In Vitro Effect of Over-the-Counter Probiotics on the Ability of Candida Albicans to Form Biofilm on Denture Strips

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Abstract

Purpose: There is a burgeoning recognition and interest in the potential of probiotics in the treatment and prevention of oral candidiasis associated with the use of dentures. Our aim was to investigate if commercially available over-the-counter probiotics can influence the ability of Candida albicans to form biofilms, which is considered a hallmark of the initiation and progression of oral candidiasis.

Methods: We conducted a 2x5 factorial in vitro study to culture C. albicans on denture strips and challenge with one of the following four commercially available probiotics in bacterial or cell-free supernatant form: Accuflora®, Align®, Culturelle® and Sustenex®. C. albicans biofilm formation was studied in triplicates in all factorial combinations of the study and assessed qualitatively with fluorescence microscopy and quantitatively with tetrazolium salt (XTT) reduction assay. Quality control measures included determination of coefficient of variation, Bland Altman plots and Pittman's test. Results were analyzed using two-way analysis of variance (ANOVA) with pairwise post-hoc Scheffe's tests.

Results: Our experimental conditions passed the quality control checks. Two-way ANOVA results indicated that cell-free supernatants provided a stronger and significant inhibitory effect on biofilm formation than their bacterial counterparts (2-way ANOVA $p=3.8x10^{-6}$). Further, Lactobacillus-containing probiotic formulations (Accuflora® and Culturelle®) significantly reduced biofilm formation especially in supernatant form.

Conclusion: Commercially available probiotics that contain Lactobacilli species interfere with the in vitro ability of C. albicans to form biofilms on dentures. The mechanistic and clinical implications of our results need to be addressed by larger in vivo studies.

Keywords: candidiasis, dentures, probiotics, biofilm, experimental studies

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Introduction

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.[12] 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 500 million colony forming units (CFU) per caplet], Align® (Bifidobacterium infantis 35624, 1 billion bacteria per capsule), Culturelle® (Lactobacillus GG, 10 billion bacteria per capsule) and Sustenex® (Bacillus coagulans BC30, 2 billion bacteria per capsule). We cultured the probiotic bacteria in Man-Rogosa-Sharpe (MRS) medium for 36 hours. C. albicans 10341 was used for the formation of biofilm on denture strips after culturing in YNB/Dextrose for 24 hours and adjusted to a concentration of $1x10^7$ cells/mL using a hemocytometer. The probiotic bacterial density was calculated with nephelometry and aimed to obtain a probiotic:candida cell ratio of 1:1. For this, the turbidity of the bacterial broth culture was adjusted to obtain a turbidity-equivalent to 0.5 McFarland standard using nephlometer at 600 to 625 nm which yields an approximate cell density of $1.5x10^8$ cells/ml. This was then diluted with PBS to obtain bacterial density of $1x10^7$ cells/ml to obtain a probiotic:candida cell ratio of 1:1. Finally, cell-free solutions from bacterial cultures were obtained by centrifuging and filtering through a filter of 0.2 µl pore size

A 2x5 factorial design was used, where the source of the probiotic material (bacteria or supernatant) and the probiotic used (none or 1 of the aforementioned 4 probiotics) constituted the study factors. The study was designed to detect the potential influence of these two study factors on the ability of C. albicans to form biofilms. This ability was studied qualitatively (using fluorescent microscopy) as well as quantitatively (using tetrazolium salt assay). All 10 combinations of the 2 factors were studied in triplicates. As additional quality control measures, a blank negative control was used (neither C. albicans nor probiotics added to the denture strip) and 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.

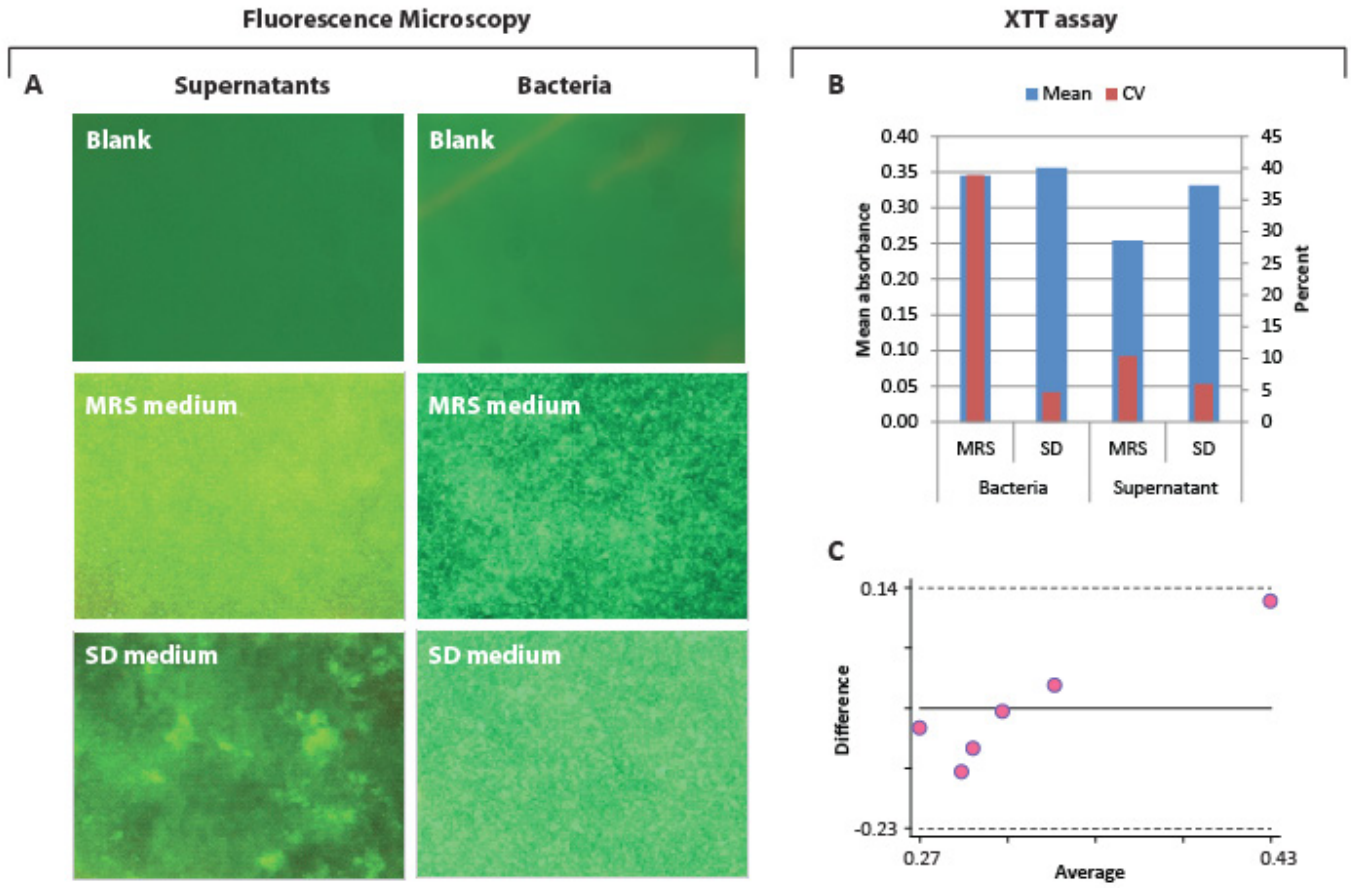
Experimental Protocols

The protocol described by Chandra et al to form biofilms on denture strips was utilized.[13] 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 1.5 cm^2 polymethylmethacrylate strips placed in a 12-well tissue culture plate. The cells were allowed to adhere to the strips for 90 minutes at 370 C. After washing away the non-adherent cells with PBS, the strips and the cells were incubated at 370 C to allow for biofilm formation. Following biofilm formation, the strips were transferred to either probiotic bacterial suspensions or to probiotic supernatants. Growth of the biofilms was quantified using the tetrazolium salt (XTT) reduction colorimetric assay.[14-16] For this, the denture strips were transferred to a new 12-well plate containing 4 ml of PBS in each well. Then 50 µl of XTT (1 mg/mL) and 4 µl menadione (1 mM) solutions were added to each well. The plate was then incubated for 3 to 5 hours at 37°C. After incubation, the solution from each well was centrifuged and absorbance measured at 492 nm using spectrophotometry as described elsewhere.[13] The morphology and architecture of the biofilm was examined using fluorescence microscopy (Zeiss, model Axio Imager Z1m; wavelength for Calcofluor white: excitation 440 nm and emission 500 to 520 nm).[13,17] Briefly, the denture strips were transferred to glass microscope slides and a drop of Calcofluor white solution was added to the slides. The slides were then incubated at room temperature for 1 minute and then examined under a fluorescence microscope. XTT and fluorescence analyses were performed by different investigators who were blinded to the results of each other.

Statistical Analysis

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

Figure 1: Quality Control of Experimental Conditions



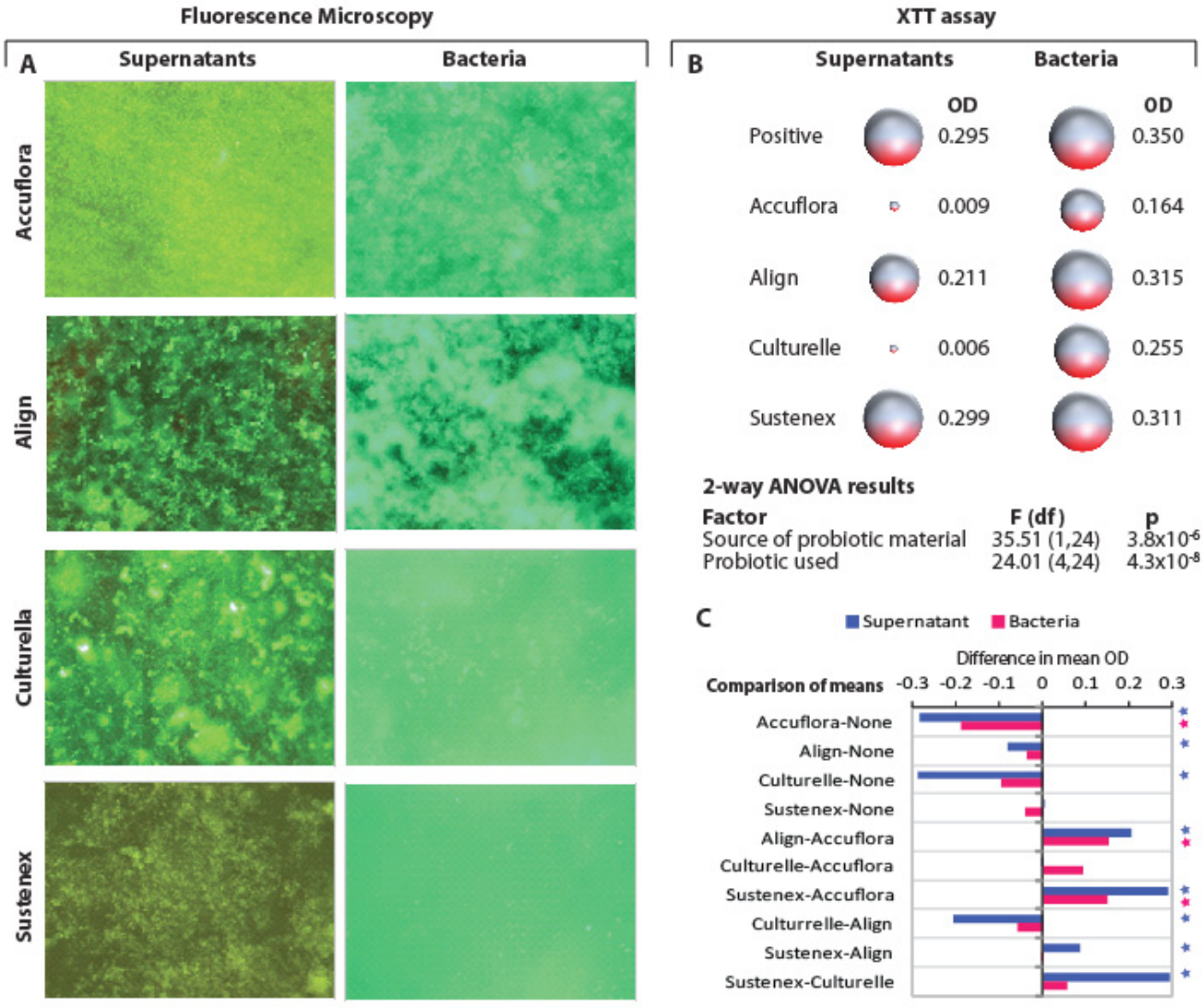
- (A) Results from fluorescence microscopy suggest that the blank strips (top row) were clear with no biofilm or C. albicans; the MRS-grown C. albicans (middle row) showed uniform biofilm matrix while the SD-grown C. albicans (bottom row) showed a dense biofilm with yeast forms.
- (B) Mean (wider blue bars) and coefficient of variation (narrow pink bars) of the absorbance optical density from XTT-assay based on the medium (MRS or SD) used for culturing C. albicans and whether the control was used in later analyses for probiotic treatment using the bacteria or the supernatant.
- (C) Bland-Altman plot for agreement between the absorbance values obtained from the MRS-grown and SD-grown C. albicans. The limits of agreement (dashed horizontal lines) were at 0.139 and -0.227 and the mean difference was -0.044 indicating slightly lower estimates of OD from the MRS-grown C. albicans. Pittman's p for equal variation in two methods was 0.008.

variance (ANOVA) and Bartlett's test for equal variances followed by post-hoc pairwise analyses using Scheffe's method. Quality control analyses included estimation of coefficient of variation (CV), Bland-Altman plots and Pittman's test for equal variances. Analyses were conducted using Stata 10.0 (Stata Corp, College Station, Texas) software and statistical significance was tested at a type I error rate of 0.05.

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 ($p=3.8\times10^{-6}$). 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 conduceted (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 hyhienic 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. lyophilization[24]) 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.

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