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PREVENTIVE TREATMENT TO COMPLEMENT INFECTION CONTROL FOR CANDIDEMIA: IDENTIFICATION OF DIETARY SUPPLEMENTS THAT EFFECTIVELY INHIBIT THE GROWTH OF *Saccharomyces* AND *B. megaterium* WHILE PROLIFERATING *E. coli*

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ABSTRACT

Fungal infections have a crude mortality rate of 40% among immunocompromised patients, who are very susceptible due to drug resistant yeast strains and delayed diagnosis. In fact, in the United States alone, annual therapeutic treatments cost $2.8 billion. This study aims to develop preventive treatments to complement established infection control for fungal infections. The hypothesis was that the combination of probiotics with cinnamon would be the most effective treatment. Various combinations and concentrations of over-the-counter supplements were evaluated in their ability to inhibit yeast and detrimental bacterial growth while proliferating beneficial bacteria. *Saccharomyces*, *E. coli*, and *B. megaterium* were grown in petri dishes to represent detrimental yeast, beneficial bacteria, and detrimental intestinal bacteria respectively. By measuring the zone of inhibition, this study shows that probiotics recreate an oxygenated, nonacidic, and beneficial bacterial habitat without interfering with the antifungal. The combination of oil of oregano (*Origanum syriacum var. bevanii*) with acidophilus was the most effective treatment, not supporting the hypothesis. Overall, taking oil of oregano and acidophilus with the antibiotic regimen may reduce the risk of developing fungal infections. Further research into understanding the underlying mechanism of these results and evaluating the clinical implications of this combination is recommended.

Keywords: Candidemia, antifungal, antibiotic regimen, oil of oregano, acidophilus

INTRODUCTION

Despite well-established therapeutic antifungal treatment classes such as polyenes, azoles, and echinocandins (**Kauffman *et al*., 2014; Tani *et al*., 2012; Spampinato *et al*., 2013**), the estimated annual cost to treat fungal infections in the United States is estimated at $2.8 billion (**Wilson *et al*., 2002; Fridkin 2005**). About 300 in 1.5 million species of fungi are known to be dangerous to humans (**Hawksworth, 2001**). However, only a few classes of antifungal agents are currently available in oral and intravenous forms. Most deadly fungal infections either originate in a hospital setting (nosocomial) or target patients with weak immune systems (**Zautis *et al*., 2005; Alangaden, 2011; Puzniak *et al*., 2004**). The latter class includes premature babies, the elderly, patients with preexisting conditions such as the Human Immunodeficiency Virus (HIV), and patients taking immunosuppressive drugs for cancer treatment or organ/stem cell transplantation (**Warnock, 2007; Mikulska *et al*., 2012; Bassetti *et al*., 2011; Horn *et al*., 2009**).
An untreated or drug resistant yeast strain entering the blood stream can lead to Candidemia. The gastrointestinal tract is the predominant source of Candidemia (**Nucci *et al*., 2001; Miranda *et al*., 2009**). Intestinal yeast overgrowth is commonly induced through antibiotics or immunosuppressive usage, where beneficial bacteria are killed off, providing the ideal acidic environment for yeast to grow. The resulting inflammation and intestinal permeability leads to Candidemia (**Kumar *et al*., 2013; Jensen *et al*., 2014**). In critically ill patients, a corroboration has been established between high exposure to antibiotics and increased risk of invasive Candida infections (**Mikulska *et al*., 2012**). A significant percentage of Candidemia goes undetected in its early stages due to non-specific symptoms and a low diagnostic yield of traditional blood cultures (**Zautis *et al*., 2005; Mikulska *et al*., 2012**). Thus, there is a need for effective natural preventive treatments that can be adapted to peoples' diets without side effects. Potential side effects of therapeutic antifungal treatments range from abdominal pain, diarrhea, and indigestion to allergic reactions **(NHS choices, 2015)**, but natural antifungals tend to have only minor, less fatal side effects. This study addresses preventive treatment to complement established infection control and preemptive treatment strategies for combatting yeast infections.
Different combinations and concentrations of over-the-counter supplements were evaluated to determine the treatment that would inhibit yeast and detrimental bacterial growth while aiding the proliferation of beneficial intestinal bacteria. *Saccharomyces* was used to represent fungi. *B. megaterium*, a common bacteria found in dirt, was chosen to represent detrimental bacteria. *E. coli* was utilized to represent beneficial intestinal bacteria.
We hypothesized that optimal results would be achieved when yeast and bacteria are treated with the combination of probiotics and cinnamon (*Cinnamomum verum*). Cinnamon and probiotics are widely advertised as over-the-counter dietary supplements that cure fungal and intestinal health ailments. However, researchers have documented mixed findings on the health benefits of both cinnamon (**Wang *et al*., 2012; Wang *et al*., 2005; EBSCO Complementary and Alternative Medicine Review Board, 2014**) and probiotics (**Gorbach, 2006; Lucak, 2010; Rolf, 2000; Collado *et al*., 2009; Ballakrishnan *et al*., 2012**). Garlic and oil of oregano have been reported to display antifungal properties (**Bakker, 2012**). Lactobacillus acidophilus (acidophilus) is an established probiotic (**Bakker, 2012**). This study aims to test the effectiveness of these substances as potential antifungals, as well as their efficacy in conjunction with probiotics.

MATERIAL AND METHODS

Antifungal and Probiotic Supplements

Garlic, cinnamon, caprylic acid, and oil of oregano were the four over-the-counter, antifungal supplements that were evaluated as treatments. Acidophilus was used as a representative probiotic supplement. Two concentrations (one and two tablets each) of these seven treatments were evaluated. In addition, two additional supplement combinations were created by compounding oil of oregano with caprylic acid and oil of oregano with acidophilus. Each of these combinations were tested with two different concentrations (Table 1). Treatment was not added to the negative control (**Burton *et al*., 1997**), and the positive control had tetracycline (antibiotic) (**U.S. National Library of Medicine, 2010; Klajn, 1996**) added to the bacterial plates. Thus, a total of 16 different treatments for bacterial plates and 15 different treatments for yeast plates were evaluated. The treatment concentrations for the supplements is listed in Table 1.

Table 1 Concentrations of the active ingredients of the treatments evaluated for this experiment along with the chemical formulas of the active ingredients are listed.

Supplement	Chemical Formula	Treatment Concentration
Garlic	$C_6H_{10}OS_2$	500 mg (1 tablet)
		1000 mg (2 tablets)
Cinnamon	$C_{11}H_{13}NO$	102.5 mg (1 tablet)
		205 mg (2 tablets)
Caprylic Acid	$C_8H_{16}O_2$	541 mg (1 tablet)
		1082 mg (2 tablets)
Oil of Oregano	$C_{10}H_{14}O$	500 mg (1 tablet)
		1000 mg (2 tablets)
Acidophilus	-	50 million (1 tablet)
		100 million (2 tablets)
Oil of Oregano with Caprylic Acid (One Tablet each)	$C_{10}H_{14}O$ + $C_8H_{16}O_2$	500 mg (1 tablet) & 541 mg (1 tablet)
Oil of Oregano with Caprylic Acid (Two Tablets each)		1000 mg (2 tablets) & 1082 mg (2 tablets)
Oil of Oregano with Acidophilus (One Tablet each)	$C_{10}H_{14}O$	500 mg (1 tablet) & 50 million (1 tablet)
Oil of Oregano with Acidophilus (Two Tablets each)		1000 mg (2 tablets) & 100 million (2 tablets)
None (Negative Control)	-	-
Tetracycline (Positive Control)	$C_{22}H_{24}N_2O_8$	5 g

To the greatest extent possible, supplements were obtained from the same manufacturer to ensure consistency of the excipients. Acidophilus, garlic, and cinnamon was manufactured by SPROUTS INC (Phoenix, Arizona, USA); caprylic acid was manufactured by SOLARAY®, and oil of oregano was manufactured by LIFETIME VITAMINS® (Park City, Utah, USA). Table 1 summarizes the concentration strength of the active ingredients, as listed on the label of the over-the-counter supplements.

Nutrient Agar Preparation

Nutrient agar solution was prepared with a concentration of 23 g nutrient agar per liter of distilled water. The solution was boiled until it turned translucent. Next, it was autoclaved and poured onto petri dishes with a surface area of 1810 mm^2 and a height of 4 mm. Plates were allowed to cool and solidify for 4 hours.

Bacterial and Yeast Culture

A sterile disc inoculated with the chosen treatment was placed in the center of each petri dish. To create a bacterial lawn, each petri dish was streaked with 100 microliters of *E. coli* (beneficial intestinal bacteria) or *B. megaterium* (detrimental bacteria). *Saccharomyces* was activated with distilled water at 30^0C. To culture yeast, 100 microliters of *Saccharomyces* (yeast) was streaked onto each petri dish. Thus, each petri dish was either streaked with *E. coli*, *B. megaterium*, or *Saccharomyces*. Petri dishes were incubated upside down at 37^0C for 4 days. *E. coli* and *B. megaterium* were obtained from Carolina Biological®. *Saccharomyces* was obtained from Fleischmann®. Each of the treatments were tested on 9 petri dishes: 3 plates with *E. coli*, 3 plates with *B. megaterium*, and 3 plates with *Saccharomyces*. Thus, each treatment was tested on each microbe in triplicate, making n=3 for each combination of microbe and treatment. Each box and whisker figure represents data from experiments performed in triplicate.

Zone of Inhibition Evaluation

After petri dishes were incubated upside down at 37^0C for 4 days, the effectiveness of the treatments was identified by measuring the surface area lacking any growth. This zone of inhibition was quantified by counting the squares of a graph paper that was cut to the exact size of the petri dish: 1810 mm^2. The graph paper was placed under the petri dish, and the percentage of the petri dish without any growth was calculated and compared across treatments.

RESULTS

Overall, oil of oregano and acidophilus were the most effective treatments used as individual supplements

Among the individual supplements that were used as treatments, oil of oregano inhibited the maximum percentage of *Saccharomyces* (as shown in Figure 1) and *B. megatarium* growth (as shown in Figure 2). Acidophilus was the second most effective treatment at inhibiting *Saccharomyces* (Figure 1) and *B. megaterium* (Figure 2) growth. However, acidophilus was more effective than oil of oregano in aiding the growth of *E. coli* (as shown in Figure 3). The negative control yielded 0% growth inhibition for all microbes, and the positive control for *B. megaterium* and *E. coli* yielded 47% and 60% growth inhibition respectively.
Garlic, cinnamon, and caprylic acid did not affect the growth of *B. megaterium* (Figure 2) or *E. coli* (Figure 3). Of these three supplements, caprylic acid was the only treatment that inhibited any *Saccharomyces* growth, but the zone of inhibition was much lower than the percentage of growth inhibition in the samples with oil of oregano and acidophilus (Figure 1).

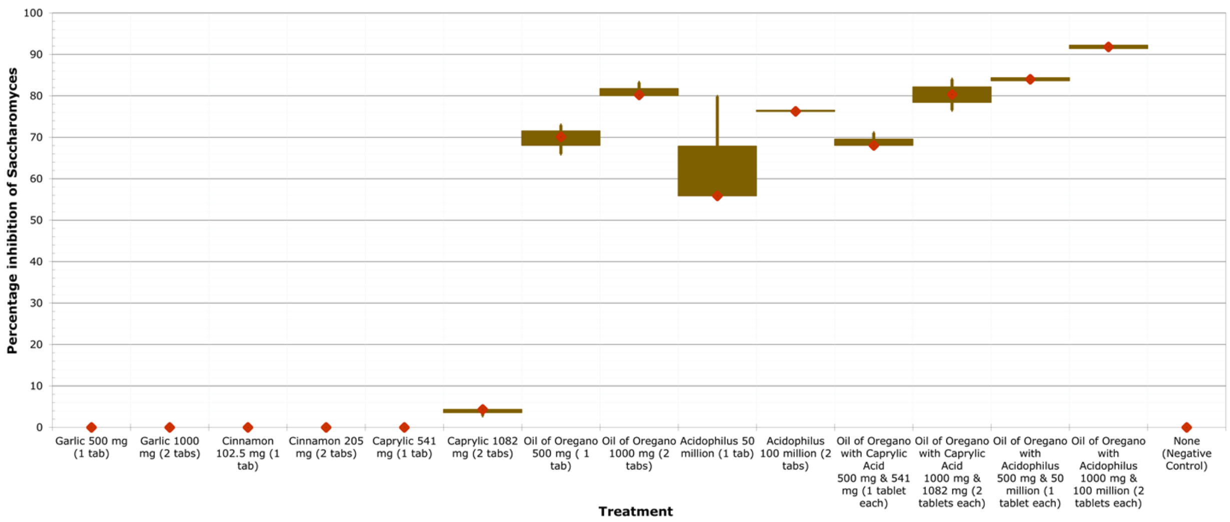

Figure 1 Effectiveness of treatments in inhibiting Saccharomyces growth. The relative frequency of inhibition in growth of *Saccharomyces* was determined by measuring the zone of inhibition in the petri dish and representing this value as a percentage. This percentage inhibition of *Saccharomyces* growth was calculated for all the treatments that were tested.

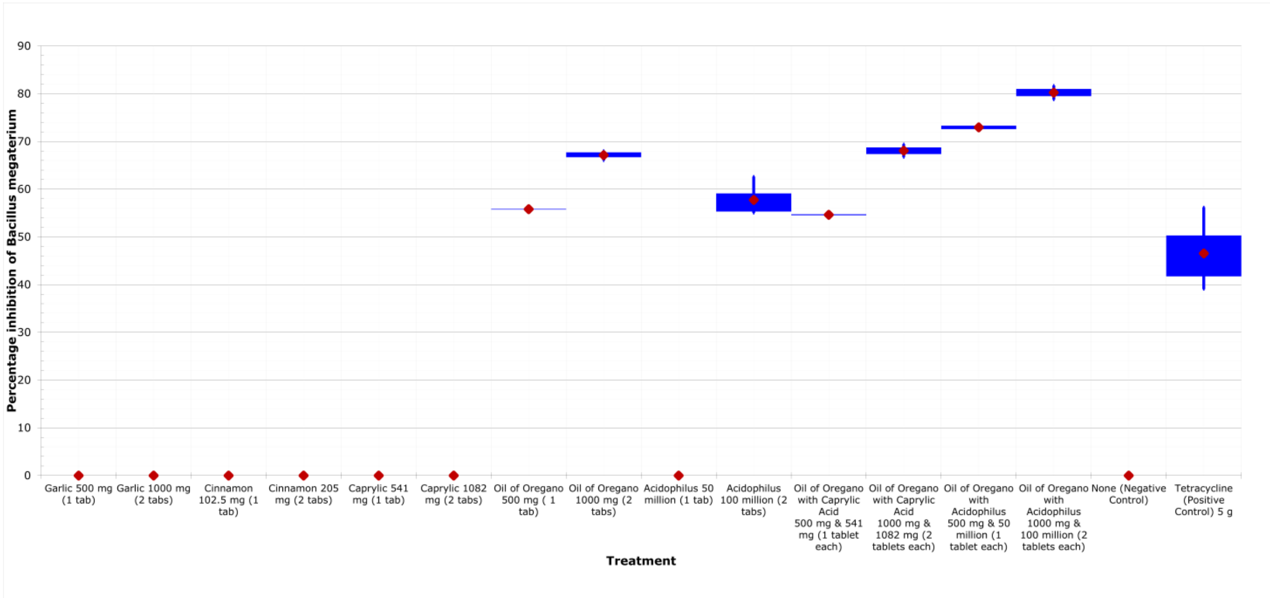

Figure 2 Effectiveness of treatments in inhibiting *B. megatarium* growth. The relative frequency of inhibition in growth of *B. megatarium* was determined by measuring the zone of inhibition in the petri dish and representing this value as a percentage. This percentage inhibition of *B. megatarium* growth was calculated for all the treatments that were tested.

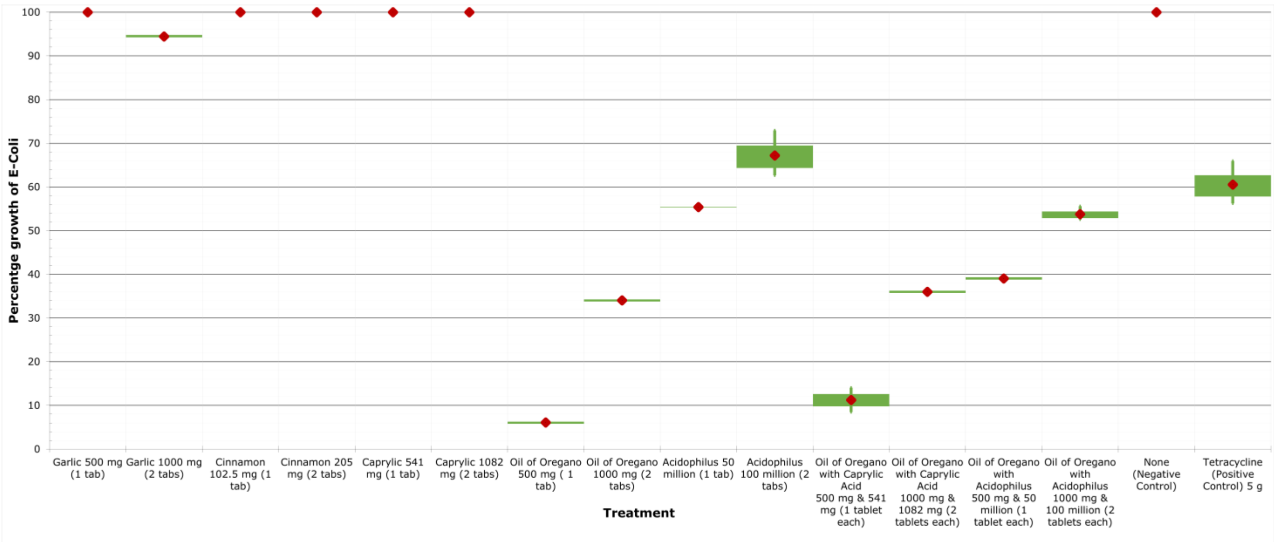

Figure 3 Effectiveness of treatments in proliferating *E. coli*. The relative frequency of growth of *E. coli* was determined by measuring the zone of inhibition in the petri dish and representing this value as a percentage. This percentage of *E. coli* growth was calculated for all the treatments that were tested.

The combination of oil of oregano with acidophilus was the most effective treatment

The highest concentration of oil of oregano with acidophilus was the most effective treatment; *Saccharomyces* growth was inhibited at 92.82% (Figure 1), *B. megaterium* growth was inhibited at 80.24% (Figure 2), and *E. coli* grew to fill 53.77% of the plate (Figure 3). Adding acidophilus to either oil of oregano or caprylic acid improved each antifungal's ability to inhibit *Saccharomyces* (Figure 1). However, adding caprylic acid to oil of oregano neither improved nor deteriorated oil of oregano's effectiveness at inhibiting growth of *Saccharomyces* (Figure 1) and *B. megaterium* (Figure 2). There was only a marginal increase in the growth of *E. coli* (Figure 3).

Increasing the concentrations of oil of oregano and acidophilus improved the potency of these treatments

Garlic, cinnamon, and caprylic acid had no impact on bacterial and yeast growth at both the lower and higher concentrations (Figure 1-3). However, doubling the concentration strength for acidophilus, oil of oregano, and their compounds improved their effectiveness by at least 10% (Figure 1-3). The most drastic effect was observed by increasing the concentration of acidophilus in *B. megaterium* cultures. Acidophilus had no impact at its lower concentration, but when the concentration was doubled, the treatment jumped from having no effective on *B. megatarium* growth to inhibiting 55.6% of growth (Figure 2).

Two methodologies were used to determine the most effective treatments overall

Table 2 summarizes the overall effectiveness of the treatments. When comparing single supplemental treatments with compounds, the most effective treatment in each category always included oil of oregano. The addition of acidophilus to oil of oregano resulted in increased inhibition of *Saccharomyces* over *B. megaterium*.

As shown in Table 2, there were 162 petri dish data samples altogether. The results of 3 samples (n = 3) for each of the 16 treatments and its associated microbe (*Saccharomyces*, *B. megatarium*, or *E. coli*) were averaged to calculate the area of growth or inhibition. The mean area of inhibition of *E. coli* was subtracted from the sum of the mean area of inhibition for *Saccharomyces* and *B. megaterium* to rank each treatment (column "Overall Rank"). An alternate overall ranking method which prioritizes *Saccharomyces* inhibition is also displayed, as the main purpose of this study to eliminate the growth of yeast. In this alternate ranking method, the area of inhibition of *Saccharomyces* growth was weighted twice over the inhibition of *B. megatarium* of growth of *E. coli* (column "Alternate Overall Rank").

Statistical Analysis

Table 2 Two methodologies to rank the overall effectiveness of treatments

Treatment and Concentration	Zone of Inhibition (mm^2) (Average of Mean of the 3 samples per treatment)			Overall assessment arithmentic (x + y - z)	Overall Rank (1 is most effective)	Alternate Overall assessment arithmentic (2x + y - z)	Alternate Overall Rank (1 is most effective)
	Saccharomyces (x)	Bacillus Megaterium (y)	Escherichia Coli (z)				
Garlic 500 mg (1 tab)	0	0	0	0	10	0	10
Garlic 1000 mg (2 tabs)	0	0	0	0	10	0	10
Cinnamon 102.5 mg (1 tab)	0	0	0	0	10	0	10
Cinnamon 205 mg (2 tabs)	0	0	0	0	10	0	10
Caprylic Acid 541 mg (1 tab)	0	0	0	0	10	0	10
Caprylic Acid 1082 mg (2 tabs)	69	0	0	69	9	138	9
Oil of Oregano 500 mg (1 tab)	1256	1010	1697	3963	1	1825	7
Oil of Oregano 1000 mg (2 tabs)	1485	1212	1193	3891	4	2989	5
Acidophilus 50 million (1 tab)	1234	0	808	2042	8	1659	8
Acidophilus 100 million (2 tabs)	1385	1017	555	2957	7	3232	3
Oil of Oregano (500 mg) with Caprylic Acid (541 mg) - One tablet each	1260	990	1610	3860	6	1900	6
Oil of Oregano (1000 mg) with Caprylic Acid (1082 mg) - Two tablets each	1491	1225	1158	3875	5	3049	4
Oil of Oregano (500 mg) with Acidophilus (50 million) - One tablet each	1520	1315	1104	3938	2	3251	2
Oil of Oregano (1000 mg) with Acidophilus (100 million) - Two tablets each	1661	1440	834	3935	3	3929	1
Tetracycline 5 g *(positive control)*	Not applicable	942	710	Not applicable		Not applicable	
No treatment *(negative control)*	0	0	0				

There were 162 petri dish data samples altogether. Each of the 16 treatments (Table 2) was tested on 9 plates; there were three plates (n = 3) for *Saccharomyces*, three plates (n = 3) for *B. megaterium*, and three plates (n = 3) for *E. coli*. Thus, each treatment had a total of 9 samples distributed across the 3 cultures. The box and whisker plots representing growth of each microbe for each treatment (Figures 1-3) display the mean, and the solid boxes represent values of the second and third quartile. The whiskers indicate values in the first and fourth quartile.

DISCUSSION

The highest concentration of oil of oregano with acidophilus was the most effective treatment, not supporting our hypothesis. In fact, oil of oregano by itself was an effective antifungal. This corroborates with other contemporary documented research of oil of oregano, which support its effectiveness as a bio fungicide against phytopathogenic fungi (**Soylu *et al*., 2007**) and against Candida (**Manohar *et al*., 2001**). Oil of oregano contains thymol, which causes cell membrane permeability in fungal cells and alters hyphal morphology. Eating a diet rich in oil of oregano (*Origanum syriacum var. bevanii*) and acidophilus or taking them as dietary supplement, especially with antibiotics, may reduce the risk of developing a yeast infection, as this combination resulted in minimal yeast and *B. megaterium* growth while greatly proliferating *E. coli*.

This study did not find the over-the-counter cinnamon supplement to be an effective antifungal. These findings could be another data point to consider in the scientific debate that questions the effectiveness of cinnamon as an effective antifungal (**EBSCO Complementary and Alternative Medicine Review Board, 2014**).

Our results indicate that the addition of bacterial probiotics in conjunction with an antifungal compound was the most effective treatment at curtailing fungal growth. Probiotics added friendly bacteria to recreate the ideal, oxygenated, nonacidic bacterial habitat; antifungals only killed off yeast without altering the acidic environment. In addition, probiotics did not interfere with the antifungal and increased friendly bacterial growth. In the absence of bacterial probiotics, a hazardous opportunity seemed to be opening up for the detrimental bacteria (i.e. *B. megaterium*) to proliferate. Thus, this study provides further evidence in the scientific debate supporting the addition of bacterial probiotics with antifungals to restore the balance of intestinal microbial.

Nevertheless, this study, only evaluated bacterial probiotics and did not explore those probiotics that contain yeast microbes as well. Medical practitioners have questioned the usage of probiotics with yeast in immunocompromised patients (**Benchimol *et al*., 2005**). In particular, several studies have identified probiotics containing *Saccharomyces boulardii* (Ultralevura manufactured by Bristol-Myers Squibb, New York City, New York, USA) as a risk factor for infection with *Saccharomyces cervisiae* (**Munoz *et al*., 2005; Riquelme *et al*., 2003; Lestin *et al*., 2003; Cesaro *et al*., 2000**).

Still, it is important to consider potential sources for error in this study. To accurately measure the zone of microbial inhibition, the outline of microbes was traced against a graph paper template, and the area was computed. The growth agar could have had unintended interference with the functionality of the treatments, although this is highly unlikely due to the ubiquitous nature of this growth medium with various types of microbes. Also, measuring the zone of inhibition is limited to growth inhibition and does not indicate whether microbes were killed or whether they were not allowed to grow in the first place. For these experiments, *Saccharomyces* and *B. megaterium* were used to represent detrimental yeast and bacteria respectively. Alternatively, samples of microbes cultured from the intestines of a sample of Candidemia patients would yield more accurate results pertaining to Candidemia infections specifically. Nonetheless, as many of these sources of error have minimal consequences or are highly unlikely to have significantly affected the results, findings form this study have relevance and important implications for future studies.

CONCLUSIONS

This study determined the combination of oil of oregano with acidophilus to be the most effective of all tested treatments in inhibiting *Saccharomyces* and *B. megaterium* growth while proliferating *E. coli*. The scope of this study did not include identifying the underlying mechanism that contributed to the observed results. A follow up study to comprehend the cellular mechanics that contribute to the effectiveness of the compound of oil of oregano and acidophilus against fungi is needed, as well as a more in depth exploration of the optimal concentration of each supplement. Results also encourage expanding the study to include other essential oil supplements, such as the oil from Fennel seeds. In the future, clinical evaluation of the treatment combination of oil of oregano and acidophilus with different concentrations is recommended, a necessary step before considering the incorporation of oil of oregano and cinnamon as part of the regular antibiotic regimen prescription.

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