Anti-cariogenic Effects of S. *cerevisiae* and S. *boulardii* in S. *mutans*–C. *albicans* Cross-Kingdom In Vitro Model

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Posted Date: 11 January 2024

doi: 10.20944/preprints202401.0894.v1

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Article

Anti-Cariogenic Effects of *S. cerevisiae* and *S. boulardii* in *S. mutans–C. albicans* Cross-Kingdom In Vitro Model

Dina Yousif [1,†], Yan Wu [1,2,†], Alexandria Azul Gonzales [3], Christa Mathieu [4], Yan Zeng [1], Sample Lee [1], Sabrina Terando [5], Ting Li [1,6] and Jin Xiao [1,*]

[1] Eastman Institute for Oral Health, University of Rochester Medical Center, Rochester, NY, 14642, USA. D.Y. dina_yousif@urmc.rochester.edu; Y.W. yan_wu@urmc.rochester.edu; Y.Z. yan_zeng@urmc.rochester.edu; S.L. lee_sample@urmc.rochester.edu; T.L. ting_li@urmc.rochester.edu
[2] Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430042, China
[3] School of Medicine and Dentistry, University of Rochester Medical Center, Rochester, NY, 14642, USA. A.G. alexandria_gonzales@urmc.rochester.edu
[4] Virginia Commonwealth University, Richmond, Virginia, 23284, USA. C.M. chmathieu2021@gmail.com
[5] University of Rochester, Rochester, NY, 14627, USA. S.T. sterando@u.rochester.edu
[6] Key Laboratory of Laboratory Medical Diagnostics, Ministry of Education, Chongqing Medical University, Chongqing, 400016, China
* Correspondence: jin_xiao@urmc.rochester.edu; Tel.: +1 585-273-1957
† These authors contributed equally to this work.

Abstract: Despite well-documented health benefits of probiotics *Saccharomyces*, its application in oral health has not been comprehensively assessed. Dental caries is a transmissible disease initiated by acid production of cariogenic bacteria and yeast, such as *Streptococcus mutans* and *Candida albicans*, on tooth enamel and followed by subsequent enamel demineralization. Here, we investigated the effect of two *Saccharomyces* strains (*Saccharomyces boulardii*, *Saccharomyces cerevisiae*) on *S. mutans-C. albicans* cross-kingdom interactions using a cariogenic planktonic model. Viable cells, pH changes and gene expression were measured. *S. cerevisiae* and *S. boulardii* inhibited the growth of *C. albicans* either in dual-, or multi-species conditions. *Saccharomyces* also inhibited *C. albicans* hyphal formation. Furthermore, *Saccharomyces* reduced the acidity of the culture medium which usually plummeted below pH 5 when *S. mutans* and *C. albicans* were present in the model. The presence of *Saccharomyces* maintained the culture medium above 6 even after overnight incubation, demonstrating a protective potential against dental enamel demineralization. *S. boulardii* significantly downregulated *S. mutans atpD* and *eno* genes expression. Overall, our results shed light on a new promising candidate, *Saccharomyces*, for dental caries prevention due to its potential to create a less cariogenic environment marked by a neutral pH and reduced growth of *C. albicans*.

Keywords: *Saccharomyces cerevisiae*; *Saccharomyces boulardii*; *Candida albicans*; cross-kingdom interaction; pH; dental caries

1. Introduction

Early childhood caries (ECC) is the most common chronic childhood disease worldwide[1]. Untreated ECC has a negative impact on the oral health-related quality of life for children and their families[2,3]. Oral microorganisms are associated with ECC etiopathogenesis, for example, *Streptococcus mutans* is the well-known pathogenic bacteria for dental caries due to its acidogenicity and aciduric properties[4]. Recent advances in pediatric caries research also revealed the cariogenic role of fungi in ECC[5–7]. Specifically, *Candida albicans* has been shown to enhance cariogenicity through its synergistic interactions with *S. mutans* in producing acid, forming biofilms, and causing

 doi:10.20944/preprints202401.0894.v1

more severe caries[5–7]. Additionally, high levels of *Candida* species have been frequently reported in children with ECC[8–10].

Conventional measures, including oral hygiene management and pharmaceutical interventions, have been adopted for ECC prevention and treatment[3,11–13]. However, children remain at high risk for recurrent caries, due to either low adherence to positive oral hygiene habits, or ineffectiveness of antimicrobial applications[14–16]. More effective preventive strategies are critically needed. As a result, other alternative treatments, such as probiotics, have been investigated for their effects on oral health.

Probiotics are non-pathogenic live microorganisms that, when administered in appropriate quantities, can be beneficial to the health of the host [17]. Studies have shown beneficial effects of probiotics microorganisms in the oral cavity by inhibiting the abundance of pathogens[17]. For example, our previous work demonstrated the ability of *Lactobacillus plantarum* 14917 to inhibit the growth of *S. mutans* and *C. albican*s and the cariogenic biofilm formation[18,19]. These studies elicit the potential of probiotics to inhibit cariogenic polymicrobial interactions and prevent ECC. However, the inhibitory effect of *L. plantarum* on *S. mutans* and *C. albican*s was dependent on a higher dosage of *L. plantarum* that poses challenges to clinical application[18]. Thus, given this background, it is worth exploring the potential of other probiotics in disrupting cariogenic cross-kingdom interactions.

Saccharomyces boulardii is a variant of *Saccharomyces cerevisiae* and is a probiotic agent. *S. boulardii* is stable over a wide range of pH levels, temperatures, and exposures to bile salts and gastrointestinal enzymes [20]. *S. boulardii* is also incapable of promoting antibiotic resistance, as exchanging antibiotic-resistant genes between fungi and bacteria is unlikely[21,22]. Moreover, *S. boulardii* is absent from the natural gut microbiota but has been extensively studied in several gastrointestinal and systemic diseases. For example, studies have shown evidence that *S. boulardii* can prevent antibiotic-associated diarrhea[23] and prevent *Clostridium difficile*-associated colitis and traveler's diarrhea[24,25]. *S. boulardii* has also demonstrated effectiveness in treating urinary tract and vaginal yeast infections, high cholesterol levels, lactose intolerance, teenage acne, and fever blisters [26–28].

Regarding oral health, two randomized controlled clinical studies[29,30] provide supporting evidence of using probiotics *S. boulardii* as an adjunct to mechanical therapy that is used to manage periodontal disease. Moreover, Deshmukh *et al.* [31] assessed the impact of formulations with *S. boulardii* on oral health and found similar efficacy between chlorohexidine and probiotic mouthwashes on reducing dental plaque accumulation and promoting gingival health.

Saccharomyces cerevisiae, commonly known as Brewer's yeast, is a unicellular fungus [32]. Studies have revealed the benefits of *S. cerevisiae* strain to both systemic and oral health. For example, daily supplements of *S. cerevisiae* were found to significantly reduce gastrointestinal symptoms of irritable bowel syndrome in both mice and humans [33,34]. *S. cerevisiae*-based treatments also accelerated the clearance of *C. albicans* in mice with vaginal candidiasis [35]. Concerning oral health, *S. cerevisiae* has been shown to decrease *C. albicans* load and virulence in mice infected with oropharyngeal candidiasis [36]. Moreover, Premanathan *et al.* [37] observed a shorter recovery time from oral candidiasis in patients treated with topically applied *S. cerevisiae*.

Interestingly, *S. cerevisiae* shares several genes with *S. boulardii* that are involved in probiotic phenotypes[38]. These genes include *HSP150* and *YGP1* which regulate responses to stress and acidic pH tolerance; *HSP26* and *SSA4* which regulate heat responses; and *ARO9* and *ARO8* which are involved in the biosynthesis of aromatic alcohols, such as phenylethanol and tryptophol [38,39]. The ability of these aromatic alcohols can inhibit the virulence of *C. albicans*[40]. Moreover, *S. boulardii* has been reported to secrete medium-chain fatty acids, mainly capric acid, with bioactivity against *C. albicans* hyphae and biofilm formation [41,42].

With the above-mentioned characteristics of *S. cerevisiae and S. boulardii*, these two species demonstrate the potential to influence cariogenic microorganisms. However, the effect of *S. boulardii* and *S. cerevisiae* on cariogenic *S. mutans and C. albicans* cross-kingdom interactions has not been assessed. Our study aims to fill this gap by examining the effect of probiotic *S. boulardii* and *S. cerevisiae* on the growth of *S. mutans* and *C. albicans* in a cariogenic planktonic model that mimics a

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high-caries-risk clinical condition. The study results will provide insight to the role of *S. cerevisiae* and *S. boulardii* on cariogenic cross-kingdom microorganisms, and expand preventative and treatment options for dental caries, such as ECC.

2. Materials and Methods

2.1. Bacterial Strains and Starter Preparation

The microorganisms used in the study were *S. mutans* UA159, *C. albicans* SC5314, *and S. boulardii* ATCC MYA796, *S. cerevisiae* ATCC 204508. *C. albicans*, *S. mutans*, and *Saccharomyces* were recovered from frozen stock using YPD agar (BD Difco™, San Jose, CA, USA, 242720), blood agar (TSA with sheep blood, Thermo Scientific™, Waltham, MA, USA), and Yeast mold agar (BD Difco™, 271210), respectively. After 48 h incubation, 3-5 colonies of each species were inoculated into 10 ml of broth for overnight incubation (5% CO_2, 37°C). *C. albicans*, *S. boulardii, and S. cerevisiae* were cultured in YPD broth (BD Difco™, 242820); *S. mutans* was cultured in TSBYE broth (3% Tryptic Soy, 0.5% Yeast Extract Broth, BD Bacto™ 286220 and Gibco™ 212750) with 1% glucose. On the following day, 0.5 ml of the overnight starters was added to individual glass tubes with fresh broth and incubated for 3-5 h to reach the mid-exponential phase with desirable optical density. The morning starters were then ready for the preparation of the planktonic model described below.

2.2. Planktonic Model

Interactions between *C. albicans*, *S. mutans*, and *Saccharomyces* species were first evaluated in planktonic conditions. The inoculation quantity of *C. albicans* (10^3 CFU/ml) and *S. mutans* (10^5 CFU/ml) was chosen to simulate high caries risk conditions in the clinical setting. The inoculation quantity of the two *Saccharomyces* (10^7 CFU/ml) is the lower dose range of the probiotics used in the commercial probiotic products (10^9-10^{10} CFU as a single dosage).

Mono-species, dual-species, and multi-species models were used to assess the interaction between *C. albicans*, *S. mutans*, and *Saccharomyces* (either *S. boulardii* or *S. cerevisiae).* The planktonic models consist of three types: mono-species, dual-species, and multi-species. For the mono-species model, *C. albicans*, *S. mutans*, or *Saccharomyces* were incubated in 10 mL of TSBYE broth with 1% glucose for 20 h (5% CO_2, 37°C). For the dual-species model, either *C. albicans* or *S. mutans* were co-cultured with one of the *Saccharomyces* species for 20 h under the same conditions. For the multi-species models, *C. albicans*, *S. mutans*, and one of *Saccharomyces* species were cultivated for 20 h under the same circumstances. The colony-forming unit per milliliter (CFU/mL) and pH value were measured at 0, 2, 4, 6, and 20 h for each model.

Inhibition of *C. albicans* hyphae and pseudohyphae formation was evaluated by observing the culture mixture under a light microscope (Olympus BX43, 214, Tokyo, Japan) with a 100X oil objective (Olympus UPlanFL N 100X, Tokyo, Japan) at 0, 6, and 20 h. A quantity of 20 µL of the culture medium was placed on the glass slide and visualized without staining.

2.3. RT-PCR and Real-Time Quantitative RT-PCR (Real-time qRT-PCR)

The RT-PCR was performed in a thermal cycler (Applied Biosystems, Waltham, MA, USA), following the instructions provided by the manufacturer to assess the amplication of genes of interest. The primers used in this study are shown in Table S1 [43]. First, DNA extractions of *Saccharomyces* species and *C. albicans* were performed using MasterPure Yeast DNA Purification Kit (LGC Genomics, Berlin, Germany). The PCR was performed in a 50-volume containing 25 µl PCR Master Mix (2×) (Thermo Fisher Scientific, Bermen, Germany), 1 µl DNA template, 5 µl for each primer, and 14 µl nuclease-free water. The reaction was performed at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 1 min, with one final extension cycle at 72 °C for 10 min. The product of the PCR was run on a pre-cast 2% agarose gel (E-gel® Ex agarose gel from Invitrogen (Carlsbad, CA, USA) along with a DNA ladder (E-gel® 1 kb plus DNA ladder). The gel was run for 10 min and then visualized under UV light, and the picture was saved for documentation.

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Real-Time qRT-PCR was conducted to validate the expression of particular genes related to *C. albicans* and *S. mutans* virulence factors or viability. The primers used in this study are shown in Table S1. First, RNAs were collected and extracted from 4 mL culture media at 20 h. Then,1-4 μg of purified RNA were used to synthesize complementary DNAs (cDNAs) with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA)). Negative controls and the resultant cDNA were quantitatively amplified using Applied Biosystems™ PowerTrack™ SYBR Green Master Mix and a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Wilmington, DE, USA). Each 20 μL of PCR reaction comprised cDNA template, 10 μM of each primer, and 2× SYBR-Green mix (SYBR-Green and Taq DNA Polymerase). Three replicates were set up, and relative gene expression was determined using the comparative ΔΔCt method. Unique core genes of *S. mutans* (gyrA) was utilized as the housekeeping genes for gene expression comparisons.

2.4. Statistical Analysis

To compare the abundance of *C. albicans*, *S. mutans*, and *Saccharomyces* species in planktonic models, the CFU/mL values were first converted into natural log values before analysis. Zero values were retained as zero. Normality tests were conducted to assess the data distribution among the variables including pH value, natural log-converted CFU/mL value, and 2−ΔΔCT (real-time qRT-PCR value) at selected time points. When data were normally distributed, the difference between groups was examined using Student's t-test for two groups and one-way ANOVA for more than two groups followed by a post hoc test. Nevertheless, when data were not normally distributed, the Mann–Whitney U test was used to compare the results of the two groups, whereas the Kruskal–Wallis test was used to compare the results for more than two groups. Tests of statistical significance were two-sided with a significance level of $p < 0.05$. All analyses were performed in SPSS Version 24 (SPSS Statistics for Windows, Version 24.0; IBM, Armonk, NY, USA).

3. Results

3.1. Growth profile of Saccharomyces species

The growth curves of *S. cerevisiae* and *S. boulardii* in YPD or TSBYE with 1% glucose are shown in Figure 1. During the initial 8 hours, *S. cerevisiae* grew faster than *S. boulardi*, and both reached a plateau at 10 h. *S. cerevisiae* showed similar growth curves either in YPD or TSBYE with 1% glucose (Figure 1A). However, the growth of *S. boulardi* in TSBYE with 1% glucose was lower than the growth in YPD at 4 and 20 h (Figure 1B).

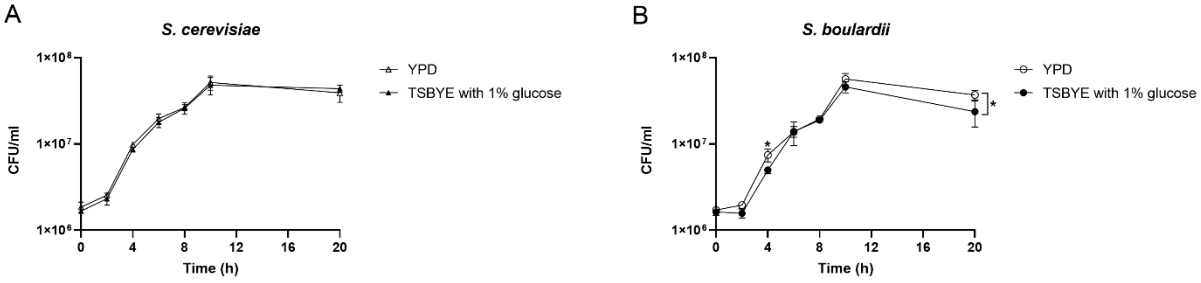

Figure 1. Growth curve of planktonic *Saccharomyces cerevisiae* (*S. cerevisiae*) (A) and *Saccharomyces boulardii* (*S. boulardii*) (B) in two culture mediums: YPD and TSBYE with 1% glucose. Data are presented as mean (± standard deviation) of three independent experiments performed in triplicates. * Indicate a significant difference in CFU/ml between different culture media, with $p < 0.05$.

3.2. The Impact of Saccharomyces Species on C. albicans and S. mutans in Dual- Species Conditions

Both *S. cerevisiae* and *S. boulardii* significantly inhibited the growth of *C. albicans* by 1 log at 4 h, 2 log at 6 h, and 6 logs at 20 h (Figure 2A). *S. boulardii* significantly inhibited the growth of *S. mutans* at 6 and 20 h. *S. cerevisiae* significantly inhibited the growth of *S. mutans* at early 2 and 4 h but failed at a later stage (Figure 2B). In contrast to the inhibited growth of *C. albicans* and *S. mutans*, *S. boulardii*

 doi:10.20944/preprints202401.0894.v1

grew better in the presence of *C. albicans* or *S. mutans* at 20 h (Figure 2C). However, the growth of *S. cerevisiae* in dual-species conditions declined at 20 h (Figure 2D), which may explain the loss of its inhibitory effect on *S. mutans* at a later stage.

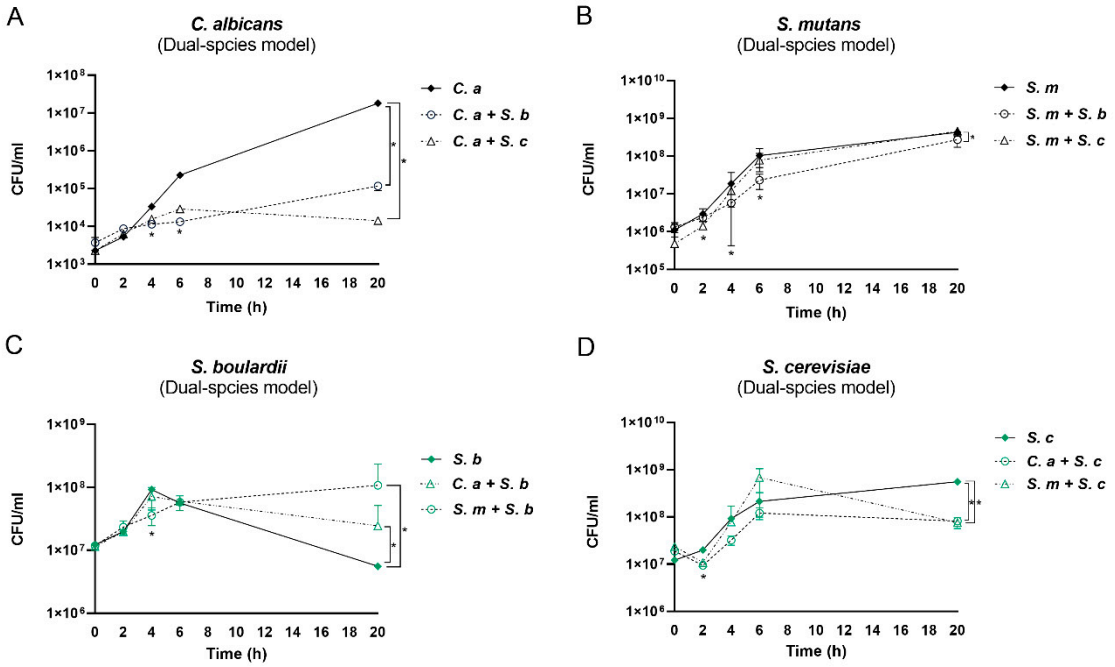

Figure 2. Interactions between *Saccharomyces* species and *C. albicans* or *S. mutans* in dual-species conditions. (A) The growth of *C. albicans* cultured with or without *Saccharomyces* species. (B) The growth of *S. mutans* cultured with or without *Saccharomyces* species. (C) The growth of *S. boulardii* cultured with or without *C. albicans*/*S. mutans*. (D) The growth of *S. cerevisiae* cultured with or without *C. albicans*/*S. mutans*. * Indicate a significant difference in CFU/ml between mono- and dual-species, with $p < 0.05$. C. a: *C. albicans; S. m: S. mutans; S. c: S. cerevisiae; S. b: S. boulardii.*

3.3. The Impact of Saccharomyces species on C. albicans and S. mutans in multi-species conditions

Intriguingly, both *S. cerevisiae* and *S. boulardii* significantly inhibited the growth of *C. albicans* at all time points (2, 4, 6, and 20 h) among multi-species conditions (Figure 3A). However, *Saccharomyces* species had a dampened inhibitory effect on *S. mutans* (Figure 3B). Compared to the *C. albicans-S. mutans* duo-species control, *S. mutans* grew faster when together with *Saccharomyces* at the early stage, however grew with a reduced speed between 12-20 h, although the viable counts at 20 h between groups have no statistical significance ($p>0.05$). Between the two *Saccharomyces* species in the multi-species model, *S. cerevisiae* had a slower growth speed than *S. boulardii* at 2 h, while a faster growth rate at the mid and late stages (6-20 h) (Figure 3C).

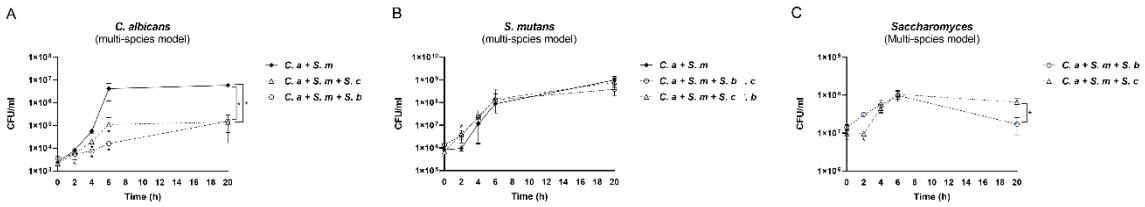

Figure 3. Interactions among *Saccharomyces* species, *C. albicans*, and *S. mutans* in multi-species conditions. (A) The growth of *C. albicans* in control (*C. a + S. m*) *and Saccharomyces* species treated groups. (B) The growth of *S. mutans* in control (*C. a + S. m*) *and Saccharomyces* species treated groups. * Indicate that $p < 0.05$ when comparing control with *Saccharomyces* species treated groups. (C) The

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growth of *Saccharomyces* species in multi-species conditions. * Indicate that $p < 0.05$ when comparing *S. cerevisiae* and *S. boulardii* treated groups.

3.4. Compositional changes of Saccharomyces species, C. albicans and S. mutans in multispecies model

Next, we assessed the *Compositional changes of the multispecies model over time*. When *S. cerevisiae* and *C. albicans* grew together, *S. cerevisiae* shows its dominance from beginning to the end, due to initial concentration of *S. cerevisiae* (10^7 CFU/ml) higher than *C. albicans* (10^3 CFU/ml) (Figure 4A). When *S. cerevisiae* and *S. mutans* grew together, the initial concentration of *S. mutans* is 10^5 CFU/ml. *S. cerevisiae* still seize its dominance from 2 h to 6 h, however until 20 h, *S. mutans* was able to prevail after a fierce competition with *S. cerevisiae* (Figure 4B).

Next, when *S. cerevisiae* grew with *C. albicans* and *S. mutans* in multi-species condition, compositional switch occurred. As shown in Figure 4C, at the beginning, *S. cerevisiae* took the lead due to its highest initial concentration. Shortly, *S. mutans* displayed rapid growth rates, took over the race and became the dominant species from 6 h to 20 h. *C. albicans* growth remained low over time. Intriguingly, compared to *S. mutans- S. cerevisiae* dual-species, *S. mutans* in multi-species model when *C. albicans* was present showed much stronger competitiveness against *S. cerevisiae*. This indicates an interspecies synergistic relationship between *C. albicans* and *S. mutans*, as well as synchronous antagonism between *S. cerevisiae* and *S. mutans*. A similar scenario was seen in the dual- and multi-species conditions when *S. boulardii* was present (Figure 4D–F).

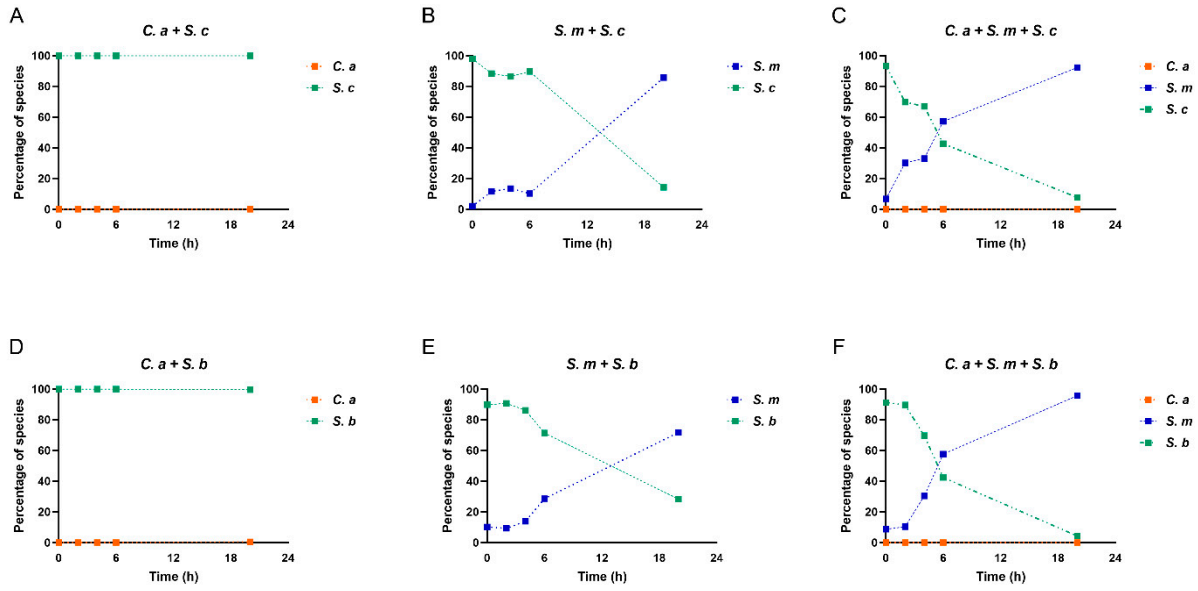

Figure 4. Changes in species composition in dual- and multi-species. (A–F) The composition of each microorganism in dual- and multi-species conditions.

3.5. Dynamic changes of culture pH in Mono-, Dual- and Multi-Species Conditions

Figure 5 shows the effect of *Saccharomyces* species on the environmental pH in mono-, dual- and multi-species models. Overall, the culture medium pH was lowered over time in all groups, particularly with a significant drop to pH 4.0 at 20 h in *S. mutans* mono-species and *C. albicans- S. mutans* dual-species. Significantly, the addition of either *S. cerevisiae* or *S. boulardii* neutralized the acidic environment and maintained the culture pH at 6.0 over the 20 hours period, which is above the well-known critical pH 5.5 for enamel demineralization.

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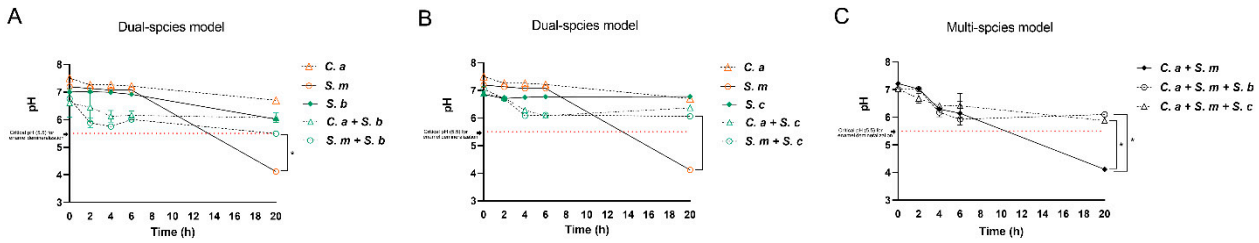

Figure 5. Dynamic changes of pH in the culture medium. (A) pH in mono-species condition and *S. boulardii* present dual-species condition. * Indicates that $p < 0.05$ when comparing dual-species (*S. b* + *S. m*) with mono-species (*S. m*) at 20 h. (B) pH in mono-species condition and *S. cerevisiae* present dual-species condition. * Indicates that $p < 0.05$ when comparing dual-species (*S. c* + *S. m*) with mono-species (*S. m*) at 20 h. (C) pH in control (*C. a* + *S. m*) *and Saccharomyces* species treated groups. * Indicate that $p < 0.05$ when comparing *Saccharomyces* species treated groups with control at 20 h.

3.6. Regulation of S. mutans and C. albicans Virulence Genes by Saccharomyces species

To evaluate the differential gene expression between the control and the experimental conditions with added *Saccharomyces* species, qRT-PCR was conducted at 20 h. To minimize the bias from gene expression crosstalk between *C. albicans* and *Saccharomyces species*, we first examined the expressions of *ACT1, EGR4, ECE1, and CHT2* in *C. albicans, S. cerevisiae* or *S. boulardii*. PCR amplification products confirmed that the above genes are expressed by *C. albicans* only, not by any of *Saccharomyces* species (Figure S1).

Next, as shown in Figure 6A, compared to *C. albicans- S. mutans* duo-species control, *S. boulardii* reduced the expression of *S. mutans* genes *atpD* (stress response gene related to ATPase complex and acid tolerance) and *eno* (associated with degradation of carbohydrates via glycolysis) by 1.4-fold ($p < 0.01$) and 2-fold ($p < 0.001$), respectively. In contrast, *lacC* and *lacG*, the genes involved in galactose metabolism, were significantly up-regulated when *S. boulardii* was added ($p < 0.0001$). The addition of *S. cerevisiae* had a negligible effect on the expression of *S. mutans* genes.

For *C. albicans* gene expression (Figure 6B), *S. cerevisiae* up-regulated the expression of *HWP1* and *ECE1* that are associated with hyphal growth, by 27.2-fold and 74.63-fold, respectively ($p < 0.001$). Whereas *S. boulardii* significantly upregulated the expression of another *C. albicans* virulence gene, *CHT2*, which is associated with fungal wall remodeling. EGR4, however, related to antifungal medication resistance is not affected by either *S. cerevisiae* or *S. boulardii*, statistically.

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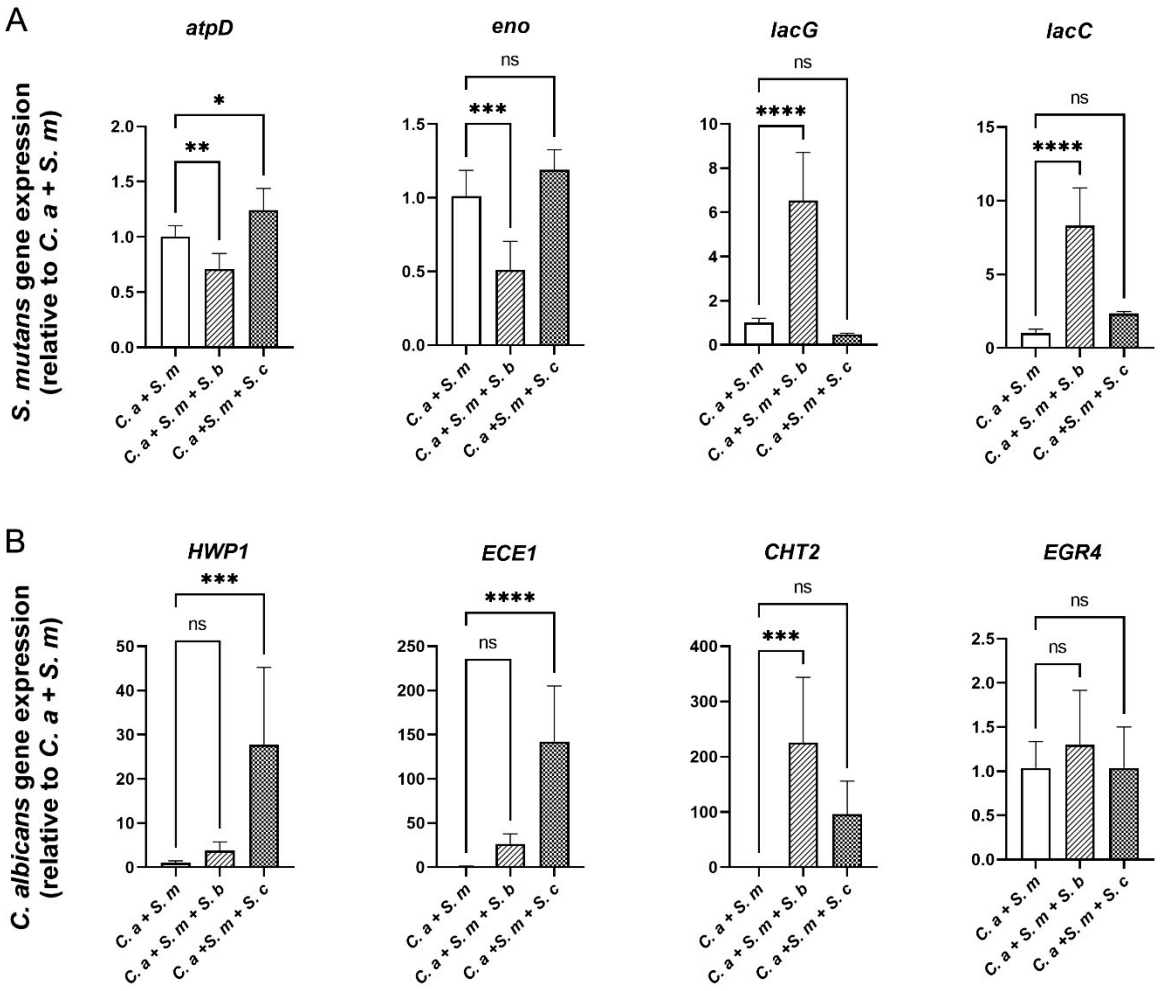

Figure 6. Effect of *Saccharomyces* species on the expression of *S. mutans* and *C. albicans* genes in multi-species model. qRT-PCR was performed for *S. mutans* (A) and *C. albicans* (B) genes of interest for mixed-species culture at 20 h. Relative mRNA levels were presented as ratios relative to control group *(C. a + S. m)*. Results are reported as the mean ± SD of 3 independent experiments. *p* values were determined by one-way ANOVA with post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.7. Inhibition of C. albicans Hyphae/Pseudohyphae Formation by Saccharomyces species

Inhibition of *C. albicans* hyphae or pseudohyphae formation was assessed by observing the culture mixture at 0 h, 6 h, and 20 h under a light microscope. In the *C. albicans- S. mutans* duo-species condition, *C. albicans* had a typical *Candidal* pseudohyphae formation at 6 h and elongated hyphal formations at 20 h. In comparison, the addition of *S. cerevisiae* or *S. boulardii* inhibited the growth of *C. albicans* in both yeast forms and the transition from yeast to hyphae or pseudohyphae form. The quantitative reduction of *C. albicans* by *S. cerevisiae* or *S. boulardii* observed in Figure 7 is consistent with the growth inhibition measured by CFUs.

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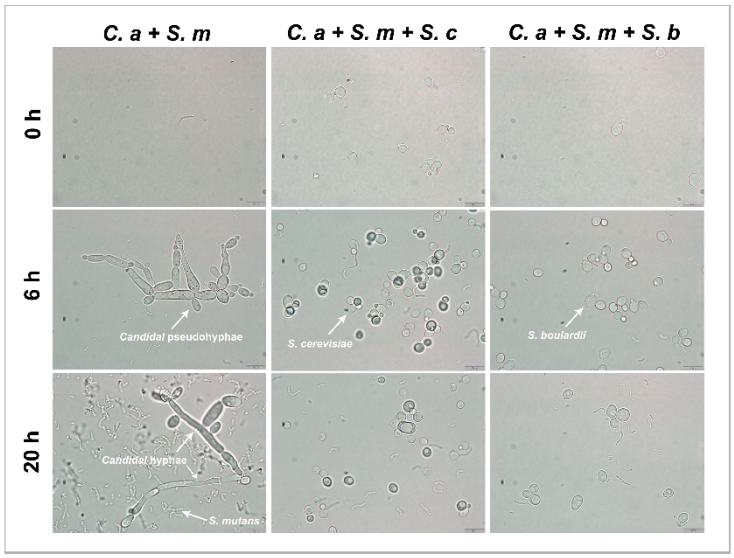

Figure 7. Inhibition of *Candidal* hyphae formation by *Saccharomyces* species in multi-species model at × 100 magnification. *C. albicans* that contains yeast-form, pseudohyphal and hyphal cells can be found in control group *(C. a + S. m),* but not in *Saccharomyces* species treated groups. These are representative images of multiple fields of view. Scale bars = 10 μm.

4. Discussion

While various treatment options have been employed to control ECC, mainly by targeting cariogenic pathogens[18,19], limited studies have assessed probiotic yeast on interrupting cariogenic bacteria–fungi cross-kingdom interactions. Our study revealed novel findings that the oral health effects of *S. boulardii* and *S. cerevisiae* are not solely limited to inhibition of the growth of oral pathogens, such as *C. albicans* and *S. mutans*, but also modulation of culture medium pH, influence on *C. albicans and S. mutans* virulence gene expression.

Kellis *et al*. and Wolfe hypothesized that the fermentative ability of this yeast complex could have evolved around the time that fruit-bearing plants with their abundant sugar appeared in the environment[44,45]. Results of the sequencing of the *S. cerevisiae* genome partially support this theory by revealing the presence of abundant genetic redundancy with a large number of genes devoted to sugar metabolism[46]. Today, *S. boulardii,* a probiotic yeast, is well known to interact with its host and exhibits antimicrobial activity, antitoxin and immune regulatory effects and provides various health benefits in humans[47].

In our models, the inhibitory effect of *Saccharomyces* species on growth of *C. albicans* was notable in both the dual- and multi-species conditions. A study by Krasowska, *et al*. show that the addition of live *S. boulardii* cells to *C. albicans* culture negatively affects two major virulence factors of this pathogenic fungus; S. *boulardii* excretes into the medium factors having an antagonistic effect on adhesion and filamentation of *C. albicans*[48]. *S. boulardii* strain has been found to inhibit *C. albicans*'s adhesion to mucosal cell lines and its extract reduces cytokine-induced inflammatory responses in Caco-2 cells as revealed by suppressed IL-8 expression[49]. *S. boulardii* strains have also been found to diminish filamentation, biofilm formation, and *C. albicans* translocation[48,50]. In our study, both *S. cerevisiae* and *S. boulardii* significantly inhibited the growth of *C. albicans* at all the time points especially among multi-species conditions. The competition for resources was apparent. *Saccharomyces* species competed with *C. albicans* and *S. mutans* for available nutrients. By utilizing sugars in the environment, *Saccharomyces* may limit the substrate available for the acid production by *S. mutans* and potentially reduce the metabolic activities of *C. albicans[51].*

Saccharomyces species, including *S. boulardii* and *S. cerevisiae*, are known for their fermentation activities. They metabolize sugars and produce organic acids (such as acetic acid and lactic acid) along with carbon dioxide and ethanol[52]. The genus can be described as the "sugar fungus", especially

 doi:10.20944/preprints202401.0894.v1

because they naturally occur in sweet-based substrates, such as nectar and fruits. This may explain their similar growth curve either in YPD or TSBYE with 1% glucose. The optimum pH for the growth of *Saccharomyces* species is 4.5–6.5 and oxygen is important to maintain viability, but they survive under microaerophilic conditions[53]. *C. albicans* occupies diverse ecological niches within the host and must tolerate a wide range of environmental pH. The plasma membrane H+-ATPase Pma1p is the major regulator of cytosolic pH in fungi. The maintained neutral pH in the culture medium when *S. boulardii* and *S. cerevisiae* interact with *S. mutans* and *C. albicans* could be attributed to several potential mechanisms, detailed below.

The production of organic acids (such as acetic acid and lactic acid) along with carbon dioxide and ethanol act as buffers, counteracting the acid produced by *S. mutans*, thus helping to maintain a higher pH[54]. Moreover, *Saccharomyces* may limit the substrate available for the acid production by *S. mutans* and potentially reduce the metabolic activities of *C. albicans[51]. Saccharomyces* species have been reported to produce antimicrobial compounds that could potentially inhibit the growth or metabolic activities of *S. mutans* and *C. albicans*, indirectly contributing to pH regulation[55]. Furthermore, *Saccharomyces* species could also influence the expression or activity of these virulence factors, indirectly impacting the acid production and biofilm formation of *S. mutans* and *C. albicans[48]*. This is verified by our PCR results, which showed *S. boulardii* significantly reduced the virulence gene expression of *S. mutans* (*atpD* and *eno*). The gene *atpD* is the acid-adaptive and related to acid stress tolerance response, while *eno* related to the degradation of carbohydrates via glycolysis. Lastly, the capability of *Saccharomyces* species for biofilm formation and matrix production may indirectly impact the local pH environment[52].

The expressions of several genes associated with *S. mutans* virulence were altered in multi-species models when *Saccharomyces* species were added. *S. boulardii* upregulated two virulence genes of *S. mutans*, *lacC* and *lacG*. The tagatose 6-phosphate kinase (*lacC*) and intracellular 6-phospho-β-galactosidase (*lacG*) both participated in galactose metabolism by *S. mutans[56]*. Liu *et al*. reported that *S. boulardii* strain could assimilate galactose but at a much lower rate than other *S. cerevisiae* strain[57]. This lower galactose utilization by *S. boulardii* was attributed to a single point mutation, G1278A. However, G1278A mutation is beneficial for *S. boulardii* cells to grow on glucose[57]. When *S. boulardii* and *S. mutans* were co-cultured for carbon source utilization, to maintain energy efficiency and competitiveness, *S. boulardii* selectively utilize more rapidly metabolizable glucose and *S. mutans* favor galactose. This may explain why *S. mutans* expressed higher levels of galactose metabolism related genes with *S. boulardii* rather than with *S. cerevisiae*.

Finally, we found *Saccharomyces* has a strong inhibitory effect on the crucial virulence factors of *C. albicans*, i.e., the ability to form filaments. The characteristic of *C. albicans* is that it can exist in three phases, budding yeast, pseudohyphae, and hyphae[58]. *C. albicans* is known to switch to a filamentous morphology upon incubation in serum at 37 °C and this ability is indispensable for the virulence of this organism. The plasticity of the mycelial form is a determinant factor of drug resistance and is also an important form during the infection stage[59]. In addition, the transformation of *C. albicans* from yeast to hypha can help fungi escape the phagocytosis of macrophages, resulting in an increased likelihood of invading host tissues and causing greater damage[60]. In this study, hyphae/pseudohyphae formation of *C. albicans* was assessed in the *C. albicans-S. mutans* duo-species condition, *C. albicans* had a typical pseudohyphae formation at 6 hours and elongated hyphal formations at 20 hours. The addition of *S. cerevisiae* or *S. boulardii* inhibited the growth of *C. albicans* in both yeast form and the transition from yeast to hyphae or pseudohyphae form. Similar to our present study, Krasowska, *et al*.[48] further demonstrated the inhibitory effect of live *S. boulardii* cells on the filamentation of *C. albicans* strain is proportional to the amount of *S. boulardii* added. *S. boulardii* live cells and the extract from its culture filtrate had a very strong inhibitory effect on *C. albicans* filamentation and biofilm formation. Worth noting, despite the reduction of *C. albicans* hyphae formation by *S. cerevisiae* or *S. boulardii observed under microscope*, the virulence gene expression of *C. albicans* (*HWP1*, *ECE1*, and *CHT2*) were found up-regulated by *Saccharomyces* species. This discrepancy between the gene expression and the observed hyphae formation reduction phenomena could be explained that *S. cerevisiae* or *S. boulardii* might have

 doi:10.20944/preprints202401.0894.v1

impacted the translation and protein synthesis process, which deserves further investigation in future studies.

Overall, our results provided evidence to an important gap in dental caries research by examining the effect of probiotic *S. boulardii* and *S. cerevisiae* on the growth of *S. mutans* and *C. albicans* in a cariogenic planktonic model that mimics a high-caries-risk clinical condition. The following limitations are recognized with the intriguing findings: although our study results indicated the interactions between *Saccharomyces* species, *S. mutans* and *C. albicans*, other cariogenic factors such as biofilm formation, enamel demineralization need to be assessed in biofilm and animal models. Second, our study introduced glucose as the sugar challenge in the planktonic model and future studies should assess other forms of carbohydrates, such as sucrose. Third, we used qRT-PCR to assess several virulence gene; however, high throughput methods such as RNA sequencing would offer more comprehensive understanding the global transcriptomic changes of *S. mutans* and *C. albicans* when they are interacting with *Saccharomyces* species, for which we plan to assess in future investigations.

5. Conclusion

To the best of our knowledge, this is the first study demonstrated the inhibition of *S. cerevisiae* and *S. boulardii* on the growth of *S. mutans* and *C. albicans* in a cariogenic planktonic model. The results shed light on a new promising candidate, *Saccharomyces*, for dental caries prevention due to its potential to create a less cariogenic environment marked by a neutral pH and reduced growth of common cariogenic pathogens.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, The Table S1, Primers used in PCR; Figure S1, PCR amplification products obtained with 4 types of primers.

Author Contributions: Conceptualization, D.Y., Y.Z. and J.X.; Methodology, D.Y., Y.W., Y.Z., and J.X.; formal analysis, D.Y. and Y.W.; funding acquisition, J.X.; investigation, D.Y., Y.W., A.G., C.M., S.L., S.T., T.L.; Data Curation, Y.W., and J.X.; project administration, Y.W. and J.X.; resources, J.X.; supervision, Y.W. and J.X.; validation, Y.W. and J.X.; writing—original draft Preparation, D.Y., Y.W. and A.G.; writing—review and editing, J.X. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by J.X. NIH/NIDCR K23DE027412 and J.X. R01DE031025. The funding agencies had no role in the study design, data collection, analyses, decision to publish, or preparation of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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