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Investigation Of The Synergistic Effect Of Select Phytochemical Containing Oils And Amphotericin B To Inhibit Fungal Biofilm Growth In Rhizopus Oryzae

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Abstract

Opportunistic pathogens, specifically those caused by fungus, have shown a significant increase over the last decade in infections by the Mucor, Rhizopus and Absidia organisms. These infections, which are sometimes fatal, are often made more difficult to treat by the formation of fungal biofilm. These biofilms result in increased resistance to antifungal agents as compared to planktonic cells. This study investigates the antifungal susceptibility of the primary species known to cause mucormycosis infections, *Rhizopus oryzae*. The combined effects of amphotericin B and various plant oils, (Oregano, Lemongrass, Cinnamon, Clove) were tested on fungal biofilms. Results indicate that *R. oryzae* is capable of forming biofilms and that these biofilms were significantly inhibited by all of the oils. The MIC_{50} of Amphotericin B on *R. oryzae* biofilm was 0.25 μg/mL. When applied as a single treatment, the MIC_{50} of each oil was as follows: cinnamon oil 0.2 μL/mL, clove oil 0.2 μL/mL, oregano oil 0.05 μL/mL, and lemongrass oil 0.025 μL/mL. When using Amphotericin B and an individual oil in combination treatment, the MIC_{50} of all four combinations was reduced substantially at an oil concentration of 0.2 μL/mL or higher. These results indicate that these four oils can be used as an antifungal agent against *R. oryzae* biofilms as well as to reduce the Minimum Inhibitory Concentration of Amphotericin B needed to treat infections.

Keywords: Biofilm disruption, Mucormycosis, Antifungal Oil

1. Introduction

Biofilms are, "highly, organized, surface associated communities of microorganisms that are protected within an extracellular matrix[1, 2]." Free-floating organisms land and attach to a surface area before excreting the extracellular matrix to form the protective biofilm. Biofilms can be formed on living and non-living surfaces and are very difficult to treat due to the protective nature of the extracellular matrix. Biofilms are almost always formed in nature; an estimated 98% of microbial agents tested have demonstrated the ability to form biofilms[3, 4].

R. oryzae is a species of fungus belonging to the order Mucorales. Members of this order are responsible for various opportunistic infections, with *R. oryzae* being one of the most common infectious agents. Due to the filamentous nature of *R. oryzae*, there are few examples in present literature that indicate that *R. oryzae* could form biofilm. Recent findings have shown that it is possible for surface filamentous fungi to form a biofilm, allowing for an emerging field concerning the microbial nature of any filamentous fungi, especially *R. oryzae*[3]. Due to the commonality of *R. oryzae* and the severity of damage it incurs on its host, the study of the behavior of *R. oryzae* at a cellular level and its modes of infection could lead to greater success in combating mucormycosis.

Mucormycosis is a unifying term used to describe any fungal infection caused by the order of mucorales. *R. oryzae*, being a part of this order, is known to cause up to 70% of all mucormycosis infections. Other common mucormycosis-causing species include *Absidia corymbiferia* and *Rhizomucor pussilus*[5]. The order mucorales is ubiquitous in nature and commonly found in dead organic matter such as household foods. The opportunistic organisms infect individuals who are immunocompromised, like those diagnosed with cancer, HIV, blood disorders, diabetes, or those receiving organ transplants[6]. Rhizopus organisms contain an enzyme, ketoreductase, that allows them to thrive in hyperglycemic and acidic conditions - commonly occuring in diabetic patients in ketoacidosis. Ketoacidosis, in conjunction with ketoreductase, stimulates the growth of Rhizopus organisms. The serum found in healthy individuals will inhibit the growth of the fungi[7].

Once an individual is infected with mucormycosis, there is an overall mortality rate of 54%. The mortality rate is dependent on the original infection site, but if the infection disseminates, the mortality rate increases to 96%[8]. Since the 1950s, Amphotericin B (Amp B) has been the first line of defense against this infection. Upon administration, Amp B reacts with ergosterol found in the fungal cell membrane creating pores that will leak the contents of the cell, ultimately causing cell death[9]. Amp B does have some serious negative side effects, such as headaches, fevers, chills, loss of appetite, stomach pain, muscle pain, and weight loss. The toxicity of Amp B has been known to cause hepato and nephrotoxicity, which can be fatal, even at the minimum inhibitory concentration. Because of the mortality rates from mucormycosis and the toxicity found in Amp B, new methods to break down the biofilm are crucial.

Antifungal agents can be found in many sources. One source of interest is the phytochemical constituents of plants. Various studies have been performed on the antimicrobial properties of essential oils. Analyses of these studies identified four essential oils with promising antimicrobial capabilities, namely: *Cymbopogon flexuosus* (lemongrass)[10, 11], *Eugenia caryophyllata* (clove)[12, 13], *Origanum vulgare* (oregano)[14, 15] and *Cinnamomum zeylanicum* (cinnamon)[16, 17] oils. Each of these oils contain key phytochemical constituents that have strong antifungal abilities. Cinnamon, lemongrass and clove oils were tested directly against fungal species of *Candida*, a yeast fungus associated with the highest rate of fungal infections worldwide. The studies found strong reason to believe that the essential oils inhibited fungal cell growth.

None of the studies mentioned above analyzed synergistic effect of essential oils with Amp B against fungal species. Combination therapy has promising antifungal activity and decreases the toxicity of Amp B. Previous studies looked at the combination of Amp B and polyene-caspofungin, another antifungal agent. The combination of the antifungals resulted in a 100% survival rate compared to the 25% survival rate found in monotherapy. This route was chosen for testing as a way to mitigate current Amp B treatment by diluting treatment with safer compounds. Additionally, previous studies did not include mucormycosis causing fungi species such as *R. oryzae* or *Absidia corymbifera*[18]. The focus of our study involved the treatment of *R. oryzae* with essential oils and Amp B both synergistically and separately. As a control, separate treatment of biofilms was used to see the preferred method of treatment to optimize cell growth inhibition.

2. Materials and Methods

R. oryzae cells were suspended at a concentration of $1*10^5$ cells/mL in RPMI in a 96-well plate with short, flat-bottom wells[19]. The study utilized three plates testing Amp B alone, three plates testing oil alone, and nine synergism plates for each oil tested. Amp B plates and oil plates consisted of condition rows in triplicate (Figure 1). Amp B plates were treated in serial 2:1 dilution with high concentrations of Amp B to low concentrations from Columns 1-10, column 11 as the positive control containing RPMI only, and column 12 treated with DMSO/RPMI only. Oil-only plates were treated in serial 2:1 dilution with high concentrations of the specific oil to low concentrations from Columns 1-10, column 11 as the positive control containing RPMI only, and column 12 treated with DMSO only. Synergism plates were treated with Amp B again in serial 2:1 dilution with high to low concentrations from Rows A-H and high to low concentrations of the specific oil from columns 1-10. Column 11 served as positive control rows, containing RPMI only. Column 12 was the negative control row, containing no media.

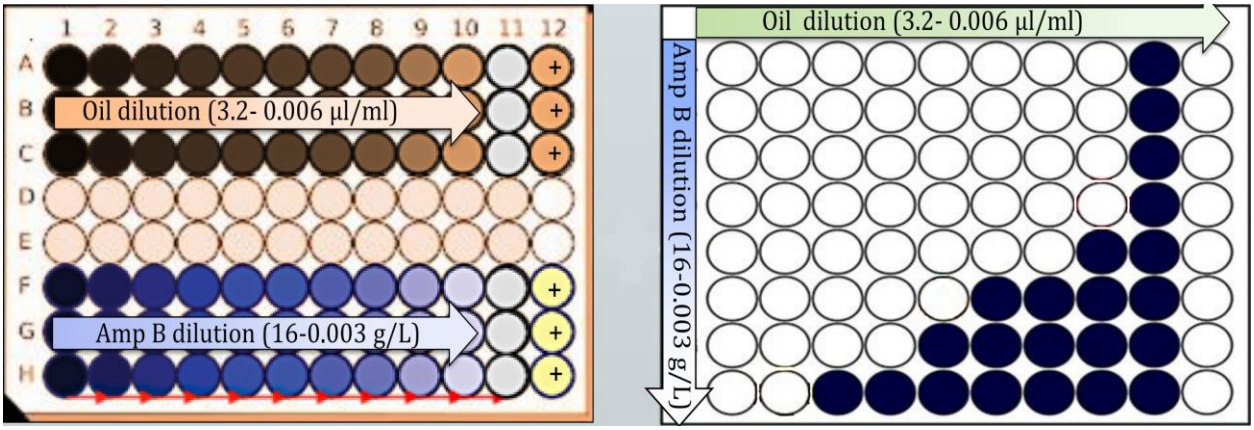

Figure 1. Schematic view of single treatment plates (left) and combination treatment plates (right).

Every well had 100 microliters of the cell suspension applied to it. The plates were placed in a shaking incubator at 75 rpm and 37 ℃ for 12 hours to form biofilms. Wells were washed with 100 microliters of sterile PBS three times and aspirated to remove any excess media and planktonic cells. Plates were treated with 100 microliters of the corresponding treatment dilution of oil, Amp B, combination of Amp B and oil, or control RMPI. After the treatment application, plates were placed in a shaking incubator for an additional 12 hours at 75 rpm and 37 ℃. Plates were washed with sterile PBS and aspirated in the same method as above. Wells were then treated with 200 µL of a 10:1 XTT and menadione solution which is used to measure the survivability of the organism. Absorbance was measured with a synergy H1 Hybrid Multi-mode Microplate Reader two to three hours after application to measure cell density, which has been shown to correlate with cell activity. Inhibition percentages were calculated by dividing the difference of each data point from the average of each treatment biofilm growth by that same control. Biofilm control data points were established as wells that received no antifungal treatment. To help simplify the data set, certain series were selected that met the criteria of both showing a difference from the Amp B baseline as well as a low concentration of antifungal. The concentration of 0.2 µL/mL was chosen to satisfy both these criteria.

In combination therapy tests, data from tests using Amp B in the absence of essential oils was used as a standard to compare with tests employing combination of Amp B with an essential oil. Data for this curve was taken from the mean of twelve duplicated experiments where the organism was treated with serial dilutions of amphotericin B. The minimum inhibitory concentration was 50% of the organism (MIC_{50})

3. Results

After a single treatment using serial dilutions, each oil exhibited antifungal capabilities with an inhibition curve similar to that of Amphotericin B at about 20% of the concentration. Minimum Inhibitory Concentrations (MIC) for each of the oils ranged from 0.025-0.1 µL/mL (Figure 2). Clove, oregano, and lemongrass all exhibited a simple saturation across the various concentrations with the cinnamon treatment experiencing a steep drop after 0.1 µL/mL and oregano treatment after 0.025 µL/mL. The organism seems to be increasingly sensitive to those two treatments after reaching those minimum concentrations Cinnamon also reached a maximum inhibition after 0.2 µL/mL never reaching the same maximum inhibition level of the other three oils.

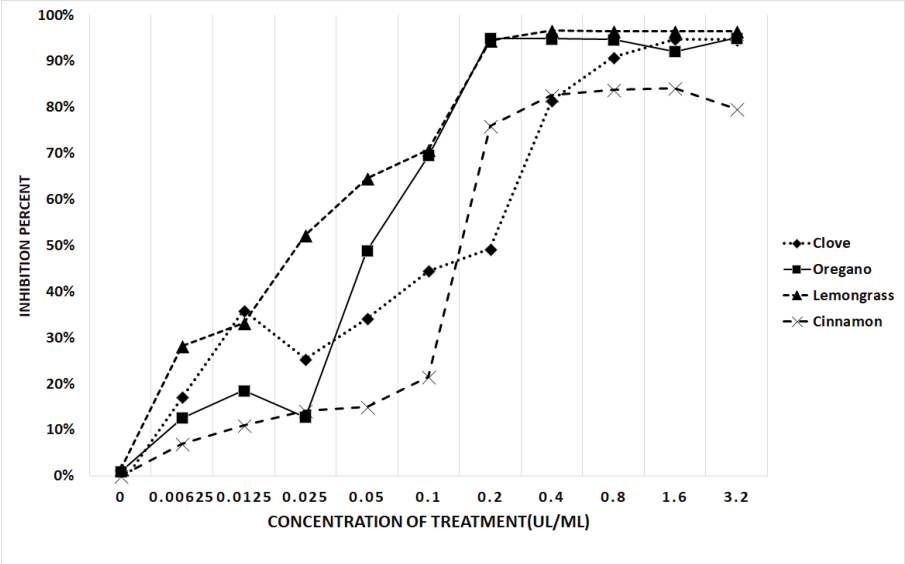

Figure 2. Percent inhibition of *R. oryzae* by concentration(µL/mL) of oil applied.

The typical behavior of R. oryzae when exposed to serial dilutions of Amphotericin B can be seen in Figures 3-6. The combination treatment with oregano oil (0.2 µL/mL) (Figure 3) produced an inhibition rate of no less than 79% across all concentrations of Amp B (0.00625 – 3.2 µL/mL). The MIC50 of the combination treatment was well below 0.0625 µg/mL and could not be properly extrapolated from this data set. Using higher concentrations of oil treatment with Amp B resulted in the MIC50 decreasing even further in concentration.

Lemongrass(0.2 µL/mL) when combined with Amp B performed similarly to oregano combination treatments inhibiting the organism no less than 73% and moving the MIC50 below the lowest concentration of Amp B used (Figure 4). This combination treatment has the highest inhibition of all the four oils when utilized with the highest concentration of Amp B (8 µg/mL) showing an inhibition rate of up to 98%.

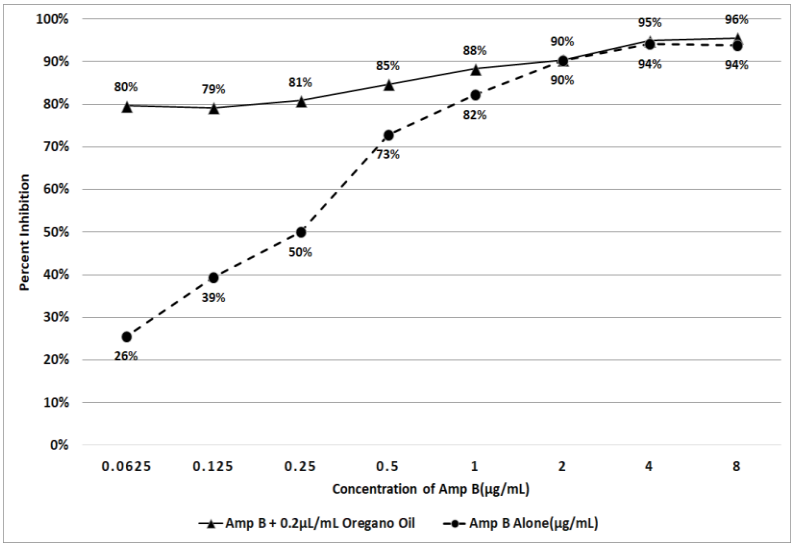

Figure 3. Effect of Amp b alone with the effect of oregano oil (0.2µL/mL) combined with amp b on the growth of *R. oryzae* biofilms.

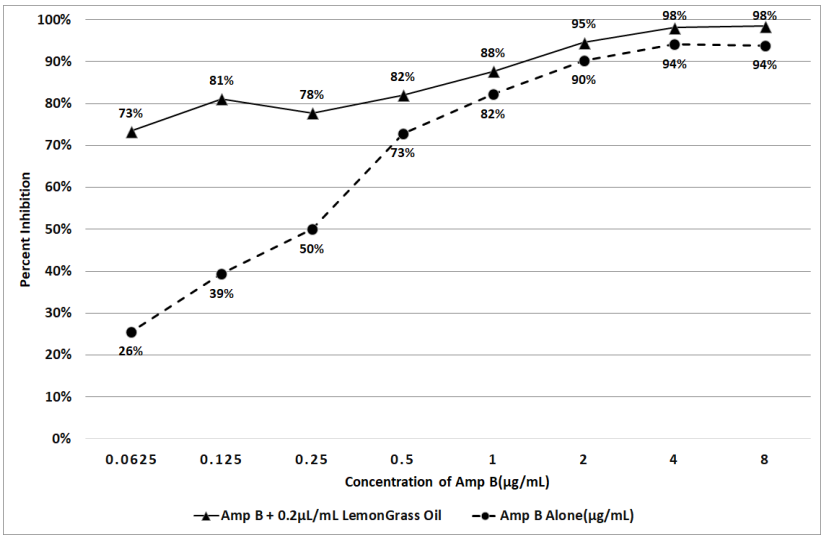

Figure 4. Effect of Amp b alone with the effect of lemongrass oil (0.2µL/mL) combined with amp b on the growth of *R. oryzae* biofilms.

Cinnamon oil combination treatment resulted in an inhibition rate of no less than 60% at the lowest combined concentration of Amp B and the same concentration of oil as used previously (Figure 5). Additionally, the MIC_{50} of this combination treatment was below the lowest concentration of Amp B used.

When Amp B was combined with clove oil at a concentration of 0.2 µL/mL, the MIC_{50} of the combination treatment moved to 0.125 µg/mL of Amp B. However, this concentration was too low to see a substantial difference between the combination therapy and the Amp B alone, so the next higher level concentration of clove oil (0.4 µL/mL) is shown in Figure 6. When combined with this concentration of oil, the treatment exhibited an inhibition of no less than 56% at the lowest concentration of Amp B.

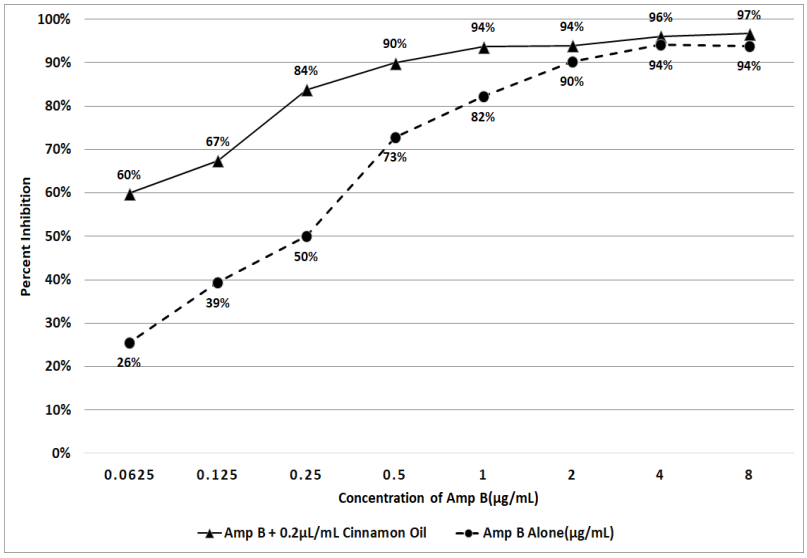

Figure 5. Effect of Amp b alone with the effect of cinnamon oil (0.2µL/mL) combined with amp b on the growth of *R. oryzae* biofilms.

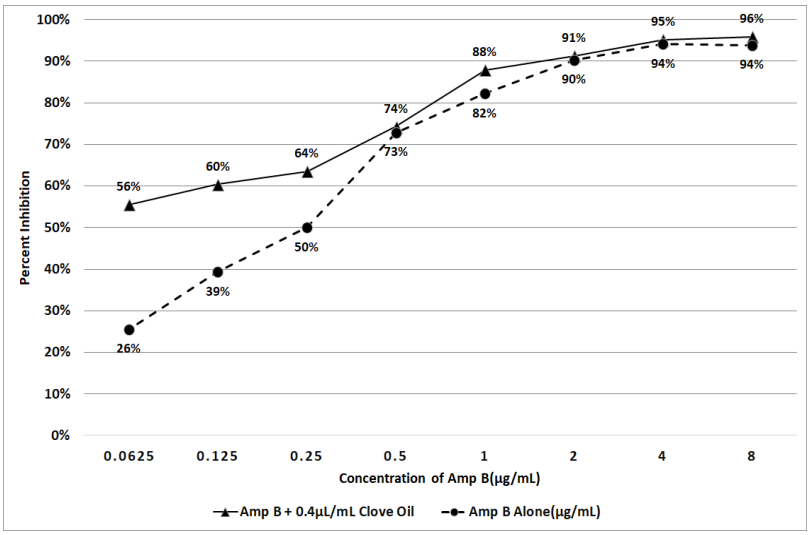

Figure 6. Effect of Amp b alone with the effect of clove oil (0.4µL/mL) combined with amp b on the growth of *R. oryzae* biofilms.

4. Discussion

While lemongrass[11], clove[13], and cinnamon[17] oils have all previously been tested against other *Rhizopus* organisms, this seems to be the first instance where they have been tested against *R. oryzae*. Chemical constituents in oregano[15] oil have previously been tested against *R. oryzae* as planktonic cells, but not while the organism was in the more common biofilm stage[4]. In all the previously referenced studies, the treatment was only tested against planktonic cells, indicating that for all methods of treatment it is possible this is the first instance in which an antifungal oil has been used to treat fungus that has formed biofilm. Due to the fact that the biofilm stage of the fungus is highly resilient to antifungal treatments and that biofilms will typically form on dead or living tissue[4], this is of particular relevance. All oils exhibited antifungal capability when exposed to the fungus in a single treatment, depicting that the oils can be used as a treatment even while the fungus is protected by biofilm. At the specified concentrations (0.2-0.4 µL/mL), all combination treatments showed a decrease in absorbance, indicating that the proliferation of cells was inhibited. This points to the conclusion that the two treatments can work additively if not synergistically. This suggests that an antifungal oil can be used to increase base effectiveness of Amp B against a fungal organism that has formed a biofilm without needing to increase the concentration of Amp B to toxic levels.

Oregano oil combined with Amp B outperformed all the other oils. At the combination concentration of 0.2 µL/mL of oil and 0.0625 µg/mL of Amp B, 80% of the growth of the fungus was inhibited. These results are similar to other results collected in previous literature[15], but were obtained while using Oregano oil constituents without Amp B and at considerably less concentration by a factor of thousands. This inhibition only increased as the concentration of Amp B increased. As a comparison, Amp B at the same concentration in the absence of antifungal oil inhibited only 20% of the growth of the organism. This shows an increase of antifungal capability of about 4 times over the single treatment. Thus, oregano oil can be used in combination with Amp B to reduce the minimum inhibitory concentration of Amp B against *R. oryzae* after biofilm formation.

Neither the lemongrass nor the cinnamon oil combination treatments were as inhibitive as oregano, but still increased the effectiveness of Amp B in similar fashions. For both treatment methods the MIC_{50} was lowered well beyond the lowest concentration used in this protocol. Lemongrass has been previously shown to inhibit sporulation in several organisms at concentrations as low as 25 ppm or 25 µg/mL[11] which is ten times greater than the concentration utilized in this study. Cinnamon oil has been shown to inhibit growth of various organisms anywhere from 1600ppm to 30,000ppm[17]. The greatest difference in inhibition for cinnamon oil and Amp B was found to be between 0.125 and 0.25 µg/mL of Amp B at the 0.2 µL/mL concentration of oil, with an increase of 17% inhibition. For future protocol design, it would be advisable to use a lower concentration of Amp B as well as more sensitive dilutions. Utilization of more sensitive dilutions would allow for more accurate measurement of the minimum inhibitory concentration.

Despite requiring a higher concentration, clove oil still increased the baseline effectiveness of Amp B when used in combination. However, this may not be beneficial as clove oil has been shown to have a high level of cytotoxicity to certain human cells at concentrations as low as 3 parts per 10,000[20]. Cinnamon oil has been postulated to be moderately toxic, but without a precise measurement of minimum toxic concentration and a wide range for lethal dosage[21]. Lemongrass has also been shown to be toxic to human cells at high concentrations[22]. Future experimentation for any of these oils should include toxicity and human cell viability assays to determine the safety of using any of these oils for treatment.

The successful findings of this research have led to queries which deserve further pursuit. These include investigations into the effectiveness of these oils against *Absidia corymbifera*, testing the potential benefits of combining technologies (such as ultrasound, extracorporeal shockwaves, and laser light biomodulation) with the synergistic treatment of oils and Amphotericin B, and to study the breakdown of fungal biofilm when integrated with human tissues or cells. Additionally, it would be of worth to continue the study of the phytochemical constituents of the oils tested to discover which compounds are producing antifungal results. All four oils exhibit antifungal activity against biofilms of *R. oryzae* which indicates it is possible to be used for the treatment of fungal infections. Furthermore, at 0.2 μL/mL any of these oils can be used to reduce the minimum inhibitory concentration of Amp B when used in conjunction. It is recommended that future research incorporate smaller levels between concentrations due to the sensitive nature of the combination treatments. Additionally, it was shown here that Amp B and each of these oils have at least an additive interaction but it would be beneficial to quantify if they have synergistic interactions.

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