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A Tri-culture Model for Examining Polymicrobial Interactions

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A Tri-culture Model for Examining Polymicrobial Interactions

By

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Abstract

Candida albicans is a fungal microbe that is often present inside of humans in the mouth and gastrointestinal tract. It shares a mostly commensal relationship with its hosts but can develop into an opportunistic pathogenic infection under conditions of immune suppression. Oral thrush or candidiasis is an uncomfortable condition resulting from excessive growth of *Candida albicans* in the oral cavity. Candidiasis is prone to progressing into more threatening symptoms without proper treatment. There are few effective antifungal medicines used for treatment and the problem of antimicrobial resistance is growing. *Alcaligenes faecalis* is a bacterial microbe that does not pose a significant threat to human health in many cases. It is also present in the human gastrointestinal tract and shares an inhibitory relationship with *Candida albicans*. *Streptococcus mutans* is also a bacterial microorganism present in the oral cavity and GI tract of humans. It is one of the primary factors related to dental decay, one of the most common modern health issues humans face. In cases of dental caries, *Candida albicans* and *Streptococcus mutans* have been found to have positive correlation in the biofilm coating teeth. This study examines the effects on microbial growth under the presence of all three organisms in a tri-culture. The results of this experiment could help better understand how to inhibit growth of *Candida albicans* and *Streptococcus mutans* and promote oral health.

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Contents

Introduction

Candida albicans

Candida albicans (*C. albicans*) is a microbial fungus that lives inside most humans as a member of the normal microbiome present, especially in the gastrointestinal tract and skin (Nobile & Johnson, 2016). It has been found that 30% to 45% of healthy adults and as much as 74% of older people provide an oral environment for *Candida albicans* (Patel 2022). Under normal conditions, the presence of *C. albicans* does not pose a threat to human health; however, in compromised states, including conditions of suppressed immune function and damage to the intestinal barrier, it can lead to overgrowth and harmful infections (Kumamoto et al., 2020). When conditions are altered, such as when antibiotics are introduced into an individual's body or a person has an immune system disorder, the typical microbial environment is disturbed, providing a potential for overgrowth and harmful effects. Conditions including skin and mucosal infections, diaper rash, and vaginal infections are often resultant (Nobile & Johnson, 2016). Growth in the oral cavity can cause a condition known as oral candidiasis or oral thrush which often presents with uncomfortable symptoms like soreness, loss of taste, and characteristically white patches in the mouth. This infection may also spread into the bloodstream or further into the gastrointestinal tract and cause far more severe outcomes, with a mortality rate reaching as high as 79% in cases of systemic candidiasis (Patel 2022). Patients in the intensive care unit, as well as patients with damaged epithelial barriers face the greatest risk of nosocomial fungal infection from *Candida* rather than any other fungus (Kumamoto et al., 2020). Patients that spend significant time in the ICU or undergo transplant or abdominal surgery have an

increased likelihood of intraabdominal candidiasis (Kumamoto et al., 2020). The reason for *C. albicans*' ability to cause such infections is in part due to its ability to produce biofilms, which helps spread infection throughout the body (Nobile & Johnson, 2016). *Candida* biofilms are very common on medical devices such a urinary catheters and are also found on contact lenses, pacemakers, and dentures (Nobile & Johnson, 2016). These biofilms are problematic especially for their ability to withstand treatment from antifungal drugs, which causes the need for potentially damaging large doses of medications or removal of some surgically implanted devices. Another key factor enabling the pathogenesis of *C. albicans* is its ability to convert between morphologies of yeast and hyphal forms (Metwalli et al., 2013).

Alcaligenes faecalis

Alcaligenes faecalis (*A. faecalis*) is a bacterium often found in natural environments including water and soil, as well as healthcare locations on tools associated with moisture like respirators and intravenous fluids (Huang 2020). *A. faecalis* is gram negative and obligately aerobic. It is both oxidase and catalase positive but does not perform fermentation. It has been discovered in many different mediums such as stool, blood, urine, and sputum. This is indicative of its presence inside the gastrointestinal tube. *A. faecalis* is known for its resistance to antibiotics like anti-pseudomonas penicillin and quinolones (Huang 2020). Humans are threatened by opportunistic infections from *A. faecalis* as it is not yet well understood how to treat effectively with antibiotic therapy. Many diseases are associated with these infections, including meningitis, endocarditis, otitis media, and pneumonia (Huang 2020).

Streptococcus mutans

Streptococcus mutans (*S. mutans*) is a gram-positive pathogenic bacterium found in the oral cavity of humans (Metwalli et al., 2013). It was named so for its resemblance to streptococci but with characteristically oval shaped cells, which were thought to be resultant from mutation (Lemos et al., 2019). Dental caries or tooth decay is the most common disease that plague the mouth and the second most common disease found in humans (Metwalli et al., 2013). *S. mutans* has been identified as one of the major contributing factors to this oral disease (Krzyściak et al., 2014). It is found within dental plaque; plaque is a biofilm composed of many species of bacteria that coats the teeth (Lemos et al., 2019). *S. mutans* is effective at destroying the enamel of teeth as a result of its production of glucans (which allow for colonization on the surface of enamel) and lactic acid which helps lower pH, demineralizing teeth and suppressing other microbial members of the oral flora. This also allows other microbes which prefer EPS-rich and acidic environments to colonize and contribute to pathogenic effects as well. (Metwalli et al., 2013; Lemos et al., 2019). *Streptococcus mutans* also have a wider range of pathogenicity, as it has been linked to bacterial endocarditis, cerebral microbleeds, IgA nephropathy (Berger's disease), as well as atherosclerosis (Lemos et al., 2019). *S. mutans* also associate with other microbes to form complexes before they expand both in population growth and colonization throughout of the oral cavity. When integrated with other microbial species in plaque biofilm, *S. mutans* becomes much more difficult to remove mechanically with a toothbrush even (Cui et al., 2019). This biofilm production also serves to form a protective seal that guards against pH neutralization from water and saliva, maintaining the optimal acidic conditions of the

environment. Antibiotic effects are also reduced as a result of biofilm secretion, so *S. mutans* infections possess increased resistance to treatment (Cui et al., 2019).

Polymicrobial Interactions

The oral cavity and gastrointestinal tract is home to a plethora of different microbial organisms that are all in competition and collaboration with each other. Each set of microbes have a distinct relationship with unique qualities that allow them to fight for survival in their shared environment. Not all microbes are in competition with each other, looking to eliminate their cohabitants, however. *Candida albicans* and oral streptococci like *Streptococcus mutans* existing in tandem improves pathogenic progression; *C. albicans* aids in biofilm production, furthering dental decay, while *Streptococci* help *Candida* infections to become more detrimental to oral tissue (Koo et al., 2018). *C. albicans* is also linked with bacterial infections found colonizing periodontal spaces in gingiva and under dental prostheses (Koo et al., 2018). The mutual presence of *S. mutans* and *C. albicans* on its own is not particularly dangerous until carbohydrates are introduced, causing greater adhesion between the microbes, acidity, and extracellular matrix formation; these conditions all accelerate dental caries (Koo et al., 2018). *S. mutans* secretes exoenzymes called glucosyltransferases, stimulating increased production of extracellular matrix around *C. albicans*, causing formation of mixed biofilms. Beta glucosyltransferases adhere to *C. albicans* and converts sucrose into extracellular polysaccharides α-glucans which allows for greater cohesion between the two microbes and infection (Koo et al., 2018). *C. albicans* also switches on *S. mutans'* competence, virulence genes, and quorum sensing molecules to release Beta glucosyltransferases. *C. albicans* helps stimulates growth of

streptococcal species by secreting growth-stimulating factors as well as providing its own biofilm to interact with *S. mutans'* biofilm (Koo et al., 2018; Sztajer et al., 2014).

Previous Theories of Research

The history of microbial infections has been very troubling for human health. The introduction of antibiotics provided an invaluable tool to reduce deadly infections to manageable, often curable conditions. However, as time has passed and antibiotic use has reached alarmingly high rates, their effectiveness has diminished. There are few novel antibiotics currently being discovered and existing pathogenic microbes are only growing in resistance. Antibiotic resistance is an ongoing problem in the health community that provides great cause for concern. The discovery was made in our lab that *A. faecalis* shares an inhibitory relationship with *C. albicans*. This inhibitory effect appears to be contact mediated through an as of yet unidentified mechanism. To further explore the relationship and the extent of inhibition between these microbes, we introduced *S. mutans*. Our goal was to observe the potential protective effects it might have on *C. albicans* in the presence of *A. faecalis*. This would help to increase understanding of their tri-culture interactions and aid in the pursuit of the inhibitory capabilities they possess for our own uses of combatting infections.

Methods

Growth Conditions and Strains

The strains utilized in the following experiments were *Candida albicans* SC5314, *Alcaligenes faecalis* ATCC 8750, and *Streptococcus mutans* ATCC 25175. These cultures were obtained from freezer stocks and routinely sub-cultured on Brain Heart Infusion (BHI) agar plates. They were then individually inoculated into non-selective BHI

liquid media and grown in an incubator at 37 degrees Celsius with shaking at 122 rpm for approximately 18-24 hours. Luria Bertoni supplemented with Kanamycin [500ug/ml] (LBK) agar plates were used for selection in co-culture experiments also and kept at 37 degrees Celsius.

Agar Plate Interactions

Fresh overnight cultures (37°C, 122rpm, BHI broth) of *A. faecalis*, *C. albicans*, and *S. mutans* were used to make confluent lawns on BHI agar plates. The two competing microorganisms were then spotted (20ul) onto the opposing lawns. Plates were allowed to dry completely and incubated for 24 hours at 37°C. Plates were then observed for zones of inhibition (ZOI).

Co-culture Growth

After inoculation and sufficient growth, 100 microliters of fresh overnight cultures were pipetted into microtubes containing 900 microliters of phosphate buffered saline (PBS). The absorbance values of these cultures were then measured using a spectrophotometer set at 600nm. The OD_{600} value was used to calculate the amount to add to achieve $1x10^6$ cells/ml concentration of each microorganism for mono, dual, or tri-culture growth. Four culture conditions were then inoculated into tubes containing 5 milliliters of liquid BHI using these values. The four culture conditions consisted of *C. albicans* only as a control, *C. albicans* and *A. faecalis, C. albicans* and *S. mutans,* and the final culture contained all three microorganisms: *C. albicans*, *A. faecalis*, and *S. mutans*. All cultures were then placed in an incubator at 37 degrees Celsius on a shaker set to 122 rpm for approximately 18-24 hours. Serial dilutions were then created using 100 microliters of each culture in 900 microliters of PBS up to 5 dilutions (10^5). 100

microliters of each dilution from the four culture conditions were then pipetted onto LBK agar plates and spread across the medium using a sterile glass rod. The agar plates were then incubated at 37 degrees Celsius for 24-48 hours. Colony forming units were then recorded for each agar plate.

Single and Co-culture Gram Staining

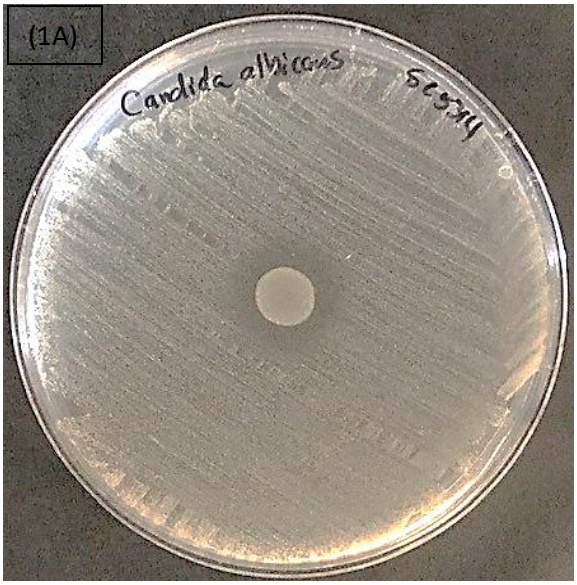
Individual cultures of *C. albicans*, *A. faecalis*, and *S. mutans* were grown in 5 milliliters of liquid BHI medium at 37 degrees Celsius. 50 microliters of each culture were placed in 2 milliliters of liquid BHI. Six cultures were grown in a six well plate: *C. albicans*, *A. faecalis*, *S. mutans*, *C. albicans* with *A. faecalis*, *C. albicans* with *S. mutans*, and *C. albicans* with *A. faecalis* and *S. mutans*. Each of these six cultures were placed on microscope slides, heat fixed, and Gram stained. A light microscope was used at 100X magnification to analyze apparent growth on each.

Results and Discussion

Alcaligenes faecalis inhibits *Candida albicans*, but not *Streptococcus mutans* on solid media.

To observe polymicrobial interactions on a solid medium, *C. albicans* was spread on BHI agar plates and *A. faecalis* and *S. mutans* were spotted on, incubated, and observed for zones of inhibition (ZOI). As seen in **Figure 1A**, *A. faecalis* produces ZOI on *C. albicans* lawns, denoting that there is an inhibitory interaction between *C. albicans* and *A. faecalis*. It has been previously shown by other researchers that *C. albicans* and *S. mutans* have a mutualistic relationship and this is evidenced by the *S. mutans* spots

on *C. albicans*. There is robust growth of the *S. mutans* colony, but no ZOI present where it interacts with *C. albicans*. Conversely, *A. faecalis* does not seem to inhibit *S. mutans* as seen in **Figure 1B** as there are no ZOI present when *A. faecalis* is spotted onto *S. mutans* lawns. Additionally, *C. albicans* and *S. mutans* do not appear to have a counter inhibition to *A. faecalis*. **Figure 1C** shows the opposite arrangement, *A. faecalis* lawn with *C. albicans* and *S. mutans* spots, and there are no ZOI on the *A. faecalis* lawn.



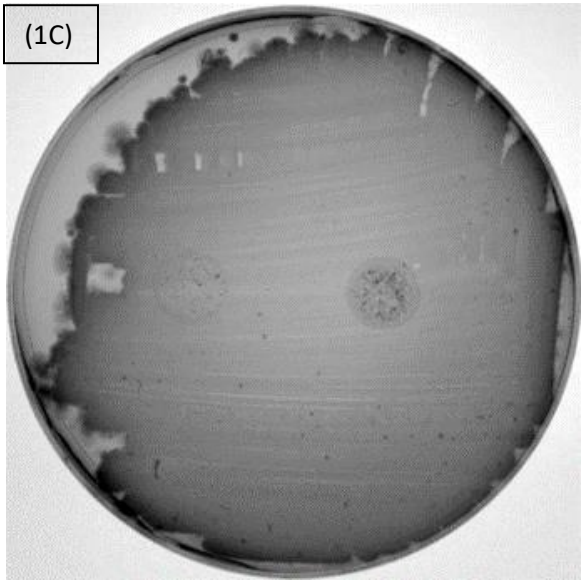


Figure 1: Microbial lawns of *C. albicans* (1A), *S. mutans* (1B), and *A. faecalis* (1C) were created on BHI agar plates and the competing microorganisms were spotted on and incubated for 24 hours.

Alcaligenes faecalis inhibits *Candida albicans*, but not *Streptococcus mutans* in liquid co-cultures.

To determine if the observations from the agar plate interactions could be applied to a liquid medium, mono and dual cultures of *A. faecalis* with *C. albicans* or *S. mutans* were created. Each microorganism was adjusted to a 1x106 cells/mL concentration, combined into BHI broth, and incubated for 24 hours. The cultures were then serially diluted and plated on selective media to enumerate colony forming units (CFUs). When cultured together, *C. albicans* growth has a 1.44 log reduction (equivalent to a 96.4 percent reduction) in the presence of *A. faecalis* as compared to the *C. albicans* only control (**Figure 2A**). However, when cultured with *S. mutans*, there is an insignificant reduction of growth by *A. faecalis* (**Figure 2B**). This further demonstrates the observations from Figure 1 that *A. faecalis* has an inhibitory effect on *C. albicans* in both agar and liquid medium but has no effect on *S. mutans*.

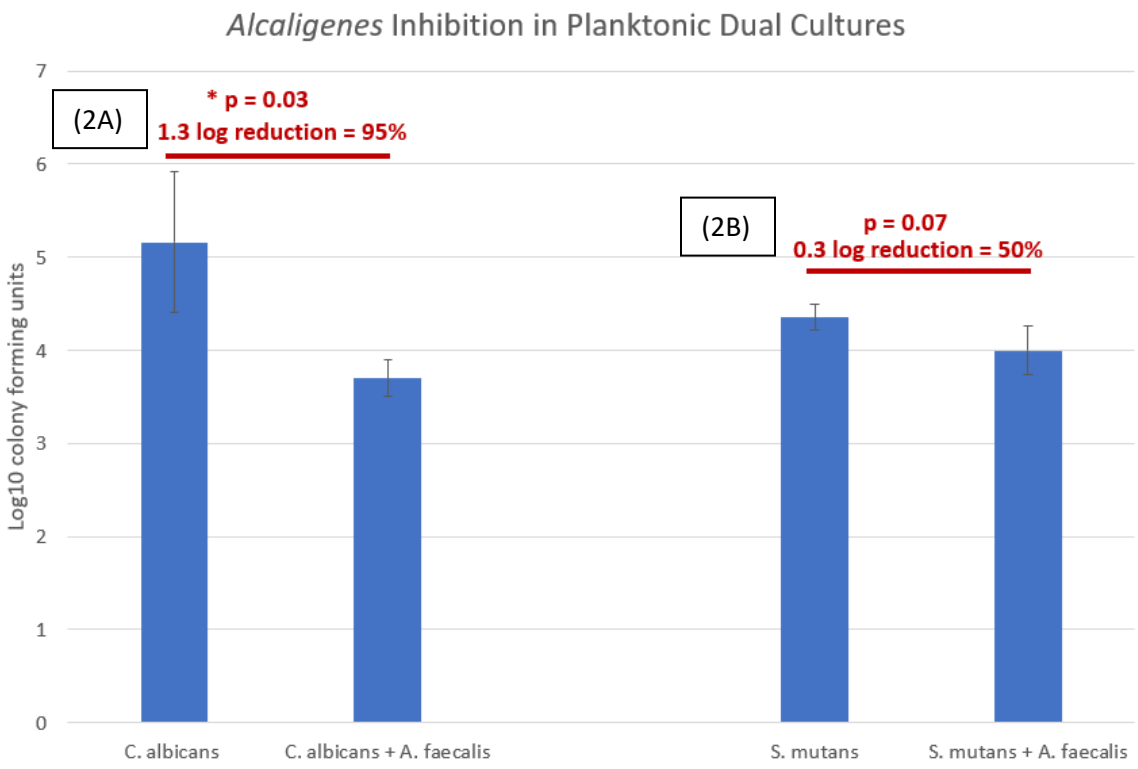


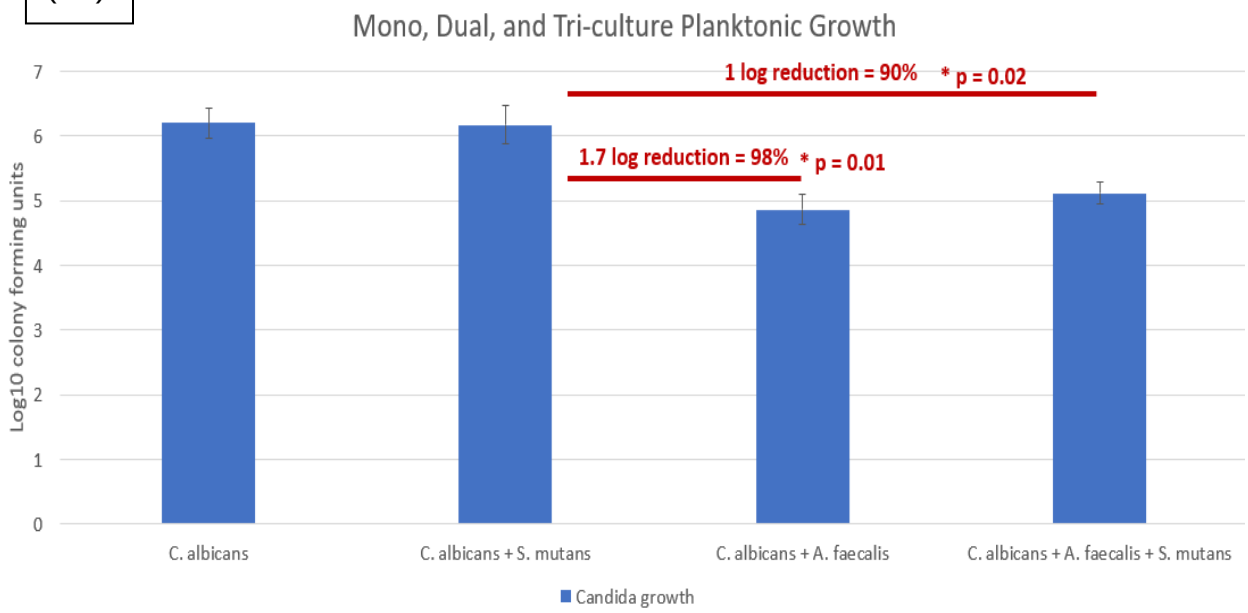
Figure 2: Mono and dual cultures of either *C. albicans* and *A. faecalis* (2A) or *S. mutans* and *A. faecalis* (2B) were inoculated and incubated for 24 hours with shaking. Cultures were serially diluted, plated on LBK, and CFUs enumerated.

Alcaligenes faecalis inhibits *Candida albicans* in both dual cultures and tricultures.

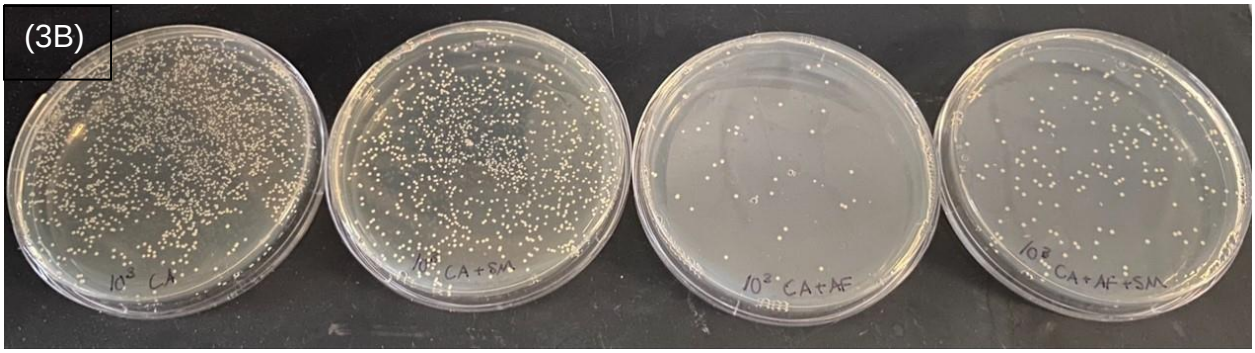
Previous research has identified a mutualistic relationship between *C. albicans* and *S. mutans* aiding in biofilm growth, invasion of host cells, and resistance to antimicrobial treatments. We wanted to take the observations from Figure 1 and 2 and determine if the inhibitory activity *A. faecalis* has on *C. albicans* is still present when a known synergistic microbe of *C. albicans and S. mutans* is included in the culture or if *S. mutans* and *C. albicans* are able to overcome the inhibitory action of *A. faecalis*. As displayed in **Figure 3A**, when cultured alone or with *S. mutans*, *C. albicans* has robust growth. When *C. albicans* is cultured with *A. faecalis*, the identified inhibition is present.

However, when in tri-culture, *S. mutans* and *C. albicans* together are not able to significantly overcome the suppressive effect *A. faecalis* has on *C. albicans* growth. The inclusion of *S. mutans* seems to provide a slight protective factor for growth of *C. albicans* in the presence of *A. faecalis*, but growth is still significantly inhibited. This can also be seen in **Figure 3B**. Plates with *C. albicans* and both *C. albicans* and *S. mutans* demonstrate excellent growth across the medium. The growth on the plate of both *C. albicans* and *A. faecalis* is visibly stunted compared with the other plates. While there is more growth on the plate containing all three microbes than just *C. albicans* and *A. faecalis,* growth is also diminished in comparison with plates containing only *C. albicans* and both *C. albicans* and *S. mutans*. To further characterize this inhibition, aliquots of the mono, dual, and tricultures were rapidly heat fixed and Gram stained in **Figure 3C**. As is seen from our previous research, *A. faecalis* inhibits *C. albicans* through an, as of yet unidentified physical mechanism. *C. albicans* is visible with typical characteristics in a single control stain. Upon introduction of *S. mutans*, there is apparent clustering with *C. albicans* in the same manner that leads to biofilm production which allows for protective effects and increased microbial durability. In the culture containing both *C. albicans* and *A. faecalis*, grouping is also visible. *A. faecalis* is present in close proximity, surrounding and aggregating with growth of *C. albicans* to allow access for its inhibitory effect on growth. The tri-culture consisting of *C. albicans*, *S. mutans*, and *A. faecalis* shows the clustering of all three microbes. *S. mutans* and *C. albicans* are growing in tandem, while *A. faecalis* is nestled close to the *C. albicans* growth to still limit fungal growth.

(3A)


Mono, Dual, and Tri-culture Planktonic Growth
1 log reduction = 90%
* p = 0.02
1.7 log reduction = 98%
* p = 0.01
Log10 colony forming units
0
1
2
3
4
5
6
7
C. albicans
C. albicans + S. mutans
C. albicans + A. faecalis
C. albicans + A. faecalis + S. mutans
Candida growth

(3B)


10² CA
10² CA+SM
10² CA+AF
10² CA+AF+SM

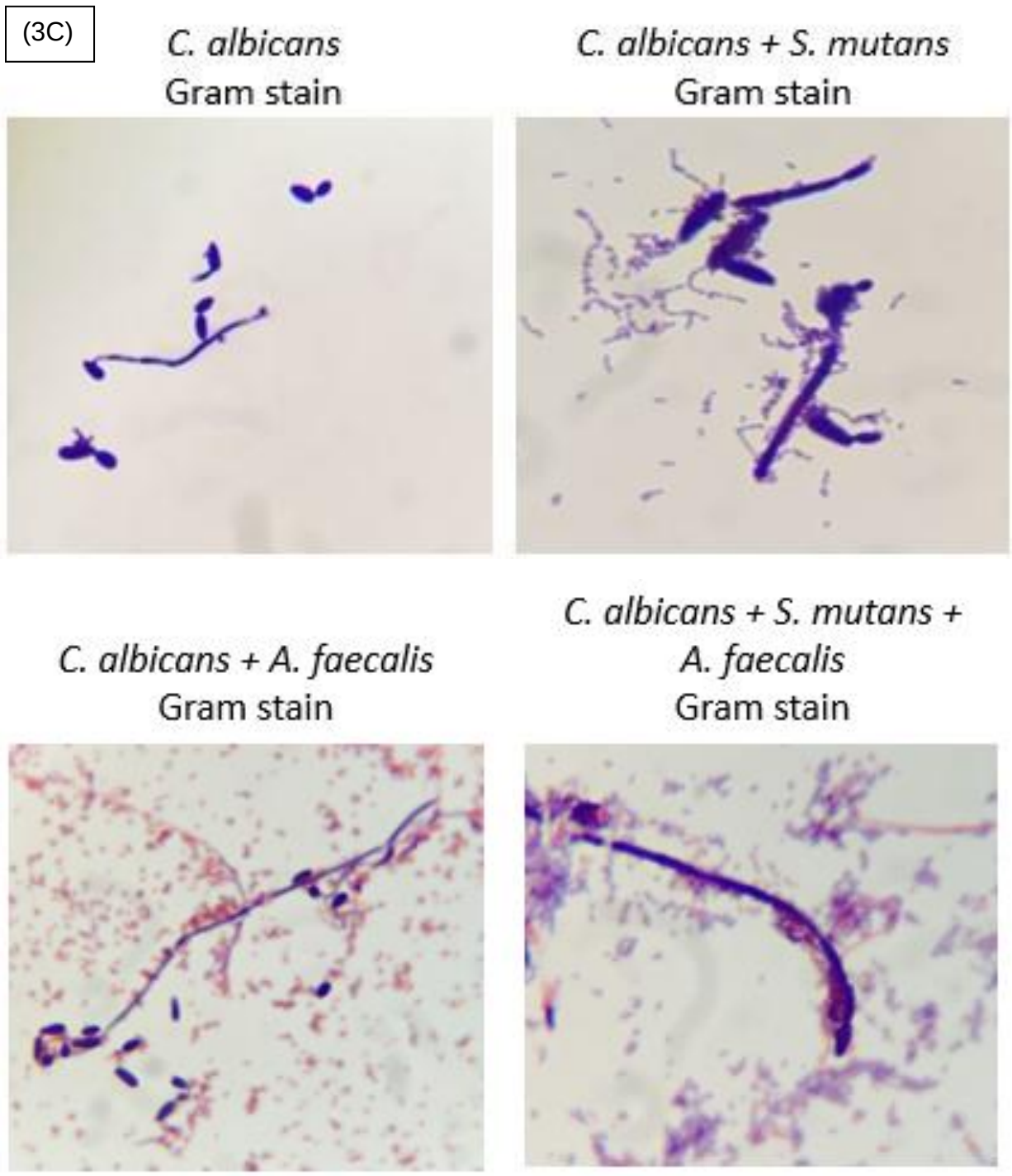


Figure 3: Mono, dual, and tri-cultures of *C. albicans*, *A. faecalis*, and *S. mutans* were inoculated and incubated for 24 hours with shaking. Cultures were serially diluted, plated on LBK, and CFUs enumerated (3A). Representative photos of the diluted plates demonstrate the inhibition of *A. faecalis* on *C. albicans* and the tri-culture is unable to overcome the inhibition (3B). Gram staining of the varying mono, dual, and tricultures shows the contact dependent nature of this inhibitory action with clusters of all three microbes aggregating together (3C).

Conclusion

Candida albicans, *Alcaligenes faecalis*, and *Streptococcus mutans* were cultured together in different combinations to better understand their effects on one another in a shared environment. It was confirmed through both liquid cultures and solid agar growth that *C. albicans* and *S. mutans* share a synergistic relationship, promoting growth. It was also confirmed through both liquid cultures and solid agar growth that *A. faecalis* has an antagonistic relationship with *C. albicans*, limiting growth. It was shown through culturing *S. mutans* and *A. faecalis* together that there is no significant antagonistic relationship between these two bacteria. When all three microbes were grown together, both liquid cultures and solid agar growth demonstrated suppression of *C. albicans* growth. The growth of *C. albicans* was greater when combined with *S. mutans* and *A. faecalis* than when it was only grown with *A. faecalis*, however, only slightly. This indicates the effectiveness of the inhibitory relationship *A. faecalis* shares with *C. albicans*. Even when *Candida albicans* and *Streptococcus mutans* are bound together, growing in tandem, *Alcaligenes faecalis* is able to penetrate their defenses and gain growth controlling access over them. In the future, more research will focus on determining tri-cultures' effects on growth with *S. mutans* as the primary variable of interest. Plating cocultures onto Phenylethyl Alcohol Blood agar (PEA) and Amphotericin B agar will allow for specific analyzation of growth from *Streptococcus* species. Determining the mechanisms of inhibition between these microorganisms would help better understand alternative methods of controlling pathogenic microbial growth during infections and provide alternatives to current antibiotics that are becoming increasingly less effective.

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