Candida albicans is a commensal saprophytic fungus that colonizes the oral cavity of humans. However, overgrowth of C. albicans can result in clinical presentation of candidiasis that includes a disturbance of the oral microbiome. Of interest, wearers of partial as well as complete dentures are at a significantly high risk of oral candidiasis.1,2 In an extensive review, Gendreau and Loewy report that 15 to 70% of denture wearers have dental stomatitis and that the oral hygiene related risk factors of this condition are significantly associated with morbidly increased colonization of C. albicans.3

Of the various virulence properties of C. albicans, formation of biofilms plays a critical role in maintenance of dental and oral hygiene.4 Biofilms represent unique niches for microbial growth, where microorganisms are encased in a self-produced extracellular matrix and are protected from the action of antimicrobial agents, saliva and immune host cells. It has been reported elsewhere that biofilm-associated C. albicans cells, compared with cells grown in planktonic form, are resistant to antifungals used to treat denture stomatitis.5 Thus, the ability of C. albicans to form biofilms on epithelial surfaces and prosthetic devices reduces its susceptibility to antifungal agents,6,7 as well as fosters accumulation of detrimental bacteria.

In this regard, probiotics have emerged as a fascinating potential intervention in the last 2 decades.8-11 It is noteworthy that several probiotics are already available for use over-the-counter. Of interest, a recent clinical trial suggests that in the elderly population, the use of probiotics can reduce...
the prevalence of oral candidiasis. Mechanistically, however, it is unclear whether this reduction of the risk of candidiasis can be attributed to the potential influence of probiotics on the biofilms formed by C. albicans. While evidence from murine models is suggestive of this mechanism, direct evidence based on denture materials is currently lacking. In this study, we therefore evaluated the in vitro effect of various over-the-counter probiotics on the ability of C. albicans to form biofilms on denture strips.

Methods and Materials

Study Design

This study was conducted in the biosafety level-2 laboratory facility of the Center for Medical Mycology, Department of Dermatology, Case Western Reserve University. Four over-the-counter probiotic supplements were used in the study namely; Accuflora® [mixture of Lactobacillus acidophilus, Lactobacillus rhamnosus, Bifidobacterium bifidum, Lactobacillus salivarius, Streptococcus thermophilus] and the 4 probiotics) constituted the source of the probiotic material (bacteria or suspension or to probiotic supernatants). Growth of C. albicans was confounded by the use of the MRS medium.

Experimental Protocols

The protocol described by Chandra et al to form biofilms on denture strips was utilized. The denture strips were first pre-coated with saliva (filter-sterilized through a 0.2 micron filter) and then subjected to formation of candida biofilms. This was achieved by application of an 80 µL quantity of standardized C. albicans cell suspension to the surface of 2 sets of positive controls (that is only C albicans without any probiotic intervention) – one using the MRS medium and another using the synthetic dextrose (SD) medium. This was done to examine if the influence of the probiotic bacteria on the biofilm-forming ability of C. albicans was confounded by the use of the MRS medium.

Statistical Analysis

Statistical methods for analysis included comparison of group means using 2-way analysis of
Results

A total of 50 dental strips were used in this study – for each source of the probiotic material (supernatant or bacteria), 1 negative control was used, 1 set of triplicates for C. albicans cultured on MRS medium, 1 set of triplicates for C. albicans cultured on SD medium, 1 set of triplicate each for each probiotic brand co-cultured with C. albicans and 6 dental strips for fluorescence microscopy. The experimental conditions were standardized by running quality control measures. Figure 1A shows that on fluorescence microscopy, the blank denture strips did not show any contamination, while C. albicans showed robust growth on both the MRS and the SD media. When the results were quantified using the XTT-reduction assay (Figure B), it was observed that, with the exception of the MRS-grown C. albicans which was used as a positive control in the experiments with bacteria, all other subgroups showed a CV<10%. The Bland-Altman plot (Figure 1C) indicated that while all observations on the MRS- and SD-grown C. albicans biofilm quantifications were within acceptable change, the MRS-grown C. albicans had slightly lower absorbance values. Pittman’s test indicated that the variances of the MRS-
Figure 2: Effect of Probiotics on Biofilm Formation by C. Albicans

(A) Qualitative results from fluorescence microscopy. Panels show that compared to the average unchallenged C. albicans (top row) Accuflora-supernatant-challenged and Culturelle-supernatant-challenged C. albicans formed thinner and patchy biofilms, respectively but the Align-supernatant-challenged and Sustenex-supernatant-challenged C. albicans biofilms were dense. On the other hand, all probiotic bacteria-challenged C. albicans showed visible and mostly dense or non-uniform biofilms.

(B) Two-way analysis of variance of mean optical density estimated from the XTT reduction assay based on source (supernatant versus bacteria) and brand of probiotic used. The bubbles are proportional to the mean optical density shown alongside.

(C) Post-hoc pairwise comparisons of mean optical density in the XTT reduction assay using Scheffe’s correction for multiple comparisons. Differences are shown as horizontal color-coded bars (blue for supernatants and pink for bacteria) and statistically significant results are identified by a color-coded star on the right.

and SD-grown C. albicans biofilms were not equal (p=0.008). Considering these results and since we aimed at having a single positive control for the ensuing analyses, the average of absorbance from the MRS- and SD-grown XTT-assays as the positive control was measured.

When the C. albicans biofilms were metabolically quantified after co-culturing with the indicated probiotic, it was observed (Figure 2B) that the mean absorbance from the XTT indicated wide variations across combinations of the study factors – source of probiotic and the brand of probiotic. Results of the 2-way ANOVA showed that both the factors contributed significantly to the inter-replicate variation in absorbance. Challenge with the supernatant was associated with a significantly lesser biofilm formation than challenge with the probiotic bacteria (p=3.8x10^-6). Therefore, to find out which probiotic brand is associated with maximum beneficial reduction of the biofilm formation, a post-hoc pairwise comparisons (using Scheffe’s correction) was
conducted separately for each source of probiotic material. When the analyses for supernatants were conducted (blue bars and stars in Figure 1C), it was found that the Accuflora and Culturelle-challenged C. albicans were associated with significantly reduced biofilms as compared to the non-challenged, Align-challenged or Sustenex-challenged C. albicans biofilms. In contrast, when the analyses were conducted for the bacterial challenge (pink bars and stars in Figure 2C), it was found that only Accuflora-challenged C. albicans was associated with a moderately reduced biofilm formation. On the other hand, C. albicans challenged with Culturelle bacteria showed mild inhibition that was not statistically significant. Results obtained from the quantitative XTT-reduction assay concurred qualitatively with those of fluorescent microscopy (Figure 2A).

Discussion

The results demonstrate that, in vitro, some commercially available probiotic formulations can reduce the biofilm-forming ability of C. albicans. Interestingly, only formulations that contained Lactobacillus species (Accuflora® and Culturelle®) appeared to have a statistically significant inhibitory effect on C. albicans suggesting that Lactobacillus species may be the sole organism responsible for the observed effect. Moreover, this effect was accentuated when the supernatants were used rather than the bacteria. To our knowledge this is the first study that demonstrates the inhibitory effect of over-the-counter probiotics on C. albicans biofilm production in vitro. Interestingly, these results are fully concordant with the series of observations in murine models of oral candidiasis.8-10,18 These results also afford indirect credence to the recent observations that probiotics can reduce the oral yeast counts in the elderly,12 as well as the growing body of evidence showing the potential use of probiotics against localized candidiasis at other sites in the body that include urogenital and gastrointestinal colonization of C. albicans.19-22

These results are important since oral candidiasis is a common condition in denture-wearers and accounts for a substantial proportion of morbidity.1-3 From a hygienic perspective, our results raise the possibility that the oral microflora may be an important contributor to oral candidiasis in denture-wearers.

An evident limitation of the study is its in vitro disposition which constrains its ready generalizability. Indeed, Bilhan et al have recently shown that the counts of C. albicans, as well as Lactobacillus, are increased in aged patients with denture-related stomatitis.23 Our findings somewhat agree with this observation since we found that the culture supernatants rather than the bacteria proffer beneficial advantage against C. albicans. However, this question cannot be directly answered by the current study. Next, the fact that supernatants rather than bacteria were more effective in inhibiting biofilm formation somewhat limits the clinical enthusiasm for a direct use of over-the-counter probiotics since some biochemical processing (e.g. lyophilization24) may be required before probiotics can be used for reduction of C. albicans biofilm. Another limitation of this study is that, by design, a commercially available probiotic formulation was used. Due to this design, however, it is not possible to estimate the relative efficacy of Lactobacillus species in inhibiting C. albicans biofilm formation. Although this places restrictions on the mechanistic interpretations from the results, it was deemed best to err on the side of clinical ease of use. In the absence of guidelines for choosing appropriate ratios of probiotics to fungal preparations, the ratio of 1:1 was chosen empirically. This is a potential limitation as it is unknown whether a different ratio might show even more significant effects of probiotics in inhibiting fungal biofilm formation. Further studies are warranted to explore the effects of different levels of probiotic to fungal load ratio.

Conclusion

Our results point towards 2 interesting directions for future research. First, simple and relatively inexpensive dietary interventions like yogurt consumption can be considered as a basis of treatment or prevention of oral candidiasis. A field trial for such intervention for diarrhea prevention has not shown encouraging results, but its value in candidiasis is unknown.25 Second, it is possible that metabolic by-products of Lactobacilli might interfere with the binding properties or the metabolic activity of C. albicans.26,27 It is also possible that the fungal growth inhibition may be consequent to the depletion of nutrients in the culture media by overgrowth of the probiotic bacteria. Future studies need to dissect out these mechanistic possibilities.

Shweta Ujaoney, MDS is currently a dental student at Virginia Commonwealth University School of Dentistry. Jyotsna Chandra, PhD is a Senior Research Associate at the Center for Medical Mycology, Department of Dermatology, University Hospitals of Cleveland and Case Western Reserve University, Cleveland, OH. Fady Faddoul, DDS, MSD is Professor and Vice-Chairman of the Department of Comprehensive Care and Director of the AEGD and Faculty Practice programs at Case Western Reserve University School of Dental Medicine. Maya Chane, D.D.S, M.S. is a Senior Instructor in dentistry.
Acknowledgments

This research was funded by Centre for Medical Mycology, Department of Dermatology (CWRU) and National Institutes of Health grant to MAG (RO1DE17846) and to PKM (R21EY021303 and R21AI074077) and the Oral HIV/AIDS Research Alliance (OHARA, grant number BRS-ACURE-S-11-000049-110229). The probiotic samples were provided by Department of Advanced Education in General Dentistry, Case school of Dental Medicine, Cleveland, Ohio.

References


