experiments, statistical significance was determined using two-tailed t-tests comparing treatment conditions to the negative control.

RESULTS

Prevention of C. albicans biofilm formation by probiotic combinations

The combined SFSs collected from overnight cultures of SD5870 and CBS N116411, both with and without the SFS of DSM 14685, significantly reduced the ability of C. albicans to adhere to polystyrene plates when incubated for 3 h under hyphae-inducing conditions. No difference was observed between samples where probiotics were grown separately versus in co-cultures overnight (Fig. 1a). Experiments performed on polystyrene were used as
‘proof-of-concept’ experiments, before utilizing the more applicable polyurethane models. The combined SFSs collected from overnight cultures of SD5870 and CBS N116411, both with and without the SFS of DSM 14685, significantly reduced the ability of *C. albicans* to adhere to polyurethane discs (Fig. 1b). Fig. 2 shows that live SD5870 and CBS N116411 cells mixed equally in a 10:1 ratio with *C. albicans* cells were able to significantly reduce adherence of the latter to polyurethane discs. The addition of DSM 14685 cells to the mixture further decreased *C. albicans* biofilm formation, but the observed effects were not statistically different from the mixture of the two lactobacilli alone (Fig. 2).

### C. albicans biofilm disruption by probiotic combinations

In addition to preventing the initial formation of *C. albicans* biofilms, the ability of combinations of SD5870, CBS N116411 and DSM 14685 to disrupt and detach preformed *C. albicans* biofilms on polystyrene/polyurethane surfaces *in vitro* was investigated. The combined SFSs collected from overnight cultures of SD5870 and CBS N116411, both with and without the SFS of DSM 14685, significantly reduced the amount of preformed *C. albicans* biofilm on the polystyrene plate. Results were consistent between samples when probiotics were grown separately versus in co-culture (Fig. 3a). Similarly, the combined SFSs collected from overnight cultures of SD5870 and CBS N116411, both with and without the SFS of DSM 14685, reduced the amount of preformed *C. albicans* biofilm remaining on the polyurethane discs, albeit in a statistically insignificant manner (Fig. 3b). The combination of live SD5870 and CBS N116411 showed the greatest ability to disrupt the preformed biofilms. The combination including DSM 14685 did not provide as great a reduction in biofilm size; however, the decrease was still statistically significant (Fig. 4).

#### Table 1. Primer sequences used for qRT-PCR (Theberge et al., 2013)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| ACT1 (actin reference gene) | F: GCTGGTAGAGACTTGACCAACCA  
R: GACAATTCTCCTTCAGCAGCATGATGGA | 87 |
| ALS3 (invasion/adhesin) | F: AATGGTCCTTATGAACTCCACTACTA  
R: GAGTTTTCTACATCATGATTTCACCAT | 51 |
| EAP1 (hyphal wall protein) | F: CTCGCTACTCACAATTGATGTCG  
R: GAACACATCGACCTTCGGGA | 51 |
| EFG1 (positive transcriptional regulator) | F: TATGCCCGAGCAAAAACCTG  
R: TGTTGTCCTGGTGCTGTGGTC | 202 |
| NRG1 (negative transcriptional regulator) | F: CACCTCAGTTGCAACCCC  
R: GCCCTGAGATGGTCTGA | 198 |
| HWI (major hyphal wall protein) | F: GTCAACTTATGCTATGCTTTATTACA  
R: GACCGCCTACCGTGTTGACAGT | 67 |
| SAP5 (secreted protease) | F: CAGAATTTCCCGTACGATGAGA  
R: CATTGTGCAAAGTAACTGCAACAG | 78 |

#### Fig. 1. Prevention of *C. albicans* biofilm formation using probiotic SFSs. Biofilm formation was assessed after *C. albicans* cells were cultured on (a) 96-well polystyrene plates or (b) polyurethane discs in the presence of combined SFS for 3 h. Results are mean ± SEM representative of three separate experiments. Significant reductions in adherent cells were observed on both surfaces (***P<0.001; ****P<0.0001).
the effects of SFS on bacteria for 3 h. Results are mean ± SEM representative of three separate experiments. The addition of S. salivarius DSM 14685 to the probiotic cocktail did provide greater biofilm inhibition, although not significantly different from the lactobacilli alone (*P<0.05; **P<0.01).

Modulation of C. albicans gene expression by probiotic SFS

Based on our data showing the combined SFSs of SD5870, CBS N116411 and DSM 14685 both prevented the formation of and reduced the amount of C. albicans biofilms, the effects of SFS on C. albicans gene expression was investigated. C. albicans was incubated with two different combinations of probiotic SFS or a low dose of amphotericin B (1 μg ml\(^{-1}\)). The fungicidal amphotericin B significantly downregulated the expression of SAP5 and HWP1 genes (Table 2). Although not significant, ALS3 was consistently downregulated. In contrast, the expression levels of EAP1, EFG1 and NRG1 genes were all significantly increased (Table 2). The combination of SD5870 and CBS N116411 SFS significantly decreased the expression of ALS3 (0.294-fold), EFG1 (0.53-fold), HWP1 (0.0094-fold) and SAP5 (0.51-fold). Similar results were observed with the triple combination of SD5870, CBS N116411 and DSM 14685, except that EFG1 was not downregulated. ALS3 expression significantly decreased by 0.428-fold, HWP1 by 0.198-fold and SAP5 by 0.572-fold. The results indicated that amphotericin B and the selected probiotic SFSs downregulated several similar C. albicans genes related to adhesion, invasion and counteraction of host defences, although amphotericin B also promoted several unique mechanisms of gene modulation.

DISCUSSION

It is now clear that the transition of C. albicans from budding yeast to a filamentous hyphal morphology is pivotal to the formation of pathogenic biofilms (Harriott & Noverr, 2011). Indeed, C. albicans EFG1 knockout strains exhibit deficiencies in biofilm formation, growth and virulence (Lo et al., 1997). Furthermore, overexpression of the hyphae-repressing NRG1-encoded transcription factor inhibits filamentous growth and biofilm formation, whilst promoting yeast dispersal (Sudbery et al., 2004; Uppuluri et al., 2010). All current evidence suggests that preventing yeast–hyphae morphogenesis will prevent biofilm formation, thereby reducing the risk of oral candidiasis and more serious systemic Candida infections. Although there are several methods available for immediate treatment of oral candidiasis, including the fungicidal polyenes (amphotericin B, nystatin), fungistatic azoles (fluconazole)
and the recently emerging fungicidal echinocandins (caspofungin), there lacks a clearly defined method of ongoing prevention of oral candidiasis in susceptible populations, other than proper oral hygiene (Andes, 2003; Bal, 2010; Williams & Lewis, 2011). Long-term administration of such drugs to populations suffering from recurrent candidiasis occasionally yields toxic effects in humans and may promote antifungal resistance (Williams & Lewis, 2011). Although these problems are relatively minor, the development of a product that reduces the risk and severity of oral candidiasis infections, and that could be taken repeatedly without potential toxic side-effects, would be ideal. Many commercially available probiotic products, such as Align and Accuflora, are marketed for the prevention of gastrointestinal disorders and the maintenance of good health when administered on a regular basis. Only recently have probiotics begun to be considered for their potential to maintain proper oral hygiene. For example, probiotic cheeses and drinks such as Yakult LB have been shown to reduce salivary Candida prevalence (Hatakka et al., 2007) and increase anti-Candida salivary IgA levels (Mendonça et al., 2012). S. salivarius DSM 13084 was shown to prevent hyphal growth and biofilm formation of C. albicans in plastic Petri dishes, and protect mice from oral candidiasis (Ishijima et al., 2012). These results indicated that DSM 13084 may prevent oral candidiasis by binding C. albicans and preventing its adhesion to mucosal and abiotic surfaces.

Our study sought to expand on previous findings that probiotics such as S. salivarius DSM 13084 could prevent C. albicans hyphal growth and biofilm formation, by utilizing novel combinations of candidate probiotics. Our results demonstrated that the combination of live SD5870 and CBS N116411 could significantly reduce the ability of C. albicans to form biofilms on polyurethane surfaces when co-incubated under hyphae-inducing conditions. The inclusion of DSM 14685 in the probiotic combination further decreased the ability of C. albicans to form biofilms; however, the results were not significantly different from the combination of the lactobacilli alone. It is important to note that although a product aimed at promoting oral homeostasis and health should be designed to maximize the probability of probiotic strains colonizing the oral cavity, this may not always be the case. The natural salivary flow in the oral cavity poses a challenge to probiotic colonization, and therefore it is crucial to consider whether secreted metabolites and/or effector molecules can provide similar effects to the live probiotic cultures. To this effect, the ability of probiotic SFSs to prevent C. albicans biofilm formation was tested. Similar to the results using live probiotic cultures, the combined SFSs of SD5870 and CBS N116411 significantly reduced biofilm formation on

Table 2. Gene expression after 3 h incubation under hyphae-inducing culture conditions (YEPD media supplemented with 0.5 % FBS, at 37 °C and 5 % CO₂)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Untreated C. albicans</th>
<th>Amphotericin B</th>
<th>L. plantarum and L. helveticus combined SFS</th>
<th>L. plantarum, L. helveticus and S. salivarius combined SFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold change*</td>
<td>Fold change*</td>
<td>Fold change*</td>
<td>Fold change*</td>
</tr>
<tr>
<td>ALS3</td>
<td>1.00</td>
<td>0.707</td>
<td>0.1514</td>
<td>0.294</td>
</tr>
<tr>
<td>EAP1</td>
<td>1.00</td>
<td>3.30</td>
<td>0.0094</td>
<td>1.12</td>
</tr>
<tr>
<td>EFG1</td>
<td>1.00</td>
<td>5.76</td>
<td>0.0048</td>
<td>0.530</td>
</tr>
<tr>
<td>NRG1</td>
<td>1.00</td>
<td>11.1</td>
<td>0.0069</td>
<td>0.935</td>
</tr>
<tr>
<td>HWPI</td>
<td>1.00</td>
<td>0.237</td>
<td>0.0015</td>
<td>0.0094</td>
</tr>
<tr>
<td>SAP5</td>
<td>1.00</td>
<td>0.562</td>
<td>0.0009</td>
<td>0.510</td>
</tr>
</tbody>
</table>

*Calculated using the $2^{-\Delta\Delta Ct}$ method for relative expression. Data are presented as the fold change in gene expression relative to the untreated C. albicans negative control after normalization to the PCR product of the ACT1 endogenous reference gene.

†Obtained by t-test comparison with the untreated C. albicans control.
both polystyrene and polyurethane surfaces. The inclusion of DSM 14685 SFS in the mixture did not improve results, but importantly did not diminish the observed effects. Finally, as any multi-strain probiotic product has the inherent risk of certain strains inhibiting the observed effects of others, all three probiotic strains were incubated together overnight before harvesting the co-culture SFS. The results were similar to when the individual supernatants were combined, if not slightly more efficacious, and thus it was concluded that these strains would form an ideal combination.

Our results are consistent with newly published findings that showed that several species of lactobacilli were individually effective at inhibiting the growth of various Candida species, including C. albicans and Candida glabrata (Jiang et al., 2015). Additionally, a large-scale in vitro study recently revealed that several commercially available lactobacilli-containing probiotic formulations, such as Culturelle and Accuflora, were effective at inhibiting C. albicans biofilm formation on dentures, which our polyurethane models sought to imitate (Ujaoney et al., 2014). Although the live cell mixtures from these products were effective, the cell-free supernatants isolated from these formulations proved even more efficacious. Supporting this finding, Jiang et al. (2015) indicated that the ability of lactobacilli to inhibit growth of Candida species was most pronounced at lower pH values (~5.5), whereas the effects were diminished as the pH increased to 7.2. Although the inclusion of S. salivarius DSM 14685 cells or supernatant did not significantly improve the efficacy of our treatment groups, it did not inhibit the effects and therefore may still warrant being included in a multi-strain product designed to prevent oral candidiasis. As with S. salivarius DSM 13084, strain DSM 14685 may provide alternate mechanisms of C. albicans biofilm prevention, perhaps by binding and sequestering C. albicans cells (Ishijima et al., 2012).

Following up on our initial results, we decided to investigate whether the same combination of SD5870 and CBS N116411 cells or supernatants, with or without DSM 14685 cells or supernatant, could be used to disrupt existing C. albicans biofilms. Our data indicate that the combination of SD5870 and CBS N116411 cells significantly reduced the density of preformed biofilms on polyurethane discs. The addition of DSM 14685 to the mixture slightly diminished this effect, perhaps due to DSM 14685’s competition for nutrients, which may indirectly increase the local pH. Similar trends were observed on polystyrene when the combined probiotic SFSs were used. The addition of DSM 14685 SFS diminished the disruption effects compared with the combination of SD5870 and CBS N116411 SFS alone, except when the three strains were grown together in co-culture. On polyurethane, both the double combination and triple combination of probiotic supernatants reduced the amount of biofilm, but in a statistically insignificant manner. Taken together, Figs 3 and 4 lend credence to the efficacy of the tested probiotic combinations in disrupting preformed C. albicans biofilms. Our data appear to indicate that for the purposes of detaching existing biofilms, a probiotic product comprising the combination of SD5870 and CBS N116411 may be more or equally as effective as a combination that includes DSM 14685. However, the inclusion of DSM 14685 may be warranted in a product seeking to manage oral conditions other than just oral candidiasis, as DSM 14685 is already proven to be an effective antagonist to Streptococcus mutans – the most prominent initiator of dental caries (Burton et al., 2013). To the best our knowledge, this is the first study that has examined the ability of probiotics to not only prevent C. albicans biofilm formation, but also disrupt preformed biofilms.

As a number of studies now point to the ability of certain lactobacilli to prevent the formation of C. albicans biofilms on various surfaces, we investigated what global effects in gene regulation may be preventing C. albicans from forming a biofilm. The combined supernatants of SD5870 and CBS N116411 significantly downregulated four C. albicans genes that are normally upregulated during the yeast–hyphae transition: ALS3, EFG1, HWP1 and SAP5. Similar trends were observed with the combination of SD5870, CBS N116411 and DSM 14685; however, EFG1 was not significantly downregulated and each gene was downregulated to a lesser extent than when the lactobacilli-only combination was used. Thus, similar to the initial biofilm prevention and disruption experiments, the genetic analysis indicates that the lactobacilli-only combination may be more effective at preventing oral candidiasis. These results shed new light on the mechanism of probiotic-induced inhibition of C. albicans biofilm formation and point towards inhibition of the cAMP–Efg1p axis in the yeast cells. The EFG1 gene encodes the transcription factor Efg1p, which is a critical transcriptional activator for several hyphae-specific genes and essential for the yeast–hyphae transition (Ramage et al., 2002). Examples of such genes include ALS3, HWP1 and the SAPs, including SAP5 (Staib et al., 2002). Therefore, the observed reduction in EFG1 expression is likely responsible for the dramatic decrease in expression of these three downstream genes. Perhaps most significantly, the combined lactobacilli supernatants caused a massive 99 % reduction in HWP1 expression. The HWP1 gene encodes the hyphal cell wall protein Hwp1p, which forms covalent bonds with human epithelial cells, is critical to biofilm formation, and has been touted as an ideal target for therapeutics aimed at treating and preventing oral candidiasis (Nobile et al., 2006). This finding almost single-handedly helps explain the observed probiotic-induced inhibition of biofilm formation and lends significant support to probiotic usage as anti-candidiasis prophylactics. The decreases in expression of ALS3 (~70 % reduction) and SAP5 (~50 % reduction) indicate a role of probiotics in reducing the virulence and subsequent dissemination capability of C. albicans. The ALS3-encoded Als3 protein is involved...
in binding to epithelial cells, endothelial cells and extracellular matrix proteins, forming biofilms on prosthetic surfaces, and sequestering iron from human ferritin (Liu & Filler, 2011). It also functions as one of two hyphae-specific invasins that bind to E-cadherin and N-cadherin receptors on host cell receptors and induce endocytosis of C. albicans, which can lead to severe cases of invasive candidiasis. SAP5 is one of 10 genes encoding secreted aspartyl proteases. These are hydrolytic enzymes that increase virulence by degrading host antibodies and promoting apoptosis of epithelial cells (Gropp et al., 2009; Reinholdt et al., 1987). SAP5 was examined due to it being highly upregulated during the yeast–hyphae transition and due to its potent effects as an apoptosis inducer. Studies investigating the ability of probiotics to modify the expression of other SAP genes are warranted (Gow et al., 2012; Naglik et al., 2003; Wu et al., 2013). Comparatively, the fungicidal amphotericin B also caused significant decreases in expression of HWP1 and SAP5, and a decrease in ALS3 (although statistically insignificant). Interestingly, none of these three genes were downregulated as strongly as with the combined probiotic supernatants. Paradoxically, amphotericin B increased expression of \textit{EFG1} (5.8-fold), as well as the downstream gene \textit{EAP1} (3.3-fold), which encodes \textit{Eap1p}, a hyphae-specific cell wall protein that assists \textit{Hwp1p} in the binding of human epithelial cells and prosthetic surfaces (Li & Palecek, 2003). These amphotericin B-induced effects have likewise been documented in previous studies and thus were not surprising (Theberge et al., 2013). One unique effect of amphotericin B was that it caused an 11-fold increase in expression of \textit{NRG1}, which encodes the Nrg1p protein – a repressor of hyphae-specific genes and general antagonist to Ef1p. These effects likely outweigh those caused by an increase in expression of \textit{EFG1}, and explain the related decreases in expression of \textit{HWP1}, \textit{SAP5} and \textit{ALS3}.

The results of our study indicate that a combination of SD5870 and CBS N116411 probiotics may be a safe and effective means of preventing the formation of \textit{C. albicans} biofilms, and thus oral candidiasis. The inclusion of DSM 14685 in the combination did not drastically improve or reduce the \textit{in vitro} efficacy and may still prove a worthy inclusion in a product designed to target multiple oral indications, such as candidiasis and dental caries. Interestingly, the probiotic combinations also proved effective in disrupting preformed \textit{C. albicans} biofilms. This could have future implications in the treatment of oral candidiasis. Finally, the mechanisms by which the probiotic supernatants may act to prevent the yeast–hyphae transition and biofilm formation at the genetic level were examined. Results point to the downregulation of \textit{EFG1}, \textit{HWP1}, \textit{ALS3} and \textit{SAP5}, i.e. genes critical to the yeast–hyphae transition, biofilm formation, host cell invasion and virulence. Future studies should examine the short- and long-term clinical effects of a product containing SD5870 and CBS N116411 in populations susceptible to recurrent oral candidiasis.

ACKNOWLEDGEMENTS

The authors would like to thank Jamie Louwagie for technical assistance, and Tania Bayoud (PharmD, BCPS) for her valuable clinical and pharmacological insight. Funding was provided by an NSERC ERA grant to P. A. C. K. M. J., R. M. C. and K. W. M. are employees of Integra Medical Inc.

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