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Comparison of antimicrobial agents Ozonated Olive Oil and Chlorhexidine Gluconate - an in vitro study

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Comparison of antimicrobial agents Ozonated Olive Oil and Chlorhexidine Gluconate - an in vitro study

by

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A Thesis submitted in partial satisfaction of
the requirements for the degree
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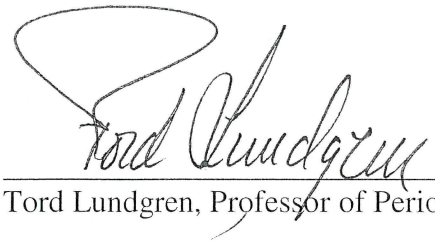
Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.



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CONTENT

TABLES

Tables Page

FIGURES

Figures Page

ABBREVIATIONS

OzOO	Ozonated olive oil
CHX	Chlorhexidine gluconate
A.a	Aggregatibater actinomycetemcomitans
F. alocis	Filifactor alocis
S. mitis	Streptococcus mitis
S. mutans	Streptococcus mutans
P. gingivalis	Porphyromonas gingivalis
T. denticola	Treponema denticola
T. forsythia	Tannerella forsythia
SRP	scaling and root planning
OD	Optical density
CLSI	Clinical and Laboratory Standards Institute
NCCLS	National Committee for Clinical Laboratory Standards
MIC	minimum inhibitory concentration

ABSTRACT OF THE THESIS

Comparison of antimicrobial agents Ozonated Olive Oil and Chlorhexidine Gluconate - an in vitro study

by

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Master of Science, Graduate Program in Periodontics
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Dr. Tord Lundgren, Chairperson

Aim: This study was aimed to compare the antibacterial effect of a commercially available OzOO with CHX 0.12% oral rinse solution against periodontal pathogens *F. alocis, Aa, S. mitis,* and *S. mutans* using a disk diffusion method.

Materials and Methods:
The antimicrobial agents OzOO and 0.12% CHX were impregnated onto sterile discs and placed onto bacterial cultures of *F.alocis, Aa, S.mutans,* and *S.mitis* to evaluate their antimicrobial activity by the disk diffusion susceptibility method. The bacterial growth inhibition zone surrounding each disc was measured in millimeters of diameter with calipers to assess the antimicrobial activity of each agent.

Results:
The mean ± SD diameter of the zone of inhibition for *F. alocis* was 24 ± 1.39mm for OzOO and 32 ± 2.64 mm for CHX. The mean ± SD diameter of the zone of inhibition for *A.a* was 0 ± 0mm for OzOO and 19 ± 1.5mm for CHX. The mean ± SD diameter of the zone of inhibition for *S. mutans* was 0 ± 0mm for OzOO and 19.4 ±1.5 mm for CHX. The mean ± SD diameter of the zone of inhibition for *S. mitis* was 0 ± 0mm for OzOO and 18 ± 1.11mm for CHX.

Conclusion: It was concluded that the antibacterial activity of CHX resulted in greater diameter of inhibition zone for the bacteria *F. alocis* compared to OzOO. The results also indicate growth inhibition for *A.a, S. mitis* and *S. mutans* for CHX but no zone of inhibition was noted for these bacteria for OzOO.

CHAPTER ONE

INTRODUCTIONA AND REVIEW OF THE LITERATURE

Hundreds of different bacterial species are found in the oral cavity. They reside in biofilms, fostering properties that would not be possible if the species existed in a planktonic state. [1,2] In the mouth, teeth provide hard, non-shedding surfaces. These are important for the development of the plaque biofilm which is considered as the primary cause of dental caries and periodontal disease.[1,2]

The main component in oral biofilm is the proliferating microorganisms. It may also contain microorganisms other than bacteria such as fungi, protozoa and some viruses.[1] The pellicle is the first step in the formation of dental plaque on the tooth surface. Once the pellicle is formed, the transition from pellicle to dental plaque is rapid. An important part of the early expansion of plaque is by the growths of different Streptococcus species.[3] The Streptococcus species are also known to be the first inhabitants of the oral cavity that can be found right after birth playing an important role in oral microbiology through-out life. [4] Elimination of the biofilms and plaque that house oral pathogenic microorganisms is consequently the most imperative part to prevent and treat caries and periodontal disease. Proficient brushing and interproximal cleaning techniques mainly eradicate the biofilm and plaque.

The oral microbial flora comprises one of the most diverse human-associated plaque biofilms and oral streptococci profoundly influence its development.[4] Oral

Streptococci may multiply to become harmful initiating disease development.[4.] The role of Streptococci in the development of oral diseases is further influenced by interactions with host epithelial cells, the immune system, and salivary components.[4]

Streptococcus sanguis and *Streptococcus mitis* (*S.mitis*) are known to be among the initial colonizers on the tooth surfaces, and they play an important role in the early plaque formation.[4] Elevated quantities of *Streptococcus mutans (S.mutans)* have been reported in patients with high caries activity.[4] When the biofilm outspreads sub-gingivally, other pathogens propagate into the plaque. The association of bacteria within biofilms is not random but rather specific.[4] *Porphyromonas gingivalis* (*P.gingivalis*), *Treponema denticola* (*T. denticola*), *Tannnerella forsythia* (*T. forsythia*), known as the red complex bacteria, have been recognized as important periodontal pathogens in many scientific studies.[6] The red complex along with *Aggregatibacter actinomycetemcomitans (A.a)* have been stated in a consensus report from the World Workshop of Periodontology to be major etiologic elements in the destruction of the periodontium and the advancement of periodontal disease.[6,7]

A newly discovered "kid on the block", *Filifactor alocis (F. alocis),* has proven with special properties.[8] It is resistant to oxidative stress, which stimulates its growth and allows it to colonize and survive with traditional periodontal pathogens. *F. alocis* has an ability to form synergistic relationships with Streptococcus species, *P. gingivalis* and *Aa* that enhances the microbial invasive capacity of the periodontal tissues.[8] The presence of

F. alocis in dental biofilm and its ability to interact with a variety of oral bacteria leading to the bacterial community development has been demonstrated through studies.[9,10,11]

The primary goal in the treatment of periodontal disease is to stop disease progress and to create long-term periodontal stability. Therapeutic regimes to achieve this objective include various combinations of oral hygiene techniques to reach an adequate level of oral cleanliness, scaling and root planing (SRP), correction of inadequate dental restorations and sometimes surgical treatment. SRP is the primary non-surgical therapy in periodontal treatment with the purpose to eliminate subgingival calculus and plaque biofilm. The caries control and periodontal therapy are frequently supplemented with the use of different topical antimicrobials. Chlorhexidine gluconate (CHX) is the most commonly used agent. It is used in concentrations ranging from 0.12% - 2% and it has been shown to be effective against many gram-positive and gram-negative pathogens.[12,13] It is also effective against some yeast such as *Candida albicans* and viruses including Herpes Simplex and HIV.[14] However, the prolonged usage of CHX is reported with some adverse effects such as tooth staining, epithelial desquamation, altered taste sensation and impairment of wound healing.[15,16]

Ozone (O_3) is an oxidant, sometimes called “activated oxygen" or "triatomic oxygen". It is a powerful oxidant and an effective antibacterial agent used to abolish bacteria, viruses, fungus and also odors.[17,18] The ozone gas has a strong odor and can be produced from oxygen in air or concentrated oxygen. It can be used in gas form or dissolved into water, oil, etc. An ozone dosage of 25 ppm for 20 minutes has been shown

to inactivate and/or eradicate many types of bacteria including *Acinetobacter Baumannii, Clostridium Difficile* and *Staphylococcus Aureus*.[19,20] The use of ozone in medicine has been widely researched and applied for clinical and therapeutic purposes for over 100 years. It has been indicated for the treatment of 260 pathologies.[21] Its first use in dentistry dates back to the 1930th in Switzerland.[22]

Ozonated olive oil (OzOO) with defined peroxide contents has been reported to have antimicrobial effects.[22] This is achieved by combining olive oil and ozone together. OzOO also has the ability to promote production of some growth factors and to activate local antioxidants that promote tissue repair.[23] Topical OzOO has been used to treat a variety of infections, such as war wounds, abscesses, anaerobic and herpetic infections, fungal disease, trophic ulcers and burns, anal fissures, furunculosis and vulvo-vaginitis.[24,25] In one study, OzOO as a mono-therapy and an adjunct to SRP for treating patients with chronic periodontitis improved the periodontal conditions.[26] In another study comparing OzOO with 0.2% CHX using a disk diffusion susceptibility test, it was noted that OzOO showed significant growth inhibition again *P. gingivalis* and *Staphyloccus aureus*.[27] Also, a significant improvement in epithelial healing and gingival health was reported after topical OzOO was placed on a free gingival graft.[28]

In a recent in-vitro study at Loma Linda University, School of the Dentistry, when comparing the antibacterial activity of OzOO against *P. gingivalis* and *Aa*, it was concluded that OzOO was superior to CHX. A noticeable finding of this project was a partial antibacterial action of OzOO against *Aa*.[29] The present in-vitro experiment was

aimed to compare the antibacterial effect of a commercially available OzOO with CHX 0.12% oral rinse solution against periodontal pathogens *Aa, F.alocis, S. mitis,* and *S. mutans* using a disk diffusion method.[29]

The null hypothesis in this research project was that no significant difference exists between the two antimicrobial agents; OzOO and CHX regarding the bacterial growth inhibition effects. Research of the agents' ability to display antimicrobial effects on certain oral bacteria will increase our knowledge of how to successfully treat dental caries and periodontal disease.

CHAPTER TWO

MATERIALS AND METHODS

Microbial Species

For this study, *F. Alocis* (ATCC 35896) was obtained from the Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University. *S. mutans* (ATCC 25175) and *A.a* (ATCC 33384) was obtained from the Center for Dental Research, Loma Linda University School of Dentistry. *S. mitis* (ATCC49456) was obtained from the American Type Culture Collection (ATCC).

Culture Conditions

Frozen and isolated *F. Alocis* and *A.a* were inoculated into culture tubes containing Brain Heart Infusion (BHI) media that consisted of 18.5 g dehydrated BHI, 5ug/ml hemin, 100ul vitamin K, 2.5g yeast extract and 0.5g DL-cystein. The tube was incubated at 37^{o} C under anaerobic conditions for 24 hours.

S.mutans and *S.mitis* were also inoculated into culture tubes containing Brain Heart Infusion (BHI) media that consisted of 18.5 g dehydrated BHI, 5ug/ml Hemin, 100ul vitamin K, 2.5g yeast extract and 0.5g DL-cystein. The tubes were incubated at 37^{o} C under anaerobic conditions for 24 hours. These liquid broth cultures were used to plate the agar plates.

The bacterial concentrations were calculated by optical density (OD). For example, at OD of 1.0 will result in $1x10^9$ bacteria/ml. The bacteria are first grown to the maximum OD, and then serially diluted to achieve countable colonies that are measured/counted.

Antiseptics

The following antiseptics were tested: OzOO (gel type, $PurO_3$, Promolife Inc, Fayetteville, AR, USA) with a peroxide value of 224 meq/kg, and 0.12% CHX (Peridex, 3M, ESPE, St. Paul, MN, USA) were used.

Figure 1. Ozonated Olive Oil ($PurO_3$, Promolife Inc, Fayetteville, AR, USA)

Figure 2. 0.12% CHX (Peridex, 3M, ESPE, St. Paul, MN, USA)

The positive controls were the CHX group and the OzOO group. Negative controls were not included in this study; it was done in the previous study at LLUSD where the results showed no inhibition against pure olive oil and saline.

Disc Diffusion Susceptibility Testing

The disk diffusion susceptibility test was used to compare and evaluate the antimicrobial efficacy of the OzOO and CHX in the bacteria culture models. Six-millimeter diameter sterile discs (antibiotic sensitivity disks, blank, sterile, Carolina Biological Supply Company, Burlington, NC, USA) were used for this study.

Figure 3. 6mm diameter sterile discs (antibiotic sensitivity disks, blank, sterile, Carolina Biological Supply Company, Burlington, NC, USA)

The sterile disks were impregnated by two antiseptics, OzOO and CHX. Fifty ul of each antiseptic was impregnated into each disk with a micropipette. The OzOO, which presents as a semi solid consistency, was heated in a bath circulator, 55^{o} C, to convert it into a liquid consistency for ease of impregnation. Each of the impregnated disks was kept for 24 hours, at room temperature, before the continuation of the research to ensure uniform impregnation.

Microbial suspension containing the bacteria *F. alocis*, *S. mitis*, *S. mutans*, and *Aa* were spread aseptically onto the petri dishes that contain specific BHI agar. The BHI agar consisted of 18.5 g dehydrated BHI, 5ug/ml hemin, 100ul vitamin K, 2.5g yeast extract and 0.5g DL-cystein and 10g bacto agar.

The discs containing the antiseptics were placed on the bacterial culture models to be evaluated for antimicrobial activity, after 48 hours of incubation. This was assessed by measuring the diameter of the zone of growth inhibition in millimeters that surrounded the sterile disks, using a Vernier caliper, measuring was done by two examiners reading

and measuring the zone of inhibition, Dr. David and Dr. Mishra (Microbiology lab, LLUSM).

The testing between OzOO and CHX with the different microbes were done in a triplicate testing manner.

Statistical Analysis

Descriptive statistics of mean and standard deviation (SD) for each bacteria, *F. alocis, A.a, S. mutans,* and *S.mitis*, using OzOO and CHX were calculated and the diameters of the zone of inhibition for each was compared using Wilcoxon Signed Rank Test at alpha level 0.05

CHAPTER THREE

RESULTS

The diameters of the zone of inhibition for all the bacteria were measured. The mean, standard deviations, sample size and p-values are presented in Table 1 along with the bar graph (figure 4).

The mean ± SD diameter of the zone of inhibition for *F. alocis* was 20.25 ± 1.39mm for OzOO and 26.78 ± 2.64 mm for CHX (figure 5). The mean ± SD diameter of the zone of inhibition for *A.a* was 0 ± 0mm for OzOO (figure 6) and 15 ± 0.33mm for CHX (figure 7). The mean ± SD diameter of the zone of inhibition for *S. mitis* was 0 ± 0mm (figure 6) for OzOO and 18 ± 1.11mm for CHX (figure 7). The mean ± SD diameter of the zone of inhibition for *S. mutans* was 0 ± 0mm (figure 6) for OzOO and 19.4 ±1.5 mm for CHX (figure 7).

The measured diameters for *A.a, S. mutans* and *S. mitis* with OzOO were 6mm, the same as the diameter of the sterile disk, showing no signs of the growth inhibition (Figures 6). The measured diameters for *F. alocis, A.a, S. mutans,* and *S.mitis* with CHX showed a clear demarcated zone of inhibition. The Wilcoxon Signed Rank Test showed a significant difference in the zone of inhibition for each of the microbes.

Table 1. Mean diameter and SD of the zone of inhibition for *F.alocis, A.a, S.mitis, and S.mutans* with OzOO and CHX

Antiseptic	*F. alocis*	*A.a*	*S. mitis*	*S. mutans*
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
OzOO	20.25 ± 1.39 (n=8)	0 ± 0 (n=9)	0 ± 0 (n=9)	0 ± 0 (n=9)
CHX	26.78 ± 2.64 (n=9)	15 ± 0.33 (n=9)	18 ± 1.11 (n=9)	19.4 ± 1.5 (n=9)
p-value	0.011	0.004	0.007	0.007

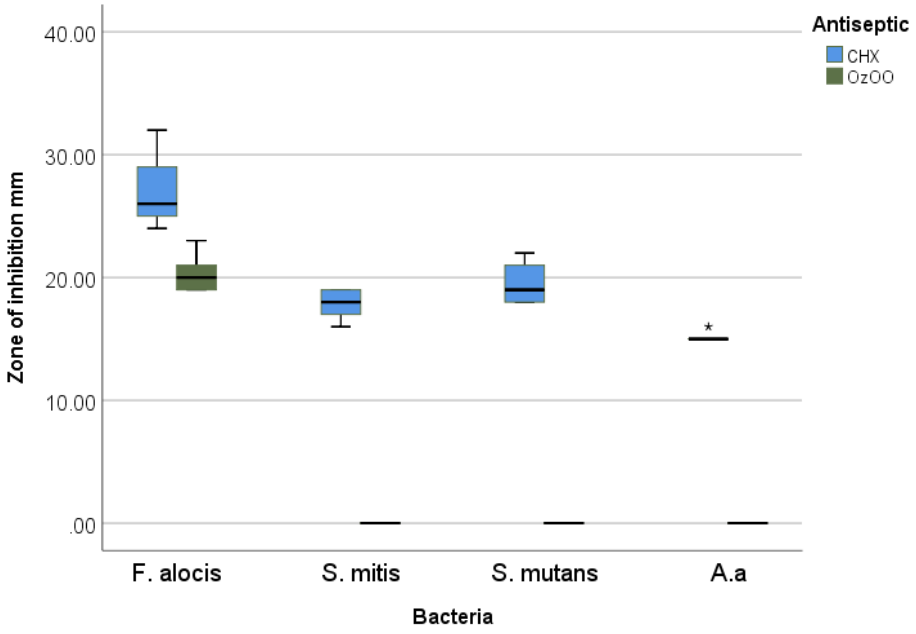

Figure 4. Box plot showing mean diameter and SD of the zone of inhibition for *F.alocis, A.a, S.mitis, and S.mutans* with OzOO and CHX

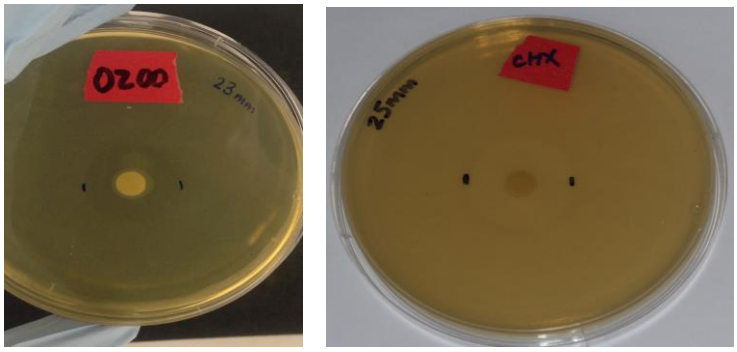

Figure 5. Petri dishes for *F. alocis* showing a well demarcated zone of inhibition with OzOO and CHX

Figure 6. Petri dishes for *A.a, S.*mitis and *S. mutans* showing no zone of inhibition with OzOO

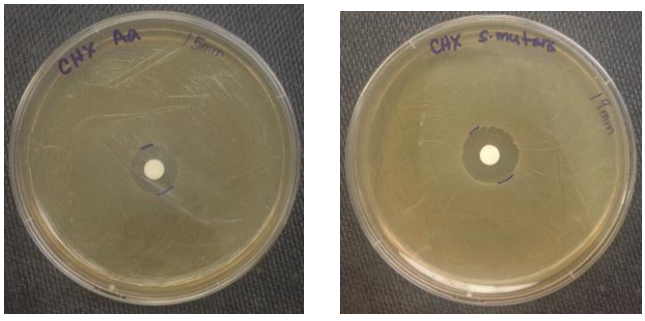

Figure 7. Petri dishes for *A.a* and *S.mutans*, showing a well demarcated zone of inhibition with CHX

CHAPTER FOUR

DISCUSSION

S. mitis are among the early colonizers of oral cavity surfaces such as teeth as well as gingiva and mucous membranes.[4] These Gram-positive bacteria's ability to attach to different surfaces is not only contributing to the early build-up of dental biofilms but is also reported to be one cause of bacterial endocarditis.[30] When the oral biofilm is growing and outspreads sub-gingival, many different potential pathogens propagate into the plaque. The association of bacteria within oral biofilms is reported not to be random but rather specific.[4]

In the consensus report from the World Workshop of Periodontology *A.a* is considered to play a major role in developing periodontal disease. *A.a is a* Gram-negative facultative anaerobe and non-motile rod with strong leukotoxic effects.[31] *F. alocis*, the other periodontal pathogen tested in this research project has proven to be resistant to oxidative stress and to form synergistic relationships with Streptococcus species[10,11]. There is evidence showing that periodontal disease and caries share many contributory factors, for example, increased colonization of *S. mutans* was reported in chronic periodontitis subjects both in saliva and sub-gingival plaque samples.[32]

As a part of controlling caries and periodontal disease the treatments are frequently supplemented with the use of different topical antimicrobials. CHX is a commonly used agent that has been shown to be effective against many gram-positive and gram-negative pathogens.[12] Caries can be managed by adding chemical therapy,

based on the assessed risk level, coupled with necessary restorative procedures. For high-risk patients, a combination of antibacterial and fluoride therapy is recommended. The addition of antibacterial plus fluoride therapy to the traditional restorative treatment plan has been shown to reduce the caries increment by about 20% to 40% in high-risk adult patients. The chemical therapy used for these patients is often a combination of daily antibacterial therapy of 0.12%- 0.20 CHX mouth rinse together with high-concentration fluoride toothpaste (5,000 ppm F), both for home use.[33]

The anti-microbial agent OzOO is created through a chemical reaction between ozone and olive oil called ozonolysis. Ozone, activated oxygen, is funneled into a container of olive oil. An electrical charge is generated and the ozone slowly infuses into the olive oil. The chemical reaction, ozonolysis, between the ozone and unsaturated fatty acids in the olive oil contributes to its antimicrobial effect as hydrogen peroxidase is formed. In the product PurO3™ that we used the manufacturer reported a content of 224 meg/Kg of hydrogen peroxide as a result of the chemical reaction. In another study done on OzOO vs CHX and povidone-iodone, a disk diffusion test, the peroxide value in the OzOO was 560/590 meg/kg.[27]

Antibacterial activity can be measured by various methods for example through diffusion, dilution, thin layer chromatography, time kill test, ATP bioluminescence assay and flow cytofluorometry.[34] One of the diffusion methods is the disk diffusion susceptibility test that was used in this in-vitro study. This test method was used in this in-vitro study to assess the antimicrobial activity by measuring the zone of inhibition that was created around each of the disks that were impregnated with antiseptics. According to the Clinical and Laboratory Standards Institute (CLSI), formerly National Committee

for Clinical Laboratory Standards (NCCLS) the results using this method could be interpreted both from quality and quantity view point.[35] The qualitative side of this test method required associating/calibrating data from CLSI. There seems to be no specific data available regarding OzOO and CHX in the CLSI panel so the results in this study were not assessed qualitatively.

In a recent in-vitro study at Loma Linda University, School of Dentistry it was concluded that the antibacterial activity of OzOO against *P. gingivalis* and *A.a* was superior to that of CHX when comparing the diameters of the growth inhibition zones. It was also reported that the growth inhibition zone of OzOO against both microbes was partially blurred especially for *A.a*. This was interpreted as a partial antibacterial activity, a potential regrowth of bacteria during the incubation period or the use of a non-purified batch of bacteria. It was recommended to repeat the *A.a* part of the testing. When OzOO was used in our study a clear inhibition zone was seen for *F. alocis* but no zone for the other tested bacteria including *A.a*. This is in disagreement with the previous LLU study and may be explained by variations of the peroxide content of the product provided by the manufacturer ($PurO_3$, Promolife Inc, Fayetteville, AR, USA). The peroxide value, 224 meq/kg, given by the company was not verified by us before the start of the research. As compared to the other studies,[27] the peroxide value in the OzOO used in this study was low. Oral streptococci are able to produce growth-inhibiting amounts of hydrogen peroxide (H_2O_2) as byproduct of aerobic metabolism. This is assumed to be a protective mechanism for the initial colonizers of the biofilm against other competing species. Therefore the species that evolve and contribute to the developing biofilm are adapted to

withstand low hydrogen peroxide levels. This may be one explanation why there was no inhibition zone seen for the *S. mitis* and *S.mutans* when using OzOO.[36,37,38]

For quantitative assessments the minimum inhibitory concentration (MIC) should be assessed. As in our disk diffusion method research we did not determine the MIC, only the diameter of zone of inhibition was compared for each of the antiseptic agents to evaluate the antibacterial activity. In a similar study by Evans et al.[39] the CHX 0.2% growth inhibition zone of *S. mutans* was reported to be between 7.4 to 7.6 mm and the minimum concentration at which an inhibition zone against *S. mutans* was seen when using a CHX 0.005% solution. As was mentioned above, the MIC was not measured in our project but the zone of inhibition for S.mutans for CHX 0.12% was 19.4 ±1.5 mm.

CHAPTER FIVE

CONCLUSION

Within the limits of this study, it is concluded that the antibacterial activity of CHX resulted in greater diameter of inhibition zone for the bacteria *F. alocis* compared to OzOO. The results also indicate growth inhibition for *A.a, S. mitis* and *S. mutans* for CHX but no zone of inhibition was noted for these bacteria for OzOO. For the clinical use of OzOO as an alternative to CHX, other research such as in vivo and clinical study should be designed and planned.

REFERENCES

1. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998;25:134-144.

2. Flemming H.C, Wingender J Griegbe, Mayer C. Physico-chemical properties of biofilms. In: Evans LV, editor. Biofilms: recent advances in their study and control. Harwood Academic Publishers; 2000;19–34.

3. Rosan B, Lamont RJ. Dental plaque formation. Microbes Infect 2000;2:1599-1607.

4. Abranches, J., Zeng, L., Kajfasz, J. K., Palmer, S. R., Chakraborty, B., Wen, Z. T.,Lemos, J. A. Biology of Oral Streptococci. Microbiol Spectr, 2018; 6(5). doi:10.1128/microbiolspec.GPP3-0042-2018.

5. Dewhirst FE, Chen T, Izard J, et al. The human oral microbiome. J Bacteriol 2010;192:5002-5017.

6. Consensus report. Periodontal diseases:Pathogenesis and microbial factors. Ann Periodontol 1996;1:926-32.

7. Proceedings of the 1996 World Workshop in Periodontics. Ann Periodontol 1996;1:1-947.

8. Aruni W, Chioma O, Fletcher HM. Filifactor alocis: The Newly Discovered Kid on the Block with Special Talents. J Dent Res 2014;93:725-732.

9. Griffen AL, Beall CJ, Campbell JH, et al. Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing. ISME J 2012;6:1176-1185.

10. Schlafer S, Riep B, Griffen AL, et al. Filifactor alocis--involvement in periodontal biofilms. BMC Microbiol 2010;10:66.

11. Wang Q, Wright CJ, Dingming H, Uriarte SM, Lamont RJ. Oral community interactions of Filifactor alocis in vitro. PLoS One 2013;8:e76271.

12. Herrera D, Sanz M, Jepsen S, Needleman I, Roldan S. A systematic review on the effect of systemic antimicrobials as an adjunct to scaling and root planing in periodontitis patients. J Clin Periodontol 2002;29 Suppl 3:136-159.

13. Wade WG, Addy M. In vitro activity of a chlorhexidine-containing mouthwash against subgingival bacteria. J Periodontol 1989;60:521-525.

14. Kawana R, Kitamura T, Nakagomi O, et al. Inactivation of human viruses by povidone-iodine in comparison with other antiseptics. Dermatology 1997;195 Suppl 2:29-35.

15. Bassetti C, Kallenberger A. Influence of chlorhexidine rinsing on the healing of oral mucosa and osseous lesions. J Clin Periodontol. 1980;6:443–56.

16. Cline NV, Layman DL. The effects of chlorhexidine on the attachment and growth of cultured human periodontal cells. J Periodontol 1992;63:598-602.

17. Romero C, Medina E, Vargas J, Brenes M, De Castro A. In vitro activity of olive oil polyphenols against Helicobacter pylori. J Agric Food Chem 2007;55:680-686.

18. K.G. Kerr, C.B. Beggs, S.G. Dean, J. Thornton, J.K. Donnelly, N.J. Todd, *et al.*Air ionization and colonization/infection with methicillin-resistant *Staphylococcus aureus* and *Acinetobacter* species in an intensive care unit Intensive Care Med 2006; 315-317

19. Saini R. Ozone therapy in dentistry: A strategic review. J Nat Sci Biol Med 2011;2:151-153.

20. Nogales CG, Ferrari PH, Kantorovich EO, Lage-Marques JL. Ozone therapy in medicine and dentistry. J Contemp Dent Pract 2008;9:75-84.

21. Stubinger S, Sader R, Filippi A. The use of ozone in dentistry and maxillofacial surgery: a review. Quintessence international. 2006;37:353-9.

22. Seidler V, Linetskiy I, Hubalkova H, Stankova H, Smucler R, Mazanek J. Ozone and its usage in general medicine and dentistry. A review article. Prague Med Rep 2008;109:5-13.

23. Oosting RS, Van Rees-Verhoef M, Verhoef J, Van Golde LM, Van Bree L. Effects of ozone on cellular ATP levels in rat and mouse alveolar macrophages. Toxicology 1991;70:195-202.

24. Bocci V. Ozone: A New Medical Drug. Dordrecht: Springer, 2005;32-5, 102-3.

25. Tara F, Zand-Kargar Z, Rajabi O, et al. The effects of ozonated olive oil and clotrimazole cream for treatment of vulvovaginal candidiasis. Altem Ther Heanth Med. 2016;22 (4): 44-9.

26. Patel PV, Patel A, Kumar S, Holmes JC. Effect of subgingival application of topical ozonated olive oil in the treatment of chronic periodontitis: a randomized, controlled, double blind, clinical and microbiological study. Minerva Stomatol 2012;61:381-398.

27. Montevecchi M, Dorigo A, Cricca M, Checchi L. Comparison of the antibacterial activity of an ozonated oil with chlorhexidine digluconate and povidone-iodine. A disk diffusion test. New Microbiol 2013;36:289-302.

28. Patel PV, Kumar S, Vidya GD, Patel A, Holmes JC, Kumar V. Cytological assessment of healing palatal donor site wounds and grafted gingival wounds after application of ozonated oil: an eighteen-month randomized controlled clinical trial. Acta Cytol 2012;56:277-284.

29. Lee, Changmin, Comparison of Antibacterial Activity of Antimicrobial Agents, Ozonated Olive Oil and Chlorhexidine Gluconate: In Vitro Disk Diffusion Susceptibility Test, Master Thesis, Loma Linda University School of Dentistry, Loma Linda

30. Murdoch D. R., Corey G. R., Hoen B., Miro J. M., Fowler V. G., Bayer A. S. Endocarditis, I. C. Clinical Presentation, Etiology, and Outcome of Infective Endocarditis in the 21st Century. Archives of Internal Medicine 2009;169(5), 463-473. doi:DOI 10.1001/archinternmed.2008.603.

31. Haraszthy VI, Hariharan G, Tinoco Em, et al. Evidence for the role of highly leuotoxic Actinobacillus Actinomycetemcomitans in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J Periodontol. 2000;71(6):912-2

32. Dani S, Prabhu A, Chaitra KR, et al. Assessment of Streptococcus mutans in healthy versus gingivitis and chronic periodontitis: A clinic-microbiological study. Contemp Clin Dent 2017 7(4):529-534

33. Featherstone JDB, Chaffee BW. The evidence for Caries Management by Risk Assessment (CAMBRA) Adv Dent Res. 2018;29 (1): 9-14.

34. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin Infect Dis 2009;49:1749-1755.

35. Karlsmose S. Laboratory Protocol: "Susceptibility testing of Enterobacteriaceae using disk diffusion". WHO Global Foodborne Infections Network Laboratory Sub-Committee. 2010.

36. Coykendall AL. Classification and identification of the viridans streptococci. Clin Microbiol Rev 1989;2: 315–328.

37. Chen L, Ge X, Dou Y, Wang X, Patel JR, et al. Identification of hydrogen peroxide production-related genes in Streptococcus sanguinis and their functional relationship with pyruvate oxidase. Microbiol 2011;157: 13–20.

38. Zhu L, Kreth J. The role of hydrogen peroxide in environmental adaptation of oral microbial communities. Oxid Med Cell Longev 2012; Article ID 717843.

39. Evans A, Leishman SJ, Walsh LJ, Seow Wk. Inhibitory effects of antiseptic mouthrinses on Streptococcus mutans, Streptococcus sanguinis and Lactobacillus acidophilis. Aust Dent J. 2015; 60 (2): 247-54