In Vitro analysis of the Antibacterial effect of *Lactobacillus casei Shirota* and *Lactobacillus paracasei* on *Staphylococcus aureus*, *Streptococcus mutans*, and *Serratia marcescens* found on smokers

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ABSTRACT

Smoking can cause disruption of oral microbiota which may lead to decrease availability of oxygen in the mouth and increased acidity of saliva, making it a more suitable environment for the growth of bacteria and acid-tolerant microbes. In this study, the researchers used strains of Lactobacillus casei Shirota and Lactobacillus paracasei obtained from commercial probiotic drinks. Agar overlay method was used to test the antibacterial effects of both Lactobacillus spp. against smoking-associated oral pathogens like Staphylococcus aurues, Streptococcus mutans, and Serratia marcescens. The experiment was done in three trials, zone of inhibition was measured and statistical treatment was employed to determine its significance. Both Lactobacillus spp. appeared to be most potent against Streptococcus mutans. Moreover, L. paracasei showed a greater zone of inhibition than that of L. casei Shirota, with a mean value of 7.67 mm, 8 mm and 6.33 mm in Staphylococcus aureus, Streptococcus mutans and Serratia marcescens respectively. Nevertheless, both Lactobacillus spp. was both resistant and is only capable of inhibiting the growth of the pathogens not necessarily kills them, making them inferior with the positive control used, Imipenem.

Key words: Antibacterial, *Lactobacillus casei Shirota*, *Lactobacillus paracasei*, *Staphylococcus aureus*, *Streptococcus mutans*, *Serratia marcescens*, Agar overlay method, zone of inhibition

INTRODUCTION

In 2016, 15.5% of adults residing in the United States aged 18 years and older smoke cigarettes. Men are more likely to be smokers than women, at a rate of 18% compared to only 13.5% with that of women. It was also discussed that current smokers are more common among the age groups of 25-44 years old with a frequency of 18%. More than 16 million of these adults can acquire smoking-related diseases. Smoking is responsible for more than 480,000 deaths per year in the United States. An average smoker dies 10 years earlier than those who do not smoke (Center for Disease Control and Prevention, 2016). In the Philippines, reports show that there are 17.3 million adult Filipino smokers, of which 14.6 million are male and only 2.8 million are females. It was also added that an average of 11 cigarettes per day is being consumed by daily smokers with ages ranging from 15-34. Health experts have discussed that an estimate of 10 Filipinos die every hour due to smoking-related disease.

Smoking is known to cause cancer, heart and lung diseases and problems in the oral cavity particularly, gum disease which is commonly caused by damage other normal microbiota or growth of bacteria (Department of Health, 2014).

Studies have shown a significant association between smoking and periodontitis. It was proven that destructive periodontal disease has five to twenty-fold increase in smokers compared with non-smokers. The disruption of oral microbiota of a person can decrease the availability of oxygen in the mouth and increase the acidity of saliva, this situation creates a more suitable environment for the growth of anaerobic bacteria and acid-tolerant microbes like Streptococcus. In a recent study, microbial DNA of swabs from a group of 1, 204 individuals marked positive to the occurrence of bacteria such as Streptococcus species. Treatment of these infections for the bacteria can be in form of oral antibiotics such as azithromycin, amoxicillin, and penicillin (World Health Organization, 2016).

Antibiotics can be initially administered to address such infections. However, antibiotic resistance can occur. This phenomenon lessens the efficacy of antibacterial agents against these microorganisms. Antibiotic resistance is known as one of the world's most insistent problem. It can cause diseases that may be treatable at first but may eventually become more infectious. Misuse and overuse of the drug can lead to this, since every time a person takes antibiotics, sensitive bacteria are destroyed yet, resistant ones are left to grow and replicate. Thus, use of repeated antibiotics or stopping the therapy early can lead to reappearances and complications. On the other hand, probiotic drinks are readily available in the market and are already proven to be consumed safely since some studies have already shown benefit regarding its antibacterial benefits. It can be taken by individuals of any age range showing minimal to no side effects while benefiting the body (Center for Disease Control and Prevention, 2017).

Probiotics are beneficial for humans considering that it can be an alternative solution to oral problems and therapies. One good example of this is the commercially available probiotic drink that contains *Lactobacillus casei Shirota*, a gram positive, catalase negative, facultative anaerobic bacilli. It is extremely tolerant against the strong gastric juices of the bile and intestines (Yakult Singapore, 2018). Another probiotic drink which contains *Lactobacillus paracasei* is a facultative gram-positive bacillus that commonly measures up to 0.8-1.0 μm which appears singly or in chains. They are usually seen in the human intestine and mouth and, also in commercially available dairy products such as probiotic drink and yoghurts (The Regents of the University of California, 2014).

Commercial probiotic drinks help the oral health and natural balance of intestines to be maintained. Also, it stimulates progressive growth of immune response and maintains a desirable amount of microorganisms to prevent any threats to the gastrointestinal tract such as irritable bowel syndrome and diarrhea, and inhibit destruction of the normal flora of the mouth preventing disorders of the oral cavity caused by excessive smoking (National Center for Complementary and Integrative Health, 2018).

Therefore, this study desires to evaluate the effectiveness of *Lactobacillus casei Shirota* and *Lactobacillus paracasei* probiotic drink in preventing the growth of selected smoking-associated oral pathogens. Also, success of this study will benefit a wider market of different age groups since probiotic drinks are known to be readily available and considered safe to all. Moreover, this study will be relevant to the field of Medical Technology by utilizing specific species of bacteria as antibacterial agent, instead of the usual natural or semi-synthetic products, and introducing a new and modified method of susceptibility testing. Most importantly, it will address a public issue such as smoking, with its harmful effects in a more convenient and easier way.

MATERIALS AND METHODS

This study used experimental method of research to analyze the effectiveness of *Lactobacillus casei Shirota* and *Lactobacillus paracasei* on different bacteria used in the study. Specifically, the study used post-test only design wherein an experimental and a control was assessed to see the effect of the treatment or the manipulation.

Probiotic drink and bacterial culture

The *Lactobacillus casei Shirota* and *Lactobacillus paracasei* was isolated from a commercial probiotic drink. The bacteria used, *Staphylococcus aureus, Streptococcus mutans and Serratia marcescens* were purchased from the National Institute and Biotechnology (BIOTECH) in University of the Philippines, Los Baňos, Laguna.

Preparation of culture media

Sabouraud Dextrose Agar was prepared by suspending 65 mg of the medium in one liter of purified water while heating with continuous agitation until completely dissolved. Afterwards, the solution was sterilized using an autoclave at 121º C for 15 minutes. Subsequently, the solution was removed from the autoclave and could cool down to 45º to 50º C, poured into 24 petri dishes and was allowed to congeal.

Isolation of probiotic microorganism

Lactobacillus casei Shirota and *Lactobacillus paracasei* from a commercial brand of probiotic drink was isolated by shaking the contents vigorously by means of centrifuging 3 mL of each sample at 3400 rpm for 10 minutes then, inoculating one loop-full of the contents to plates. The plates were incubated for 24 hours at 25-30º C in an inverted position. After the incubation period, the cultures were stored in a biological refrigerator at temperature ranging from 2º-8º C until used.

Determination of antibacterial activities

Agar overlay method was used to determine the antagonistic property of *Lactobacillus casei shirota* (LcS) and *Lactobacillus paracasei*. This approach was frequently utilized for *Lactobacillus species* since it is relatively inexpensive and requires minimal resource than any other susceptibility tests, as the technique allows production of homogenous lawn of bacteria within a thin layer of agar. Both were individually spot inoculated in a different Sabouraud Dextrose Agar plates with a loop-full approximately equal to 0.5 McFarland which is 1.5 x 108 CFU/mL of SDA broth culture incubated for 24 hours at 37^0 C. After this, SDA plates (5 mm in diameter) which contained the strains were overlapped with a soft Mueller Hinton Agar (0.8% agar) mixing 83 uL of isolated pathogenic bacterial strain using automated micropipette. Agar solidified and was incubated at 37^0 C for 24 hours. The positive control used is Imipenem disk and the negative control was distilled water. Zone diameter of inhibition values observed were interpreted as ≥16 mm: Susceptible, 14-15 mm: Intermediate and ≤13mm: Resistant (Clinical and Laboratory Science Institute, 2017).

Data analysis

Paired sample T-test was used to employ the significant differences of zone diameter of inhibition between the positive control and treatment and Analysis of Variance determined the susceptibility of *Staphyloccocus aureus*, *Streptococcus mutans* and *Serratia marcescens* to *Lactobacillus casei Shirota* and *Lactobacillus paracasei.*

RESULTS AND DISCUSSION

Staphyloccocus aureus was found to be resistant to the inhibitory activity of both probiotic cultures. The highest mean zone of inhibition was recorded for *Lactobacillus paracasei* at a mean of 7.67 mm across trials. On the other hand, the smallest zone of inhibition was recorded for *Lactobacillus casei Shirota* with a mean value of 4.67 mm. This result deviates from the results of the study conducted by Wong (2015) in which crude protein fractions from lactic acid bacteria, specifically *Lactobacillus plantarum* showed a greater inhibition with that of *S. aureus.* The bacteriocins produced by lactic acid bacteria are considered as an innovative tool for control of pathogens. In addition to this, a *p*-value of 0.007 and 0.020 was noted for *L. casei* and *L. paracasei* respectively. Therefore, both probiotic cultures are considered to have minimal inhibitory activity when compared to the standard drug, but still inferior compared to the positive control.

In relation to this, *Lactobacillus paracasei* showed antibacterial and anticandidal activities against oral pathogens like *Candida albicans* (Chuang, Huang, Yang, & Lin, 2014). Moreover, Actis and Yakuchalii (2015) discussed in one of their researches the antagonistic activity of probiotic Mean values of zone of inhibitions were noted at 7.67 and 8 for *L. casei* and *L. paracasei* correspondingly for *Streptococcus mutans*. According to the antibacterial effectivity, the values obtained are just resistant against the bacterial pathogen but still exhibits inhibitory characteristics exemplified by the presence of a zone of inhibition. A study that can affirm to this observation states that *Lactobacillus spp.* can counteract the virulence factors exhibited by bacteria similar to *S. mutans*, particularly *L. casei,* since they have the power to tolerate acid and bile salts, inhibiting growth of some bacterial pathogen such as Listeria monocytogenes (Hesari, 2014). *L. casei Shirota* was declared significant having a *p*-value of 0.004.

In contrast, *L. paracasei* with resulting *p*-value of 0.082 was considered not significant. As a result, *L. paracasei* was found to be a more potent and effective antibacterial agent than the latter. Nonetheless, the values still showed close yet substandard data when compared to the positive control. This outcome was supported by a study discussing *Lactobacillus paracasei* which was noted to have dual role of both a starter and probiotic culture utilizes its antibacterial effect by inhibiting further growth of bacteria (Sisto, 2013). It can be evidently seen that inhibitions demonstrated by *Serratia marcecens* in respect to the other two pathogenic bacteria, obtained the least level of inhibition against the two probiotic cultures. A mean value of 5 was recorded for *L. casei* zone of inhibition, while *Lactobacillus paracasei* obtained a zone of inhibition mean of 6.33 mm.

A study by Teimourian et al. (2017) reiterated that pretreatment of other *Lactobacillus spp.*, particularly *Lactobacillus acidophilus* ATCC 4356 supernatant can be resistant against *Serratia marcescens* strain, but simply had no effect on other antibiotics except ceftriaxone. This claim supports the study since L. acidophilus was known to grow in complement with *L. casei Shirota* (Pietrangelo, 2016). Both *Lactobacillus spp.* attained the same p-value of 0.005 and is deliberated statistically significant and thus, considered still effective. However, the values obtained for the probiotic cultures still remains mediocre than that of the positive control. This was supported by Shokouhfard (2015) who claimed in his study that *Lactobacillus* strains live as commensals in the human body.

It has been identified that their antimicrobial activity and capability to delay pathogens attached to epithelial cells of urinary and gastrointestinal tracts makes them more suitable to be a substitute for antibiotics. *Lactobacillus casei Shirota* has a statistically significant result, yielding a value of 0.030. Therefore, there is a variation among the susceptibility results obtained by the bacterial pathogens. A mean ZOI value of 4.67 mm, 7.67 mm, and 5 mm was recorded for *Staphylococcus aureus*, *Streptococcus mutans* and *Serratia marcescens* respectively. *S. mutans* yielded the highest mean among the three, leading to the conclusion that it is the bacteria in which *L. casei* Shirota was recorded to be the most potent to.

A study by Marquis (2015) supports this claim by stating that *Streptococcus mutans* grows on temperature ranging between 30-47^0 C and was known as a typical mesophile, making it more suitable to grow inside the hosts. The property of these bacteria can be inferior against antibacterial property of *L. casei Shirota* producing a larger zone of inhibition as recorded.

Moreover, the effectivity of this probiotic culture against the pathogens tested was claimed to be true in a research conducted by Sutula (2013), wherein it was reiterated that lactic acid produced and competitive adhesion or dislodgment of pathogenic bacteria has inhibited the growth of pathogen in dentures and been proposed to exhibit similar mechanisms seen in the gut, since mouth is the first part of the GI tract. A significance of 0.072 presents that *Lactobacillus paracasei* has a statistically insignificant result thus, there is no difference in terms of their antibacterial susceptibility. Despite of the outcome obtained, all three pathogenic bacteria exhibited substantial amount of results producing a mean ZOI of 7.67 mm for *Staphylococcus aureus*, 8 mm for *Streptococcus mutans* and 6.33 mm for *Serratia marcescens.*

However, *Streptococcus mutans* has the leverage between the other two. It can be supported by a study conducted by Chen (2017), evaluating the effect of different *Lactobacillus strains,* including *L. paracasei* on *Streptococcus salivarius* which is one of major participant in the development of dental caries similar to *Streptococcus mutans*. Additionally, Pompejus et al (2013), explained in their pilot study that *Lactobacillus paracasei* has the capability to reduce the presence of pathogenic bacteria present in the gastrointestinal tract which correlates to the study focusing on the growth of bacteria in the oral cavity.

CONCLUSION

The antibacterial property range exhibited by the probiotic cultures, *Lactobacillus casei Shirota* and *Lactobacillus paracasei* was evident as resistant. *L. casei Shirota* was recorded to be most potent against *Streptococcus mutans* while *L. paracasei* was documented to be most powerful against the same pathogen, *Streptococcus mutans* among all the other bacteria tested upon. A greater and larger zone of inhibition was recorded for *L. paracasei* in general when tested against the three pathogenic bacteria, as compared to the antibacterial property showed by *L. casei Shirota*. It can be inferred that both Lactobacillus spp. inhibit further growth of bacterial pathogens; *Staphylococcus aureus*, *Streptococcus mutans* and *Serratia marcescens* but not necessarily killing them. Therefore, making them inferior with the positive control used, Imipenem.

After conducting the experiment, the researchers recommend to look for other method of testing such as disk-diffusion, well-diffusion or broth/agar dilution for the antibacterial effect of *Lactobacillus strains*, to test other pathogenic microorganisms that can be isolated in the mouth of smokers and to use products other than probiotic drinks which contain live *Lactobacillus strains* such as yoghurt and milk.

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