

The effect of percentage combination of probiotics
and chlorhexidine on *C. albicans*



UNIVERSITY of the
WESTERN CAPE

A minithesis submitted in partial fulfillment of the
requirements for the degree of master's degree in Oral
Medicine in the Department of Periodontology and
Oral medicine

University of the Western Cape.

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chlorhexidine on C.albicans*

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KEYWORDS

C. albicans

Chlorhexidine

Colony counting

Oral candidiasis

Probiotics

Serial dilution



ABSTRACT

Background:

Candida is a normal commensal of the oral cavity; these microorganisms can be isolated in 20% to 80% of healthy individuals. Oral candidiasis (OC) is the most common fungal infection related to the oral cavity caused by the most common opportunistic fungus (*C. albicans*). The management of oral candidiasis involves removal or reduction of predisposing factors together with the administration of an antimetabolic agent either in topical or systemic form with or without chlorhexidine (CHX). The increasing number of people who are immunosuppressed and the development of antimicrobial resistance, has necessitated there is a need to explore others treatment to fight this infectious disease. This led to the exploration and use of beneficial microorganisms (probiotics) as an alternative prophylactic & therapeutic mode of treatment against *Candida* infections.

Aim:

To evaluate the effect of percentage combination of chlorhexidine (CHX) and probiotics on (PB) *C. albicans* growth.

Method:

The study was conducted in the Department of Oral Medicine and Periodontology and the Oral and Dental Research Laboratory (ODRL) at the Faculty of Dentistry, University of the Western Cape, Tygerberg campus. This in-vitro laboratory study was based on the established principle of spread plate technique followed by colony counting on selective media and non-selective media. Chlorhexidine (CHX) and probiotics (PB) were combined at different concentrations (xCHX_yPB) (percentage combination), the added sum of which equals to 100 % (the percentage is achieved by multiplication of volumes (V*V)), to evaluate their effect on *C. albicans*. The treatments were incubated at 37°C for 30 minutes and 24 hours. Serial dilution was carried out on a micro-titer plate based on McFarland standards. A fixed volume (100 µL)

of the solution was transferred into plates (selective and non-selective media). Deionized water served as a control (negative).

Results:

100 μL CHX with 0 μL PB (100 CHX 0 PB) had a mean value of (0) at both 30 minutes and 24 hours. While 25 μL CHX with 75 μL PB (25 CHX 75 PB) the mean value (9.4) at 30 minutes and (9.5) at 24 hours. 0 μL CHX with 100 μL PB (0 CHX 100 PB) the mean value was (9.6) at 30 minutes while at 24 hours the mean value was (9.9). The control (0 CHX 0 PB) showed at 30 minutes and at 24 hours slightly similar reading (mean) as for the 0 μL CHX with 100 μL PB (0 CHX 100 PB). The largest difference was between the control (0 μL CHX with 0 μL PB) (0 CHX 0 PB) versus 100 μL of CHX with 0 μL PB (100 CHX 0 PB) (9.692), $p < 0.001$. The smallest difference was between control (0 μL CHX with 0 μL PB) (0 CHX 0 PB) versus 0 μL CHX with 100 μL PB (0 CHX 100 PB).

Conclusion:

Chlorhexidine (CHX) and probiotics (PB) could be used at different combination concentration, provided that the CHX ratio is lower than the PB. Depending on the combination used, the number of colonies were slightly different. CHX (100 CHX) showed the lowest number of colonies (zero), while probiotics (0 CHX 100 PB) showed the highest number of colonies. Combination 25 CHX 75 PB showed reduction in CFU/mL. This means that CHX destroyed the organism which have already been established in literature, in comparison to probiotics which reduces the number of organisms (strain specific effect). These two effects are required when treating *Candida* infection, the treatment goal when treating *Candida* infection is to eradicate the organism but not completely, because if we completely eradicate the organism it will cause an unbalance in the normal flora of the oral cavity. Therefore, it is recommended that probiotics be administered to patients after being treated with CHX to avoid an imbalance in the normal flora. . Should these combinations be used simultaneously or subsequently is a question that requires further elucidation by research.



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DECLARATION

I, the undersigned, Sara Ali Mohamed Ahmed, hereby declare that the work contained I this dissertation titled; “The effect of percentage combination of probiotics and chlorhexidine on *C. albicans*” is my original work and has not been previously in its entirety or in any part submitted at any university for any degree or examination.

Dr. Sara Ali Mohamed Ahmed

01 November 2019



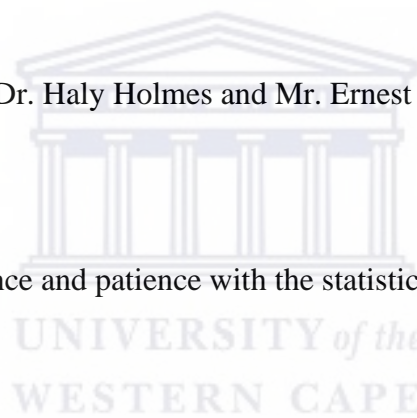
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ABBREVIATIONS

μL	: Microliter
BHI	: Brain heart infusion
<i>Candida</i> spp	: <i>Candida</i> species
CFU	: Colony forming units
CHX	: Chlorhexidine
CO ₂	: Carbon dioxide
CYP2C9	: Cytochrome P450 2C9
GIT	: Gastrointestinal tract
H ₀ :	: Null hypothesis
H _A :	: Alternative hypothesis
HIV	: Human immunodeficiency virus
MIC	: Minimal inhibitory concentration
OC	: Oral Candidiasis
ODRL	: Oral and Dental Research Laboratory
P 450 (CYP)	: Cytochrome P450
PB	: Probiotics
PBS	: Phosphate buffered saline
RCTs	: Randomized control trails
SD	: Standard Deviation



SDA :Sabouraud dextrose agar with chloramphenicol

Vs.V : Volume by Volume multiplication

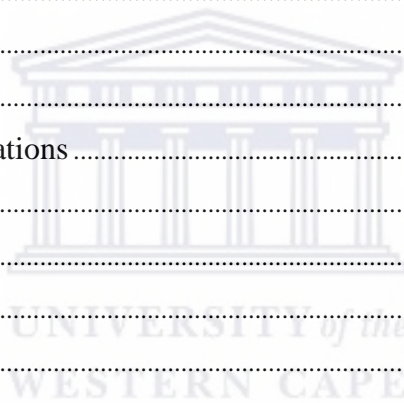
$x\text{CHX}y\text{PB}$: where x represents the value of CHX (chlorhexidine) in μL , y represents the value PB (probiotics) in μL



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Chapter one

Introduction

Candida is a normal commensal of the oral cavity and has been isolated in 20% to 80% of healthy individuals. The microorganism is also normal commensals of the vagina and digestive tract. Any imbalance (dysbiosis) in the normal environment or in the host defense system as seen in immuno-compromised and immuno-suppressed individuals provides *Candida* with the opportunity to overgrow and become pathogenic (Sardi *et al.*, 2013, Vecchiarelli *et al.*, 2012).

Oral candidiasis (OC) is the most common oral fungal infection (Arendrup 2010).

Factors attributed to oral dysbiosis include use of broad-spectrum antibiotics, low birth weight neonates, immuno-suppressed individuals such as transplant recipients, diabetics and those undergoing chemotherapy (Arendrup 2010).

The management of OC involves reduction or removal of the predisposing factors along with an antimetabolic agent in either topical or systemic form. Antimetabolic drugs are categorized into two broad entities - polyenes and azoles (Scheibler *et al.*, 2018).

Chlorhexidine (CHX) is a di-gluconate antiseptic/disinfectant agent developed in 1940 in England. Its inhibitory effect on the dental biofilm was discovered by Schroeder in 1969. Chlorhexidine (CHX) is considered the gold standard in dentistry and is available in various formulations (Balagopal, Arjunkumar 2013). Chlorhexidine (CHX) is a dose-related agent which can be either bacteriostatic or bactericidal depending on its dosage. In high concentrations the molecules exert their antibacterial property by interfering with the bacterial cell membrane, causing an increase in cell permeability with subsequent cell lysis. In lower concentrations the cellular transference of bacterial cell is damaged with the formation of pores in the cellular membrane (Machado *et al.*, 2010).

Chlorhexidine (CHX) (0.2%) concentration is utilized in clinical practice because of its wide range of activity against oral microorganisms including *Candida*. Chlorhexidine (CHX) has demonstrated an antifungal (both fungistatic and fungicidal) effects in various clinical studies where it was successful in the management of oral candidiasis (OC) (Ellepola, Samaranayake 2001). Ellepola, Samaranayake 2001 also revealed that the normal oral epithelium (mucosa) and their receptors for microbes are destroyed after exposure to chlorhexidine (CHX).

As previously mentioned CHX is also known to have both a fungicidal and fungistatic effect, which results in coagulation of nucleoproteins and alterations in cell walls permitting possible leakage of cytoplasmic components through the plasmalemma (Machado *et al.*, 2010).

Associated side effects are also related to the concentration and duration of use. Staining is less frequently seen with large quantities of dilute concentrations in comparison with smaller quantities with higher concentrations. Evidence have shown that continuous and frequent application of CHX oral rinses may have a transient effect on taste sensation, this is assumed to be due to binding of CHX to specific sodium receptors in the taste buds, which is completely different from the receptors for sour, sweet and bitter sensation. Desquamative oral mucosal lesions have been reported with continuous use of chlorhexidine (CHX). This may be explained by the precipitations of acidic mucins and proteins that coat the mucous membrane, which makes the mucosa susceptible to mechanical damage or to the cytotoxic effects of chlorhexidine (Sajjan *et al.*, 2016). Thus, lower concentrations of chlorhexidine are recommended for prolonged usage.

With the increase number of individuals who are immunosuppressed and the development of antimicrobial resistance as well as the unwanted secondary effects associated with CHX, the exploration of other treatment modalities to fight these infections warrants investigation (Huovinen 2001).

Numerous studies (Chew *et al.*, 2015, Ishikawa *et al.*, 2015) have shown probiotics to be an efficient treatment modality in the management of fungal infections caused by *Candida* species (*Candida* spp). In addition, probiotics are easy to use and well tolerated by individuals (Mundula *et al.*, 2019).

The purpose of this study is to explore the use of beneficial microorganisms (probiotics) as an alternative prophylactic & therapeutic mode of treatment against *Candida* infections (Matsubara, *et al.*, 2016).



Chapter two

Literature review

***Candida* and oral health**

The terminology *Candida* emerges from the Latin word candid, meaning white. The spores of *Candida* are symbiont, an innocuous form of a dimorphic fungus that becomes protruding and pathogenic bearing pseudo hyphae when there is a disruption in the equilibrium of the normal flora or decline in the host immunity (Byadarahally Raju, Rajappa 2011).

The transformation to a pathogenic microorganism is dependent on the host and other predisposing factors, of which the virulence of the microorganism remains the principal factor in the pathogenesis (Byadarahally Raju, Rajappa 2011).

Candida spp. are regarded as opportunistic microorganisms. There are many species of *Candida* namely, *Candida albicans* (*C. albicans*), *Candida glabrata* (*C. glabrata*), *Candida krusei* (*C. krusei*), *Candida tropicalis* (*C. tropicalis*), *Candida dubliniensis* (*C. dubliniensis*) and *Candida parapsilosis* (*C. parapsilosis*). The most encountered species from the oral cavity, in both healthy and diseased individuals, is *C. albicans*, which is reported in more than 80% of all oral yeast infections . There is an increase in interest in infections generated by the opportunistic pathogen *Candida*, partially related to the development of HIV infection as well as the global increase in the use of immunosuppressive chemotherapy.

Non-*albicans* *Candida* species also play an important role in human disease. *C. glabrata* and *C. krusei* are species of great importance due to their resistance to numerous antifungal agents. *C. dubliniensis* was first reported in 1995, when it was co-isolated with *C. albicans* from Human Immunodeficiency Virus (HIV) individuals presenting with oral candidiasis (OC) (Byadarahally Raju, Rajappa 2011).

Structure of *Candida albicans*

C. albicans is a polymorphic fungus, with various associated growth patterns. It can grow in oval -yeast shape pattern, elongated ellipsoid pattern with narrowing at the septa (pseudo hyphae) or as parallel-walled true hyphae (Figure 1). The yeast and true hyphae are frequently detected during infection. The responsibility of pseudo hyphae and switching (from commensal to pathogen) in-vivo is not clearly understood and chlamydo spores have

not been detected in patient samples (Chlamydo-spores present as thick-walled spore-like structures) (Mayer *et al.*, 2013).

C. albicans morphology can be affected by a wide range of environmental signals. At low pH (< 6) *C. albicans* grow in the yeast pattern, whereas at a high pH (> 7) hyphal growth is produced. Other conditions that promote the formation of hyphae include starvation, the presence of serum or N-acetylglucosamine, physiological temperature and carbon dioxide (CO₂). Morphogenesis has also been attributed to quorum sensing, which is a mechanism of microbial communication (Mayer *et al.*, 2013).

The alteration between yeast and hyphal growth pattern is known as dimorphism and it has been advocated that both growth patterns are significant for pathogenicity. The hyphal growth pattern is more invasive than the yeast pattern, while the smaller yeast pattern is generally accepted to be involved in primary dissemination (Mayer *et al.*, 2013).

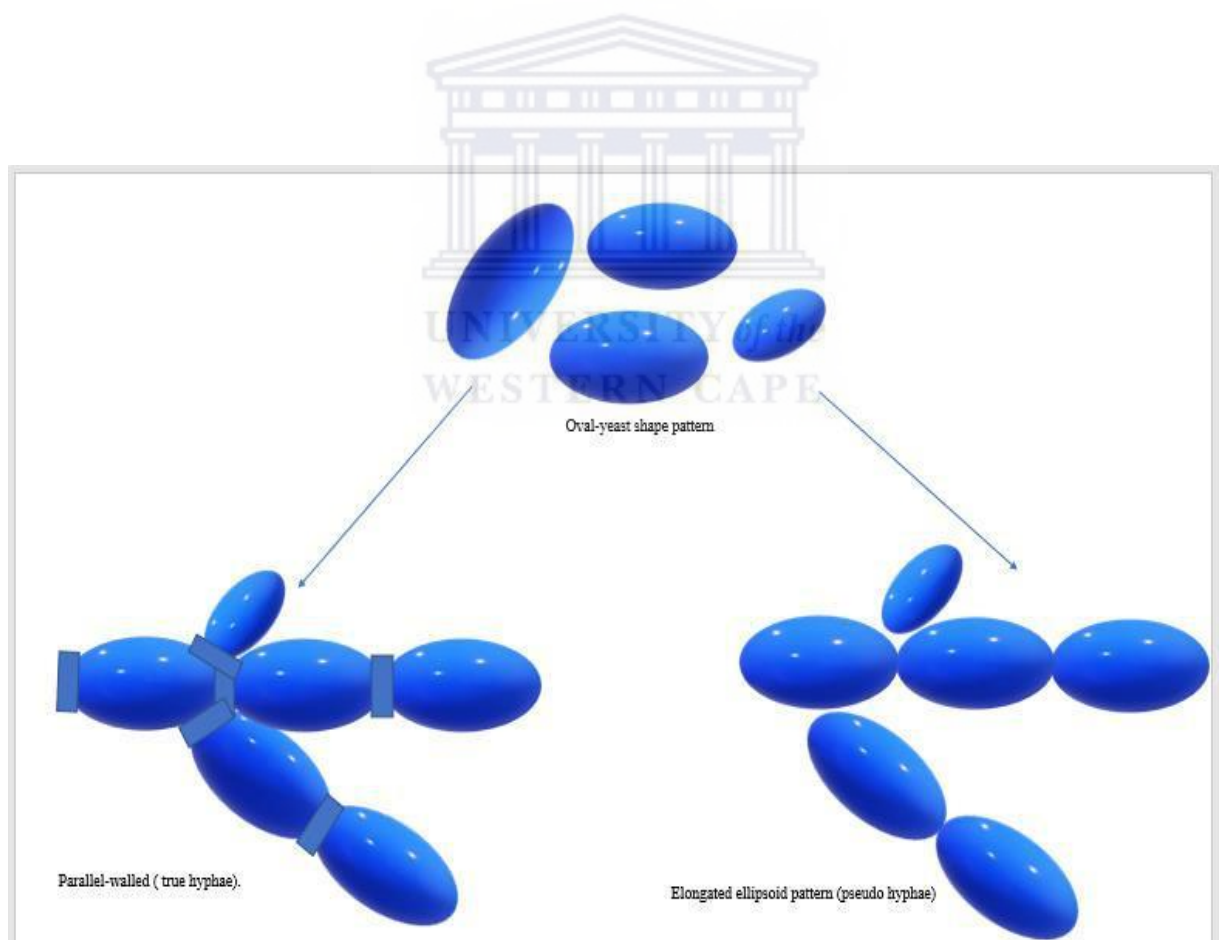


Figure 1. Shows the various growth patterns of *C. albicans* (Mayer *et al.*, 2013).

In-vitro pathogenesis of *C. albicans* biofilm

The formation of *C. albicans* biofilm in the laboratory can be effortlessly noticed on any surface. The evolution of *C. albicans* biofilm incorporate four temporal phases; phase one is the adherence to a surface in spherical yeast cells pattern, followed by multiplication to create a basal layer of anchoring cells, phase three is characterized by the growth of pseudo hyphae and hyphae. The pseudo hyphae grow in an ellipsoid cells pattern in which the cells join end to end while the hyphae grow in chains of cylindrical cells associated with creation of extracellular matrix material. Phase four is the slow dispersion of yeast-shape cells from the biofilm to seed new sites (Nobile, Johnson 2015).

C. albicans can be differentiated from the numerous fungal species by being able to produce both yeast cells and hyphae under multiple environmental set-ups (hence the early classification of this fungus as being dimorphic). They effortlessly form in planktonic cultures and are significant component of the biofilm. It is hardly surprising that the genes essential for hyphal growth in cell culture are also required for proper biofilm formation. The hyphae in the biofilms impart to the general architectural stability of the biofilm, serving as a support for yeast cells and other hyphae. Thus, the ability to produce hyphae and the ability of these hyphae to adhere to each other and to yeast cells are significant for the formation and maintenance of the normal biofilm (Nobile, Johnson 2015).

The in-vitro formed biofilm is consistent of fungal cells inserted in polysaccharide rich extracellular matrix (Nagoba, 2018).

Several factors have been associated with biofilm formation. The first factor being the fluid flow. Fluid flow is considered one of the physiological conditions associated with the infection site which influences the exchange of nutrition and structurally solidarity of the biofilm. *Candida* biofilms produced under flow conditions produces more extracellular matrix in comparison to those produced under static conditions. The second factor is the substrate; various studies have shown the importance of substrates in regulating the ability of *Candida* to produce a biofilm. The architecture, morphology and thickness of the biofilm is greatly influenced by different substrates which is greatly determined by nutrition. Nutrition is considered the third factor associated with the formation of biofilm, especially sugars. Serum and liquid are significant determinants of *Candida*'s ability to form a biofilm. Species variability is also considered one of the factors associated with the formation of biofilm. The capability to produce biofilm varies greatly among the different *Candida* spp.

C. albicans biofilm is more pathogenic than those formed by other *Candida* species. The quantity and quality of the biofilm produced by *C. albicans* is also more complex compared to that of other *Candida* spp (Nagoba, 2018).

Normal flora of the oral cavity

The mouth is made up of a community of living and non-living microorganism in which numerous amounts of species have evolved (Caglar *et al.*, 2005). Among these species *C. albicans* and other less commonly encountered *Candida* spp are found in the mouth of up to 75% of the general population. In immunocompetent individuals there is an internal equilibrium in this colonization, which remains predominantly harmless (Mayer *n et al.*, 2013).

When the immunity of an individual is compromised, they commonly struggle with refractory infections of the oral cavity. If these oral infections are associated with *Candida* spp the termed “oral candidiasis” (OC) can be used. Such infections are frequently produced by *C. albicans* and can affect the oropharynx and/or oesophagus of individuals with a malfunction of the adaptive arm of the immune system. A diversity of local and systemic factors results in the transition of *Candida* from commensal to pathogenic organism. Dentures, corticosteroids inhalers and xerostomia are considered local factors, while immunosuppressed states including Human immunodeficiency virus (HIV); leukaemia; nutritional deficiency, aging, endocrine malfunction (including diabetes); radiation and chemotherapy; the use of systemic corticosteroids, immunomodulator drugs, xerogenic drugs and broad-spectrum antimicrobial agents can cause disorganization of internal equilibrium leading to oral candidiasis (OC) (Millsop, Fazel 2016, Mayer *et al.*, 2013).

Oral candidiasis

Oral candidiasis (OC) is an oral infection caused by *Candida albicans* (Seneviratne *et al.*, 2008). OC presents clinically as either a white or erythematous lesion. Clinically identifiable white lesions include pseudomembranous candidiasis and hyperplastic candidiasis. While erythematous candidiasis is identified clinically as a red lesion. These include acute atrophic candidiasis, chronic atrophic candidiasis, median rhomboid glossitis, angular cheilitis, and linear gingival erythema. Other variants of oral candidiasis include chronic mucocutaneous candidiasis, cheilocandidiasis, and chronic multifocal candidiasis (Millsop, Fazel 2016).

The diagnosis of OC is made generally by obtaining a thorough medical history and physical examination. Confirmation of an OC diagnosis can be carried out by various methods (tests) including smear, oral rinse sample, whole saliva sample or performing an oral biopsy (Millsop, Fazel 2016).

Each of these methods (tests) have some benefits and disbenefits associated with it. The choice of the confirmatory diagnostic method utilized is largely based on the clinical presentation. For pseudomembranous candidiasis, periodic acid-Schiff (PAS) staining of a lesional cytological smear can validate the presence of *Candida* hyphae. While cytology smear is meticulous in validation of pseudomembranous candidiasis it is of no benefit in validation of other oral *Candida* lesions. In such cases, culture of fungal spores from the identified lesions on a special media is required to authenticate the existence of a mycotic infection. Sabouraud dextrose agar also known as (SDA) is commonly suggested, frequently combined with a subsequent differential medium such as Pagano-Levin agar or commercial chromogenic agar. Oxidative culturing on SDA at 37°C for 24-48 hours produces convex shaped, creamy colonies, but do not provide information regarding the *Candida* spp involved (Farah *et al.*, 2010).

The distinction between the various strains of *Candida* may be necessary in recurrent lesions or in those individuals with suppressed immunological status. Even though some media are capable of distinguishing between several species via macroscopic colony features, immunohistochemical techniques must be utilized to differentiate between the various strains of *Candida*. In addition to strain recognition, persistent lesions in an individual may benefit from antifungal sensitivity testing which aid in the determination of susceptibility and more precise antimetabolic therapy (Farah *et al.*, 2010).

Management of oral candidiasis

Oral candidiasis (OC) seldom arises in the absence of predisposing factor. Therefore, it will be of benefit to the individual if the management is guided towards identification and correction of the underlying cause that may be the primary or secondary cause. If the underlying cause is designated, pharmacologic management is compulsory (Farah *et al.*, 2010).

The first line of treatment and prevention of OC has been the same for decades. This include different forms of antimicrobial medication and antimicrobial mouth rinses such as chlorhexidine (CHX) (Ericson *et al.*, 2014).

Chlorhexidine (CHX) is an antimicrobial agent that exerts its effect on the inner membrane of the cytoplasm, therefore it is considered a membrane active substance. The molecule of CHX is symmetrical, it is made up of 4 chlorophenyl rings and 2 biguanide groups linked by a core of hexamethylene bridge (Balagopal, Arjunker 2013). It is dicationic at pH levels higher than (3.5). It also acts as antiplaque and antigingivitis as it prevents the accumulation of plaque and it diminishes the attachment of *Porphyromonas gingivalis* to epithelial cells. It is also effective against broad spectrum of bacteria including both Gram-positive and Gram-negative bacteria as well as dermatophytes and lipolytic viruses and fungi. It is considered as an anticariogenic agent as it effective against *streptococcus mutans*. Chlorhexidine (CHX) has proven to be capable of neutralizing *streptococcus aureus*, *Porphyromonas gingivalis* and *Prevotella intermedia* and additional, but significant feature is its substantivity. Substantivity also defined as oral retentiveness, is dependent on a variety of factors such as pH, concentration, temperature and the timing of adherence of the suspension with oral structures (Balagopal, Arjunker 2013).

The mechanism by which CHX exerts its effect is outlined in (Figure 2) (Balagopal, Arjunker 2013).

Chlorhexidine (CHX) is available in a wide range of formulations, including rinses, gels, spray, varnishes and in some sugar free chewing gums. The rinse (mouthwash) formulation is available as 0.2% or 0.12%. The efficacy is equal when utilized at appropriate similar dosage and recommended rinse time (between 30-60 seconds) but this is also dependent on the rate of adsorption of the mouth rinse to the oral surfaces. Ellepola, Samaranayake (2001) have shown that singular minute of rinsing with 0.2 % CHX, 30 % of the drug is retained in the mouth for 24 hours. The application of 50 % (half the concentration) of 0.2 % CHX, it attaches to the receptors within 15 seconds. 0.2 % and 0.12 % CHX have an adherence period of 12 hours therefore it is recommended to be used twice daily (morning and night). (Balagopal, Arjunker 2013, Ellepola, Samaranayake 2001).

One of the recognized side effects associated with CHX usage is extrinsic staining of the teeth and the tongue. An advanced version of chlorhexidine with anti- discoloration system also known as (CHX-ADS) has been introduced recently into dental practice. In this latest



version of chlorhexidine besides its antiseptic property it avoids the staining side effect associated with the old version (Bernardi *et al.*, 2004).



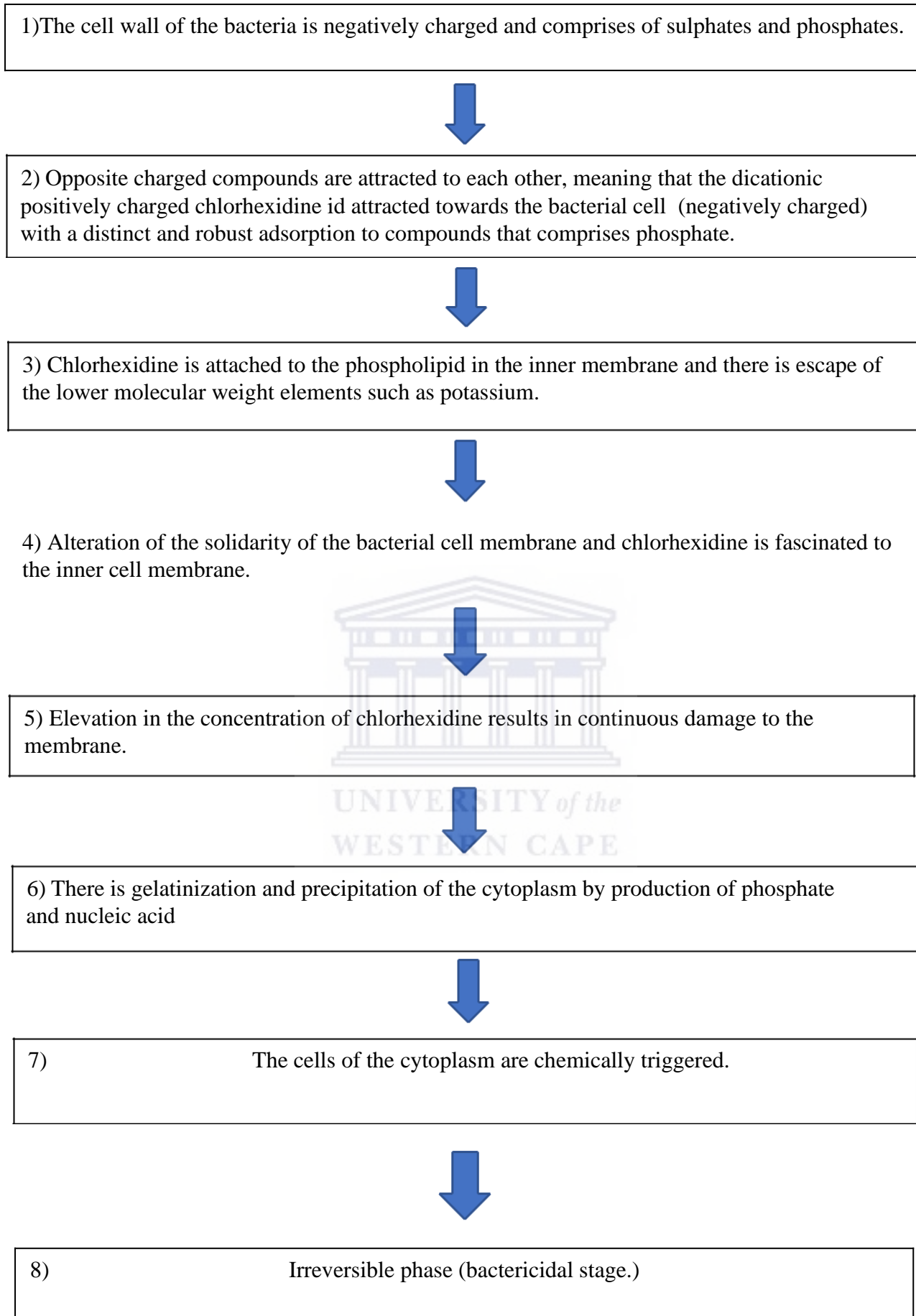


Figure 2. Shows the mechanism of action of chlorhexidine(Balagopal, Arjunker 2013).

Chlorhexidine (CHX) perform a non-specific reduction on the level of both harmless (normal flora) and harmful oral microorganisms. The oral environment restores its normal flora after being completely cleared with CHX by persister cell population. These cells are generated by *C. albicans* biofilm upon initial attachment to the tooth surface. The small subpopulation of these persister cells can tolerate high concentration of fungicidal agent (CHX), they generate new population of persisters and recover the normal flora (LaFleur *et al.*, 2010). Sherry *et al.*, (2012), Sardi *et al.*, (2013), and Coleman *et al.*, (2010) have tried the use of different natural products instead of the chemical formulations of chlorhexidine. Mendonça *et al.*, (2012), Ishikawa, (2015) and Kraft Bodi *et al.*, (2015) found that patients diagnosed with OC had a slight to moderate improvements when managed with probiotics.

The other agents that are used in combination with CHX are antifungals as mentioned previously. Antifungals are used in topical formulations in treatment of oral candidiasis. Topical antimicrobial agents are either fungicidal or fungistatic. Topical formulations are preferred as drug interaction and side effects are less common and are of no significance compared to those systemically administered. To reach the therapeutic effectiveness individuals must keep the topical antimicrobial agents as long as possible in their mouth. Polyenes (amphotericin B, nystatin), and azoles (miconazole) are regularly utilized antifungals. Each of which has its own mechanism of action, advantages and disadvantages (Fourie *et al.*, 2016).

Polyenes interfere with ergosterol component of the fungal cell membrane making it more permeable, this will allow the escape of the cytoplasmic content and thus resulting in lysis of the fungal cell (Fourie *et al.*, 2016).

Azoles prevent the biosynthesis of ergosterol with functional changes of the fungal cell wall and thus preventing the multiplication of the cell, or cell lysis. Azoles also prevent several hepatic cytochrome P450 (CYP) microsomal enzymes, including CYP2C9 which is an enzyme that participate in the metabolic breakdown of warfarin, as a result of which the blood concentration of warfarin increases, increasing the possibility for bleeding. Even topically applied miconazole oral gel is considered to be only insignificantly absorbed in the gastrointestinal tract and might result in an increase in the blood concentration of warfarin (Fourie *et al.*, 2016).

Systemically prescribed antifungals such as Fluconazole and Itraconazole are sufficiently absorbed in the GIT system. Remnants of Fluconazole are secreted in saliva providing an extra-topical effect (Fourie *et al.*, 2016).

The concept of probiotics was introduced with the increase side-effects of the conventional antimicrobial agents and with the increase resistance of these medications among individuals, this directed the scientist to explore alternative means to combat infectious diseases. (Caglar *et al.*, 2005).

Probiotics

Probiotics as defined by the food agricultural organization (FAO)/World Health Organization (WHO) are “Living microorganisms when dispensed in adequate quantity (either in food or as supplements) provide a health benefit to the individual, by establishing the intestinal microbiologic balance at an equilibrium (Hill *et al.*, 2014).

Recent literature revealed that the most frequently utilized microorganisms in probiotic industry are either Lactic-acid bacteria or Bifidobacterium (Alvarez-Olmos, Oberhelman 2001). Examples of lactic acid bacteria include; *Lactobacillus Rhamnosus GG* (LGG), *L. acidophilus*, *L. casei* (*Rhamnosus*), *L. reuteri*, *L. bulgaricus*, *L. plantarum*, *L. johnsonii* and *L. lactis* of which *Lactobacillus Rhamnosus GG* (LGG) is the most frequent used. Examples of *Bifidobacterium* species include *B. bifidum*, *B. longum*, *B. breve*, *B. infantis*, *B. lactis* and *B. adolescentis* (Alvarez-Olmos, Oberhelman 2001).

Lactic acid bacteria are viewed as Gram-positive organisms under the microscope. They lack cytochromes and grow with favourable growth in absence of oxygen. These organisms do not require oxygen for survival, nor are harmed by its presence. The most significant genera are Lactobacilli, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc and Bifidobacterium (Pradeep *et al.*, 2014).

Lactobacillus acidophilus was the first probiotic species researched by Hull *et al.*, in 1984, followed *Bifidobacterium bifidum* by Holcomb *et al.*, in 1991. (Pradeep *et al.*, 2014). These two species were researched because they are commonly found in the oral cavity including caries lesions, the authors found that these species aid to reestablish the equilibrium of the “good” bacteria and “bad” bacteria and promote the growth of healthy bacteria



Uses of probiotics

The use of probiotics has been established for decades in fermented products industry. Beneficial organisms, whether yeast or bacteria (Lactic acid bacteria) which evince in food, can have an excellent effect on individual's health (Flichy-Fernández, Alegre-Domingo *et al.*, 2010).

In 1907, a Ukrainian-born biologist and Nobel laureate Elie Metchnikoff realized that the intake of Bulgarian yogurt, consisting of Lactic acid bacteria have beneficial effects on individual's health. *Lactobacillus bulgaricus* was later introduced into sour-milk products throughout Europe and from there came the idea of using probiotics to increase human longevity (Vishnu, 2012).

Lactobacillus species in probiotics play an important role in the preservation of human health by stimulating the innate immunity of an individual (Gill & Prasad, 2008).

Medical conditions in which probiotics have shown to benefit the individual include diarrhea, gastroenteritis, short- bowel syndrome and inflammatory conditions such as Crohn's disease and Ulcerative colitis, amongst others. However, there is limited data available on probiotic usage in the oral cavity (Flichy-Fernández *et al.*, 2010).

C. albicans is the main cause of OC which is commonly encountered in the elderly and in those who are immunosuppressed (Flichy-Fernández *et al.*, 2010).

In 2007 Hatakka *et al.*, hypothesized that "cheese containing probiotic bacteria could reduce the prevalence of oral candidiasis". By using a randomized controlled double-blind, placebo study of 16 weeks duration, 276 (n=276) elderly people consumed 50 grams of either probiotic (n=136) or cheese (control) on daily basis (n=140). The results of the study showed that the salivary yeast count was reduced from 30% to 21% in the probiotic group, while increased from 28% to 34% in the cheese (control) group. Probiotics usage reduced the risk of high yeast counts by 75% which supports the hypothesis that probiotics bacteria are beneficial in controlling OC in elderly individuals.

Probiotics have shown to be safe and effective antifungal agents, for both prophylaxis and therapeutic adjuvants in the management of mucosal candidiasis (Matsubara *et al.*, 2016).

Mechanism of action of probiotics

Numerous mechanisms of action have been suggested, even though the exact mechanism remains unclear. It is proposed that, probiotics microorganisms secrete numerous antimicrobial elements such as organic acid, hydrogen peroxide (H₂O₂). Furthermore, probiotics compete with pathogenic agents for adherence to sites in the oral mucosa (Gerbaldo *et al.*, 2012). Lastly, probiotics may confer useful properties by stimulating the nonspecific immunity and controlling the humoral and cellular immune response. Probiotic strain can also be combined to intensify their beneficial properties (Bonifait *et al.*, 2009).

Only a small proportion of probiotics remains in the oral cavity when administered orally, representing about 1% of the normal flora of the oral cavity. Lactobacilli probiotic strain(s) that are available in saliva include *L. paracasei*, *L. plantarum*, *L. salivarius*, and *L. rhamnosus*, while that of the *bifidobacterial* species include *B. bifidum*, *B. dentium*, and *B. longum*. Considering the mechanisms of action, these organisms play a significant role in improving dental health care and preventing the occurrence of oral health related conditions (Figure 3) (Bonifait *et al.*, 2009, Iqbal *et al.*, 2014).

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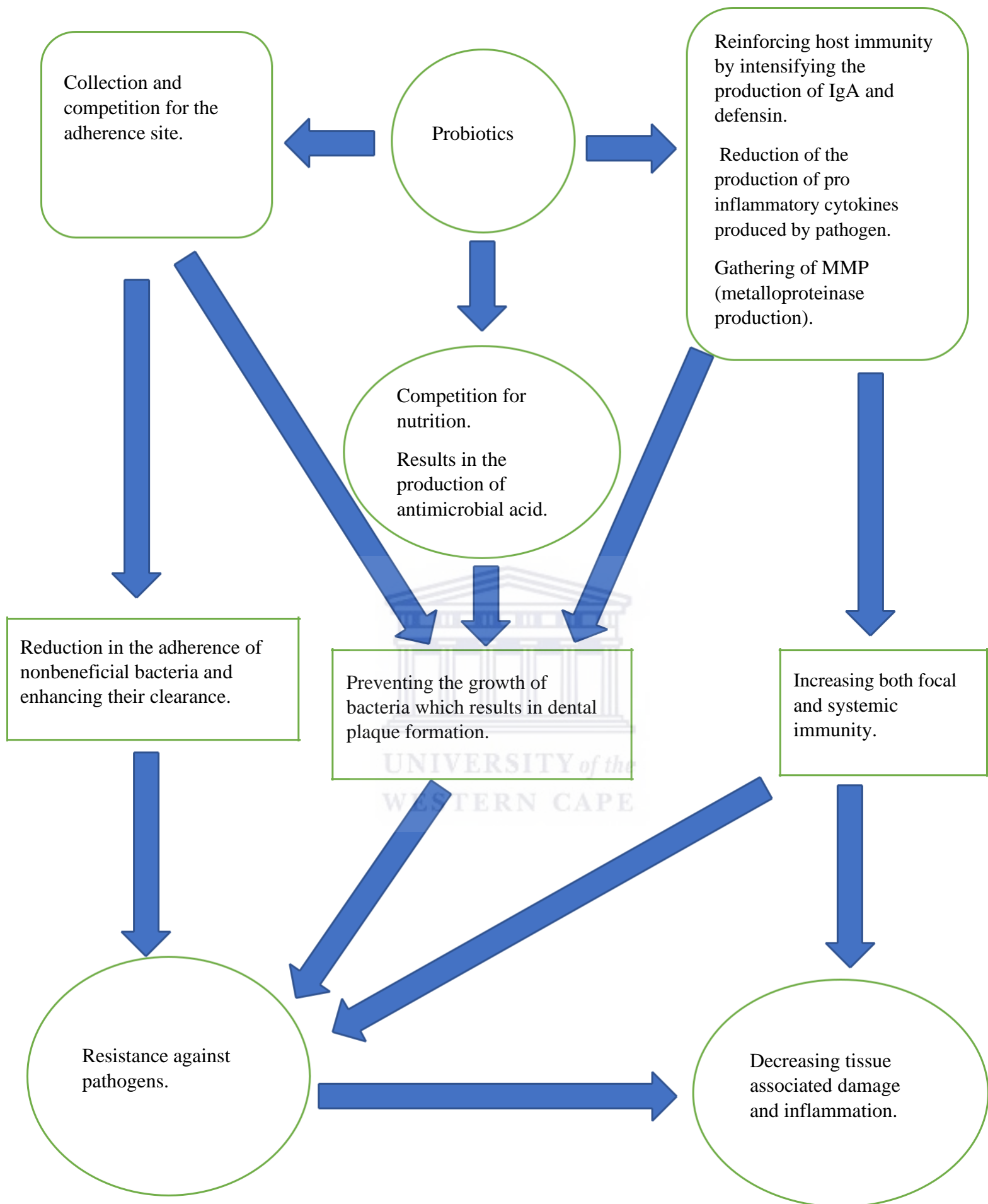


Figure 3. Mechanism of action of probiotics (Bonifait *et al.*, 2009, Iqbal *et al.*, 2014).



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Selection characteristics and requirements for probiotics

Currently, in addition to the main role of probiotics (providing essential nutritional elements for normal growth and development). Other supplementary characteristics and requirements are becoming evident, (Table 1) summarizes some of these characteristics and requirements (Markowiak, Śliżewska 2017).

Table 1. Selection characteristics of probiotic strains.

Characteristics	Requirements
Safety	Originates from humans or animals. Obtained from healthy individual GIT system. Previous history of safe use. No associated side-effects.
Functional	Maintaining the metabolic activity, survival and growth at the site of interest. Resistance to bile salt and enzymes destruction. Resistance to the acidic environment of the stomach (low pH). Competition with bacterial species occupying the intestinal environment. Resistance to by-products such as bacteriocins and acids formed by the normal flora of the intestine. Adherence and colonization at specific sites inside the host and proper survival rate in the GIT system.
Technical	Must be genetically stable. Long shelf life. Viability and stability during manufacture process. Must meet the intended sensory property of the final product, especially in food industry.

(Markowiak, Śliżewska 2017).

The characteristics of probiotics are mainly based on the strain type selected (either Lactic-acid bacteria or Bifidobacterium) The safety of the strain is determined by several factors including; origin of the strain, lack of correlation with pathogenic cultures and the antimicrobial resistance profile (Markowiak, Śliżewska 2017).

Generally, probiotics are regarded as non-harmful microorganisms that are beneficial to individual's health. Owing to the fact of being safe *Lactobacillus* species are utilized for a great time. Probiotics are classified into two classes based on their risk to individual's health. Group 1 is the no risk group and include Lactobacilli and Bifidobacterium, while group 2 is the small risk group and include *L. rhamnosus* and *Bifidobacterium dentium* (Table 2) (Iqbal *et al.*, 2014).

Table 2. Shows the risks of various species of probiotics.

Health Risk	Probiotic species
Bacteraemia, Meningitis	<i>Sacharomyces bulardi</i> , <i>Bfidobacteria</i>
Liver abscess	<i>L. rhamnosis</i>
Abdominal abscess	<i>Bfidobacteria</i> , <i>Lactobecillus</i>
Pneumonia and sepsis	<i>L. casei</i>

(Iqbal *et al.*, 2014).

Probiotics must also have a recognized pro-health property reliable with the features of the strain available in the market. Investigations verifying a specific property of a probiotic strain at a specific tested dose do not extend to the same strain at various doses (Markowiak, Ślizewska 2017).

Species and strains of probiotics

Probiotic strain can be either bacteria, yeast or molds of which bacterial species are the most common. The latter can be classified four major groups 1) Lactobacillus, Bifidobacterium, Streptococcus, 2) non-lactic acid-producing bacterial species such as *bacillus* and *Propionibacterium* 3) the non-pathogenic yeast strain include; *Saccharomyces*, 4) non-spore forming and monoflagellated rods or coccobacilli (Nagashima *et al.*, 2013).

Lactobacillus acidophilus are Lactic acid bacteria with small bulk of Lactic acid creation. The temperature at which they grow varies between 20 °C and 48 °C, with 37 °C being the optimal temperature for growth. It is a probiotics bacterium with important useful characteristics include; positive control over the metabolic activity of abdominal microorganisms, inhibition of diarrhea related to antibiotics use, protection of intestinal strength and integrity throughout radiotherapy, encouragement of immune system response intensification of iron availability, decrease in vaginal microbial flora and creation of antimicrobial elements that act against other bacteria, viruses, protozoa and fungi (Nagashima *et al.*, 2013).

Bifidobacterium are anaerobic organisms who reside in the large intestine and can survive in the absence of oxygen. Health benefits associated with these species include the digestion of lactose, production of Lactic ions from Lactic acid, vitamin production from complex

carbohydrates, production of useful short chain of fatty acids, reduce diarrhea induced by antibiotics usage as well as traveler's diarrhea, prevent constipation, improve inflammatory bowel disease and inhibition of DNA impairment and lastly, potential inhibit or postpone the initiation of cancer (Nagaraj *et al.*, 2012).

Saccharomyces is a non-colonizing Lactic acid-producing yeast. It inhibits diarrhea induced by antibiotics usage, *Clostridium difficile* (*C. difficile*) related disorders, acute and traveler's diarrhea, as well as diarrhea associated with AIDS, and deterioration of Crohn's disease. Most of these helpful species accelerate the manufacture of vitamins, decrease serum cholesterol level and have anticancer activity (Nagaraj, Ravi *et al.*, 2012).

Cildir, Germec *et al.*, 2009 studied the residence time of probiotics in the human mouth after cessation of treatment and found reduction in *Streptococcus mutans* (*S. mutans*) level after using *L. reuteri* enriched yoghurt for 2-weeks. Wolf *et al.*, 1995 observed the loss of *L. reuteri* colonization after 2-months of antibiotics withdrawal. The authors concluded that there is no continuous colonization in the oral cavity and suggest that probiotics should be constantly used for maintenance of their beneficial effect in the oral cavity.

Table 3 summarizes various in-vitro randomized control trials (RCTs) supporting the antifungal strain specific effect of probiotics (PB) (Matsubara *et al.*, 2016) while Table 4 summarizes several in-vivo studies also supporting the antifungal strain specific effect of probiotics (PB)) (Matsubara, *et al.*, 2016).

Table 3. In-vitro studies on the antifungal effects of probiotic strains.

Reference	Probiotics strain	Organism	Results	Comments
(Chew <i>et al.</i> , 2015)	<i>L. rhamnosus</i> GR-1 and <i>L. reuteri</i> RC-14	<i>C. glabrata</i> ATCC 2001	Growth inhibitory activities (bacterial cells) fungicidal against <i>C. glabrata</i> .	Lactobacilli may inhibit <i>C. glabrata</i> colonization through the creation of aggregates.
(Vilela <i>et al.</i> , 2015)	<i>L. acidophilus</i> ATCC 4356	<i>C. albicans</i> ATCC 18804	Fewer hyphae development in the presence of <i>L. acidophilus</i> cells or culture filtrate.	<i>L. acidophilus</i> formed substances with anti- <i>Candida</i> activity, giving an indirect effect on <i>Candida</i> .
(Ujaoney <i>et al.</i> , 2014)	<i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. salivarius</i> , <i>Bifidobacterium bifidum</i> , <i>Streptococcus thermophilus</i> (<i>S. thermophilus</i>), <i>Bifidobacterium infantis</i> , <i>Lactobacillus GG</i> , and <i>Bacillus coagulans</i> BC30	<i>C. albicans</i> 10341	Significant inhibitory result on biofilm development.	Reduction of nutrients in the culture media by overgrowth of the probiotic bacteria may prevent fungal growth.
(Verdenelli <i>et al.</i> , 2014)	<i>L. paracasei</i> , <i>L. plantarum</i> , <i>L. fermentum</i> , <i>L. rhamnosus</i> IMC 501, and <i>L. paracasei</i> IMC 502	<i>C. albicans</i> <i>C. glabrata</i> <i>C. krusei</i> <i>C. parapsilosis</i> , <i>C. tropicalis</i> (clinical isolates)	All lactobacilli had the ability to prevent <i>Candida</i> in different degrees.	Inhibition and coaggregation capability vary according to the <i>Lactobacillus</i> strain and the pathogen used.
(Coman <i>et al.</i> , 2014)	<i>L. rhamnosus</i> IMC 501 <i>L. paracasei</i> IMC 502 Combination of both (SYNBIO)	Gram-positive, Gram-negative Bacteria <i>C. albicans</i> <i>C. glabrata</i> <i>C. krusei</i> <i>C. parapsilosis</i> <i>C. tropicalis</i>	<i>L. rhamnosus</i> Inhibitory activity against both Gram-positive and Gram-negative bacteria, and against 2 <i>C. albicans</i> strains (ATCC 10261 and ISS7) <i>L. paracasei</i> Inhibitory action against Gram-positive and Gram-negative bacteria, especially <i>S. aureus</i> and <i>Proteus mirabilis</i> . All <i>Candida</i> spp were inhibited except <i>C. glabrata</i> and <i>C. tropicalis</i> Inhibitory activity	<i>L. paracasei</i> IMC 502: higher activity toward all the pathogens, especially <i>Candida</i> strains; strong inhibition registered for SYNBIO.

			against most of the bacteria and fungi strains, especially <i>C. albicans</i> and <i>C. krusei</i> .	
(Strus <i>et al.</i> , 2005)	4 different strains: <i>Lactobacillus fermentum</i> <i>Lactobacillus rhamnosus</i> <i>Lactobacillus plantarum</i> <i>Lactobacillus acidophilus</i>	<i>C.albicans</i> , <i>C. pseudotropicalis</i>	<i>C. albicans</i> growth was inhibited to a certain degree by all probiotics. Most lactobacilli were able at least slightly inhibit the growth of <i>C. pseudotropicalis</i> .	Anticandidal activity related to H2O2 production and alternative mechanism

(Matsubara *et al.*, 2016).

Table 4. In-vivo studies evaluating the antifungal effects of Probiotic strain.

Reference	Probiotics strain	Organism	Method	Results	Comments
(Hatakka <i>et al.</i> , 2007)	<i>L.rhamnosus</i> GG, <i>L. Rhamnosus</i> LC705, <i>Propioni Bacterium</i> <i>Freuden reichii</i> subsp <i>shermanii</i> JS	<i>C.albicans</i> <i>C. glabrata</i> <i>C.krusei</i> <i>C. tropicalis</i>	RCT:276 elderly Daily consumption of 50g of probiotic (PB group). Cheese or control cheese for 16weeks, Community periodontal Index and mucosal lesions were recorded. Sampling for oral yeasts were undertaken.	Prevalence of yeast in saliva decreased in the probiotic group and increased in the control group. Probiotic intervention reduced the risk of high yeast counts by 75%.	Probiotic reduced the prevalence of hyposalivation; No adverse events were observed.
(Li <i>et al.</i> , 2014)	<i>Lactobacillus bulgaricus</i> , <i>Bifidobacterium longum</i> , <i>Streptococcus thermophilus</i>	<i>Candida</i> spp	RCT: 65 patients with <i>Candida</i> -Associated Stomatitis Probiotic group: antifungal alone (sodium bicarbonate solution + nystatin paste) or associated with probiotic, 3 times daily for 4 weeks. Culture of saliva, and lingual dorsum swab was assessed.	Detection rate of <i>Candida</i> spp was decreased in the probiotic group; Significant relief of clinical signs and symptoms after probiotic consumption.	No adverse events were observed.
(Ishikawa <i>et al.</i> , 2015)	<i>L. rhamnosus</i> HS111, <i>Lactobacillus acidophilus</i> HS101, and <i>Bifidobacterium Bifidum</i>	<i>Candida</i> spp	RCT: 55 dentures wearers sheltering <i>Candida</i> spp with clinical absence of oral candidiasis symptoms. Probiotic group, once daily for 5 weeks (probiotic or placebo). Palatal swab sample for <i>Candida</i> cell quantification and identification.	Significant reduction of <i>Candida</i> infection after probiotic consumption. <i>C. albicans</i> was the most prevalent species before and after the probiotic therapy.	Reduction of <i>Candida</i> infection was independent of initial <i>Candida</i> level, colonizing species, or age of denture.

(Matsubara *et al.*, 2016).

Effect of probiotic on oral infection

Probiotics have been recently investigated in the management of oral disorders as such as dental caries, halitosis and periodontal related conditions such as periodontitis (Flichy-Fernández *et al.*, 2010).

Probiotics have been associated with reduction of a high level of *streptococcus mutans* (*S. mutans*) level. Hatakka *et al.*, (2001) announced the reduction of dental caries incidence among children after consuming probiotic (*L. rhamnosus GG*)-enriched milk in contrast to control group (milk without probiotics). Consistent findings were reported by Ahola *et al.*, (2007) in which bovine milk fermented with *Lactobacillus reuteri* proved to be beneficial, as it resulted in decreased risk of dental caries.

Grudianov *et al.*, (2002) studied the effect of probiotics (tablets form) on periodontal related diseases. The study revealed that probiotics usage resulted in equilibrium of the normal flora compared to the control group. Another study Flichy-Fernández *et al.*, (2010) showed that probiotics containing *L. reuteri* resulted in reduction of gingival and plaque index in scores compared to the control group. Supporting beneficial effect of probiotics consumption in periodontal related disease. Large quantities of *lactobacillus* in the normal flora resulted in 82% and 65% prevention of *Porphyromonas gingivalis* and *Prevotella intermedia* growth in old and immunocompromised individuals (Flichy-Fernández *et al.*, 2010).

Kang *et al.*, (2006) recognized a significant reduction in the level of volatile sulfide elements originating from *Fusobacterium nucleatum* after the consuming probiotics containing *Weissella cibaria*, supporting the beneficial effect of continuous use of probiotics in controlling halitosis, which might be related to the production of hydrogen peroxide (H₂O₂) by *Weissella cibaria*.

Methods for in-vitro evaluation of candida activity

Various methods have been used in-vitro to determine the effect of probiotic on *Candida* spp. Namely agar disc diffusion testing, spread plate with colony counting and colorimetric assay such as XTT assay.

The agar disc diffusion testing cannot differentiate between fungicidal and fungistatic effects of a medicament on the microorganism. It is also not suitable to determine the minimum inhibitory concentration (MIC) as it is not possible to measure the amount of antimicrobial dispersed into the agar (Seil, Webster 2012).

XTT assay with spectrophotometer measures the cell viability on surfaces and in solution by reduction of tetrazolium salts to formazan dyes. These are colorimetric inspections and the change in colour produced can be observed spectrophotometer. The differences in colour change observed relates to the effectiveness of the agent being experimented. It's a reproducible technique (method) and it is suitable for determination of MIC. It does however require a spectrophotometer device and the reagents used with this method are expensive (Seil, Webster 2012).

In the spread-plate technique, a suspension is created from the samples based on McFarland standards. After which the suspension is diluted by using serial dilution on a micro-titter plate then a small volume of the sample is taken and spread across the surface of an agar plate (this is performed by using a hockey stick and standardized spread plate technique). The agar plates are then incubated until visible colonies are seen and counted by using colony counting device. The spread plate technique (method) with colony counting, is very accurate however, it only determines the number of colony forming units (CFUs). Colony size cannot be measured by this method. Media and plates preparation as well as the colony counting is time-consuming (Seil, Webster 2012).

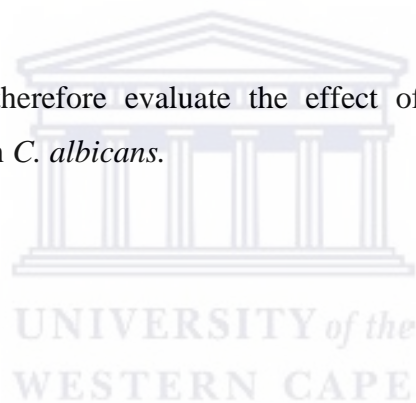
The spread plate technique with colony forming units (CFUs) is the most appropriate method for our research because of its accuracy as well as being inexpensive, but it is time consuming.

In conclusion *Candida* spp are part of the normal oral micro flora of the oral cavity; they constitute about 25%-75% of the normal microbiota in healthy individuals. They are opportunistic pathogens and any imbalance in the normal oral flora allows them to become pathogenic, causing infection. One of the virulence attributed to *Candida* spp is the formation of a community of microorganisms which contains cells that cause loss of response to antifungal agents and the immune system. The first line of treatment and prevention of OC has been the same for decades. Different forms of antimitotic medication and antimicrobial mouth rinse (chlorhexidine) (Akpan, Morgan 2000).

Recent studies have revealed that the effect of probiotic therapy can have an influence beyond that of the intestine(Akpan, Morgan 2000). Few studies have reported on the benefit of probiotics to oral health. Their mechanism of action was largely investigated in the GIT system and further studies are required to discover their applicability in the oral cavity (Akpan, Morgan 2000).

With the increase in development of resistance to *Candida* and the side effects associated with antifungal agents (Akpan, Morgan 2002). The positive results obtained from many studies regarding the use of probiotics in the GIT system has led to increase in interest in the use of probiotics as an alternative/adjuvant treatment for *Candida* infection (Kwon, Farrell 2003). Probiotics could provide a more holistic solution in controlling yeast infection. This should open up a future vision for the use of probiotics in controlling yeast infection (Hatakka *et al.*, 2005).

The proposed research will therefore evaluate the effect of percentage combination of chlorhexidine and probiotics on *C. albicans*.



Chapter 3

Aim and objectives

Aim: To evaluate the effect of percentage combination of chlorhexidine and probiotics on *C. albicans* growth.

Note: (the percentage will be determined by volume by volume (V*V) multiplication).

Hypothesis:

1. Ho: alteration in percentage combination has no effect on *C. albicans* growth.
2. Ha: alteration in percentage combination differ significantly on the growth of *C. albicans*.

Objectives:

- To determine CFU at 50 μ L chlorhexidine and 50 μ L probiotics (50 CHX 50 PB) (X1)
- To determine CFU at 100 μ L chlorhexidine and 0 μ L probiotics (100 CHX 0 PB) (X2).
- To determine CFU at 75 μ L of chlorhexidine and 25 μ L probiotics (75 CHX 25 PB) (X3).
- To determine CFU at 25 μ L of chlorhexidine and 75 μ L probiotics (25 CHX 75 PB) (X4).
- To determine CFU at 0 μ L of chlorhexidine and 100 μ L probiotics (0 CHX 100 PB) X5.
- Compare the impact of all combinations of medications on *Candida* growth.

Chapter 4

Methodology

Methodology (overview)

The study was conducted in the Department of Oral Medicine and Periodontology and the Oral and Dental Research Laboratory (ODRL) at the Faculty of Dentistry, University of the Western Cape, Tygerberg campus.

This in-vitro laboratory study was based on the established principle of spread plate technique followed by colony counting on both selective and non-selective media. CHX and PB (treatments) were combined at different concentrations ($xCHX_yPB$) to evaluate their effect on *C. albicans*. The percentage combinations had an added sum equal to 100 % (the percentage was achieved by multiplication of volumes ($V*V$)), The treatments were incubated at 37°C for 30 minutes and 24 hours after which time serial dilution was carried out on a micro-titer plate based on McFarland standards. A fixed volume (100 μ L) of the solution was transferred into plates containing selective media. Deionized water served as a control (negative).

Materials and method.

Study design: experimental comparative study.

Sample size: n= 180(Observations).

90 observations of treatments incubated for 30 minutes.

90 observations of treatments incubated for 24 hours.

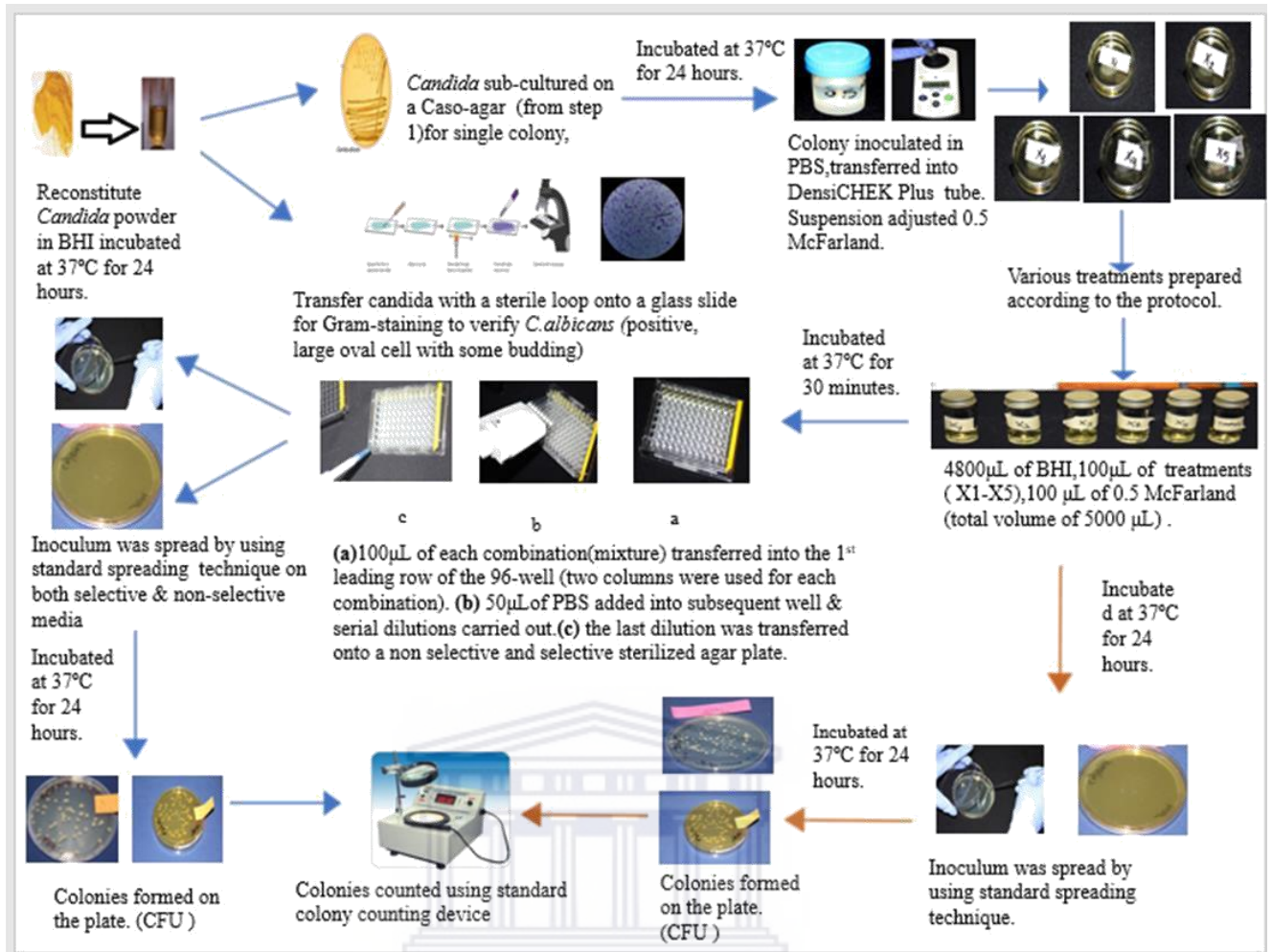


Figure 4. Flow diagram illustrating all the steps involved in this study.

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Media preparation

Table 5. Media preparation.

Media	Brand	Preparation
Phosphate buffered saline	Oxoid, BR0014G	1 tablet dissolved in 100 mL of sterile distilled water and autoclaved at 121 °C for 15 minutes.
Brain heart infusion	Sigma- Aldrich, 53286-500G	37g of media powder dissolved in 1 L of sterile distilled water, sterilized at 121°C for 15 minutes.
SDA with chloramphenicol	Sigma-Aldrich, A 6686-500G	64 g of the media powder dissolved in 500 mL of sterile distilled water, autoclaved at 121 °C for 30 minutes.

SDA with chloramphenicol selective media

The mixture was cooled to 45°C and plates were poured (Sigma-Aldrich, A6686-500G).

Preparation of cultures

Candida albicans was the test organism because of its high prevalence in causing oral infections (Byadarahally Raju, Rajappa 2011). *Candida albicans* strain (ATCC 36810) was obtained from the research laboratory. A single colony was aseptically isolated and transferred into a Caso-agar plate (CASO-A m.LTHTh-ICR 30ml. Merck Life Science GmbH. Eppelheim, Germany) using a sterile loop. This plate was incubated at 37°C for 24 hours to ensure full growth of the organism.

After 24 hours incubation, a single colony was aseptically picked (sterile loop) and transferred onto a new clean glass slide, and Gram staining was performed to verify the organism and its morphology.

Establishing the McFarland standard

The McFarland Standard is a turbidity standard method that is utilized to estimate the quantity of microorganisms within a suspension (Sutton *et al.*, 2011).

The 24-hour *C. albicans* isolate was standardized with phosphate buffered saline to 0.5 McFarland on a DensiCHEK (*BioMerieux, North Carolina, USA*) for the measurement of microorganisms' densities in liquid suspensions. A newly prepared culture was transferred

into phosphate buffered saline in order to create a suspension of the isolate and calibrated to the 0.5 McFarland standards (approximately 1.5×10^5 CFU/mL) (Andrews, 2001).

Materials

The following material were used in this study:

1. *C. albicans* type strain
2. Probiotics (bio Gaia lozenges) probiotics
3. Lactic acid bacterium, *lactobacilli reuteri prodentis* (*L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289)
4. Chlorhexidine (0.2%)
5. Brain heart infusion (BHI)
6. Phosphate buffer saline (PBS)
7. Sabouraud Dextrose Agar with Chloramphenicol (SDA with chloramphenicol)
8. Sterilizable screw-topped bottles (Bijou bottles)
9. 96 well microtiter plate
10. Micropipettes (three different types were used P100, P5000, P200 multichannel pipettes)
11. Agar plates (Sabouraud Glucose Agar with Chloramphenicol (SDA))
12. Caso agar
13. Incubator
14. Colony counter
15. Applicator (inoculating loop)
16. Hockey stick
17. Alcohol (ethanol) 70%
18. Bunsen burner
19. Autoclave

Methodology (Procedure)

The experimental phase of this study was carried out in three phases.

Phase1

- *C. albicans* strain (ATCC 36810) was obtained from the Oral and Dental Research Laboratory, Faculty of Dentistry, Tygerberg campus.

- Gram staining of *C. albicans* was carried out for authentication (Figure 5).

(The result of this Gram staining was Gram positive, large oval cell with some budding).

- Then *C. albicans* strain was sub-cultured for single colonies.

Which was done by streaking for single colonies and incubated at 37°C for 24 hours.

- The agar plates were prepared with Sabouraud Glucose Agar with Chloramphenicol (selective media) according to the manufacture instructions.

- Phosphate buffered saline (PBS) and Brain heart infusion (BHI) media were prepared in accordance with the manufacturer's instructions.

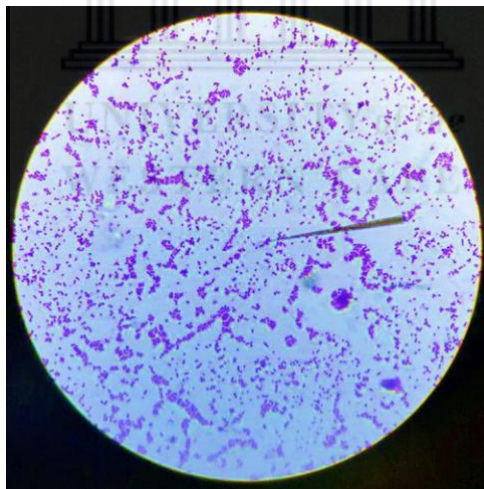


Figure 5. Gram staining confirming the presence of *C. albicans*.

Phase 2

(was carried out 24 hours after preparation of phase1 to ensure that the *C. albicans* strain have reached the log phase).

- The different concentrations of the treatments were prepared as mentioned in the protocol.

Note: The different concentrations were achieved by multiplication of the two volumes of the two treatments.

The overnight (24 hour's culture) *C. albicans* culture was adjusted to the 0.5 McFarland standards as followed: .

1. A single colony was isolated aseptically from the culture plate.
2. Then the colony was inoculated in PBS, this created a suspension.
3. The suspension was examined for turbidity and adjusted to the 0.5 McFarland standards on the 'DensiCHEK Plus'.
 - 4800 μL of BHI was added into Bijou bottles.
 - Then 100 μL of each treatment was added into the appropriate Bijou bottles.
 - 100 μL of 0.5 McFarland suspension was added to the 4800 μL BHI and 100 μL of the treatments, making up 5000 μL .
 - The Bijou bottles were incubated at 37 °C for 30 minutes.
 - After 30 minutes, 100 μL was taken from the incubated Bijou bottle and transferred into the leading row of 96 micro titter plate (two columns were used for each combination).
 - 50 μL of PBS was added into the subsequent well in each column.
 - From the 100 μL of the leading row, 50 μL was transferred in the second row of the well which resulted in 50% decrease in the concentration.
 - Keep on taking 50 μL into the subsequent well till row H.
 - The last dilution was aseptically transferred onto a sterilized agar plate (selective and non-selective media).
 - A standard technique was used to spread the inoculum over the whole plate by use of a hockey stick.
 - The plate was incubated for 24 hours or until visible colonies were formed on the plates.

Note: (at the end the dilution concentration of the McFarland standards suspension, chlorhexidine and probiotics remained unchanged as we were looking at CFU/ volume).

Phase 3

After visible growth have been manifested. Colony counting was carried out. The plates with colonies ranging between 50-250 CFU were selected (Sutton 2011) the results of which was recorded on the data sheet.

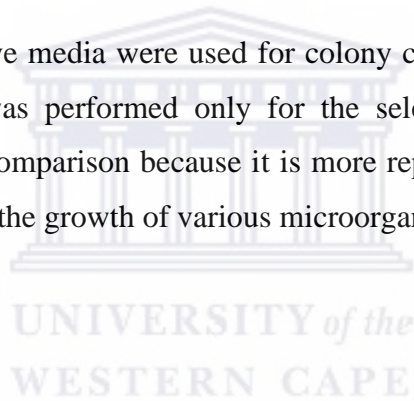
Chapter five

Results

A total of 5 combinations were used at the beginning of this study, of which 2 combinations (50 CHX 50 PB and 75 CHX 25 PB) were discarded due to their -cidal effect (Balagopal, Arjunker 2013).

The results were based on a sample of 180 observations. 90 observations each at 30 minutes and 24 hours respectively. All baseline sample readings set at 1.5×10^5 CFU/mL (0.5 McFarland standard), i.e. equivalent to $\log(\text{CFU/mL}) = 11.51$. Two combinations treatments (50 CHX with 50 PB) (75 CHX with 25 PB), were excluded from the analysis. 4 out of the 30 plates in the 100 CHX 0 PB combination CFUs were observed.

Both selective and non-selective media were used for colony counting to measure the effect of the treatments. Analysis was performed only for the selective media while the non-selective media was used for comparison because it is more representative of the oral cavity (oral environment) as it allows the growth of various microorganisms.



A two-way repeated measures ANOVA was run to determine the effect of different treatments over time on log CFU concentration and the data reported as mean with (standard deviation), unless otherwise stated.

Table 6. Comparison of the effect of treatment on log (CFU/mL) *C. albicans* over time.

		Mean (standard deviation)	Difference from Baseline	p-value	95 % Confidence Interval
100 CHX	Baseline	11.512 (0)			
	30 min	0 (0)	-11.512 (0.0059)	< 0.001	-11.709 to -11.3166
	24 hours*	0 (0)	-11.512 (0.0059)	< 0.001	-11.709 to -11.3166
25 CHX 75 PB	Baseline	11.513 (0)			
	30 min	9.391 (0.45)	-2.122 (0.0059)	< 0.001	-2.318 to -1.926
	24 hours*	9.519 (.055)	-1.994 (0.0059)	< 0.001	-2.189 to -1.797
0 CHX 100 PB	Baseline	11.513 (0)			
	30 min	9.629 (0.265)	-1.8838 (0.0059)	< 0.001	-2.08 to -1.687
	24 hours**	9.903 (0)	-1.609 (0.0059)	< 0.001	-1.805 to -1.413
0 CHX 0 PB	Baseline	11.513 (0)			
	30 min	9.693 (0.273)	-1.82 (0.0059)	< 0.001	-2.016 to -1.624
	24 hours**	9.903 (0)	-1.609 (0.0059)	< 0.001	-1.805 to -1.413

*There was no statistically significant difference between 30 minutes and 24 hours. **There was a statistically significant difference between 30 minutes and 24 hours. **Key:**

100 CHX 0 PB= 100 µL of chlorhexidine with 0 µL of probiotics.

25 CHX 75 PB= 25 µL of chlorhexidine with 75 µL of probiotics.

0 CHX 100 PB= 0 µL of chlorhexidine with 100 µL of probiotics.

0 CHX 0 PB= 0 µL of chlorhexidine with 0 µL of probiotics.

Table 7. Effects on *C. albicans* after exposure to the combination treatments over time.

	Interaction	Mean Difference	Standard Error	p-value	95% Confidence Interval
30 minutes					
	25 CHX 75 PB vs 100 CHX 0 PB	9.391	0.05	< 0.001	9.195 to 9.587
	0 CHX 100 PB vs 100 CHX 0 PB	9.629	0.05	< 0.001	9.433 to 9.852
	0 CHX 0 PB vs 100 CHX 0 PB	9.692 *	0.05	< 0.001	9.496 to 9.889
	0 CHX 100 PB vs 25 CHX 75 PB	0.238	0.05	0.005	0.042 to 0.1434
	0 CHX 0 PB vs 25 CHX 75 PB	0.301	0.05	< 0.001	0.105 to 0.498
	0 CHX 0 PB vs 0 CHX 100 PB	0.063 **	0.05	0.996	-0.132 to 0.26
24 hours					
	25 CHX 75 PB vs 100 CHX 0 PB	9.519	0.05	< 0.001	9.323 to 9.715
	0 CHX 100 PB vs 100 CHX 0 PB	9.903 *	0.05	< 0.001	9.707 to 10.099
	0 CHX 0 PB vs 100 CHX 0 PB	9.903 *	0.05	< 0.001	9.707 to 10.099
	0 CHX 100 PB vs 25 CHX 75 PB	0.384 **	0.05	< 0.001	0.1878 to 0.58
	0 CHX 0 PB vs 25 CHX 75 PB	0.384 **	0.05	< 0.001	0.1878 to 0.58
	0 CHX 0 PB vs 0 CHX 100 PB	0	0.05	1	-0.196 to 0.196

Key

* = Largest difference between the combinations (30 minutes).

** = Smallest difference between the combinations (30 minutes).

* = Largest difference between the combinations (24 hours).

** = Smallest difference between the combination (24 hours).

P-value column all readings were statistically significant expect the ones in the white boxes.

vs = versus

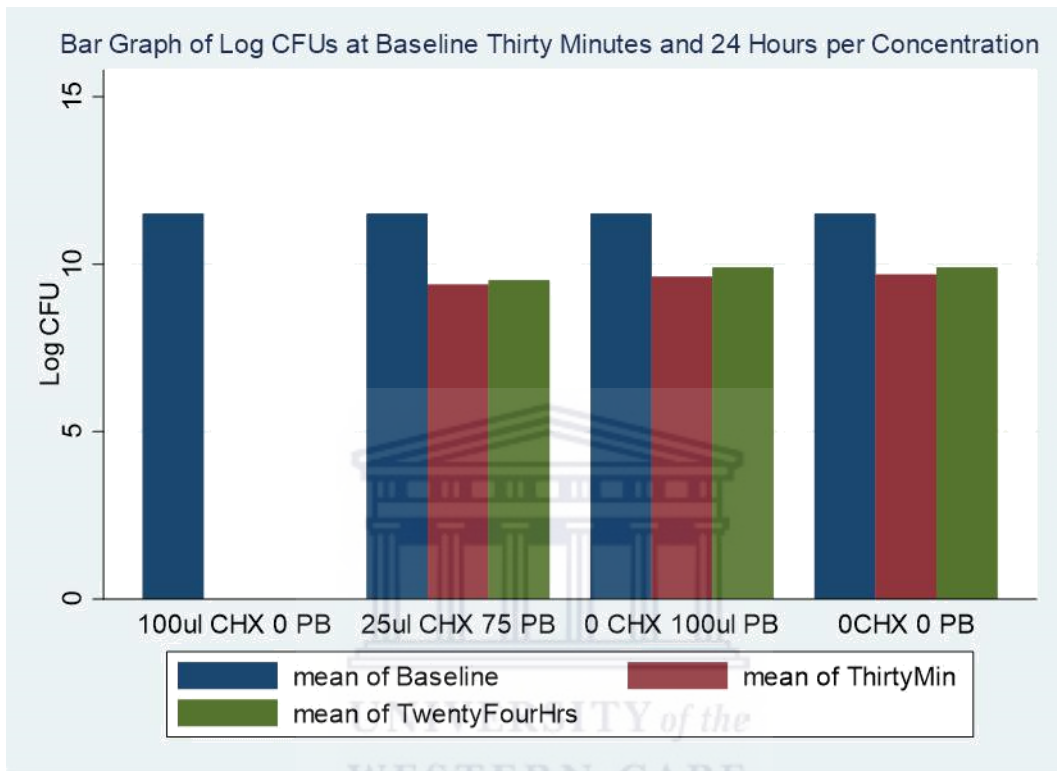


Figure 6. Shows a bar graph of log CFUs at baseline,30 minutes and 24 hours per combination.

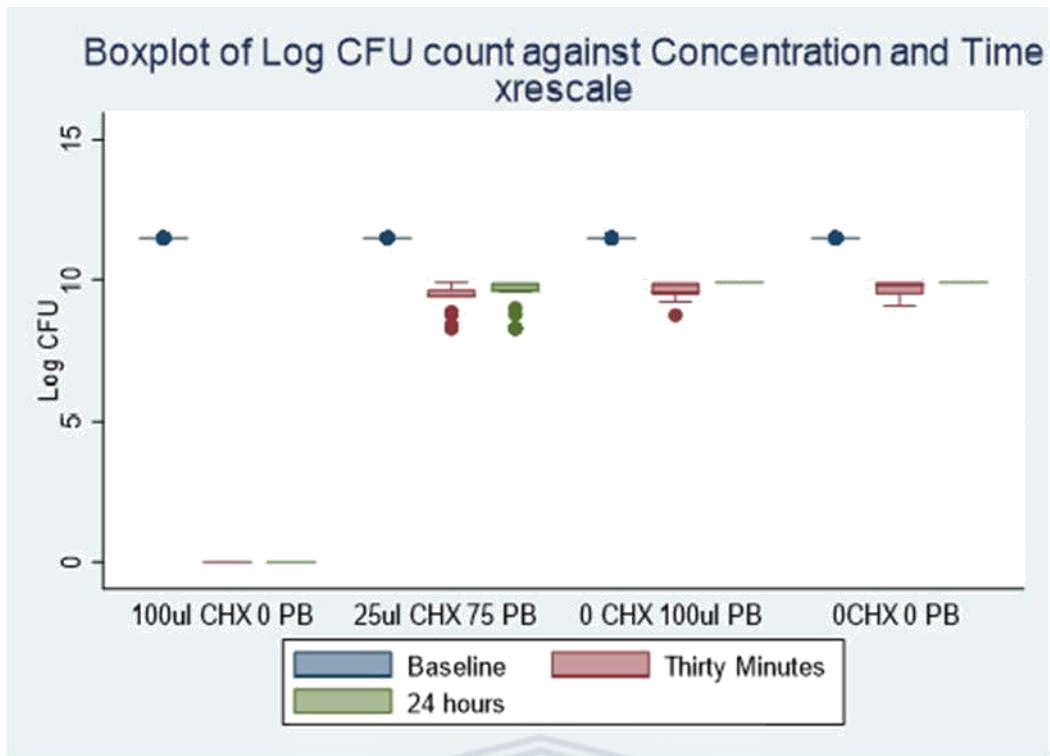


Figure 7. Boxplot presenting comparison of the effect of each combination treatments to baseline and across time.

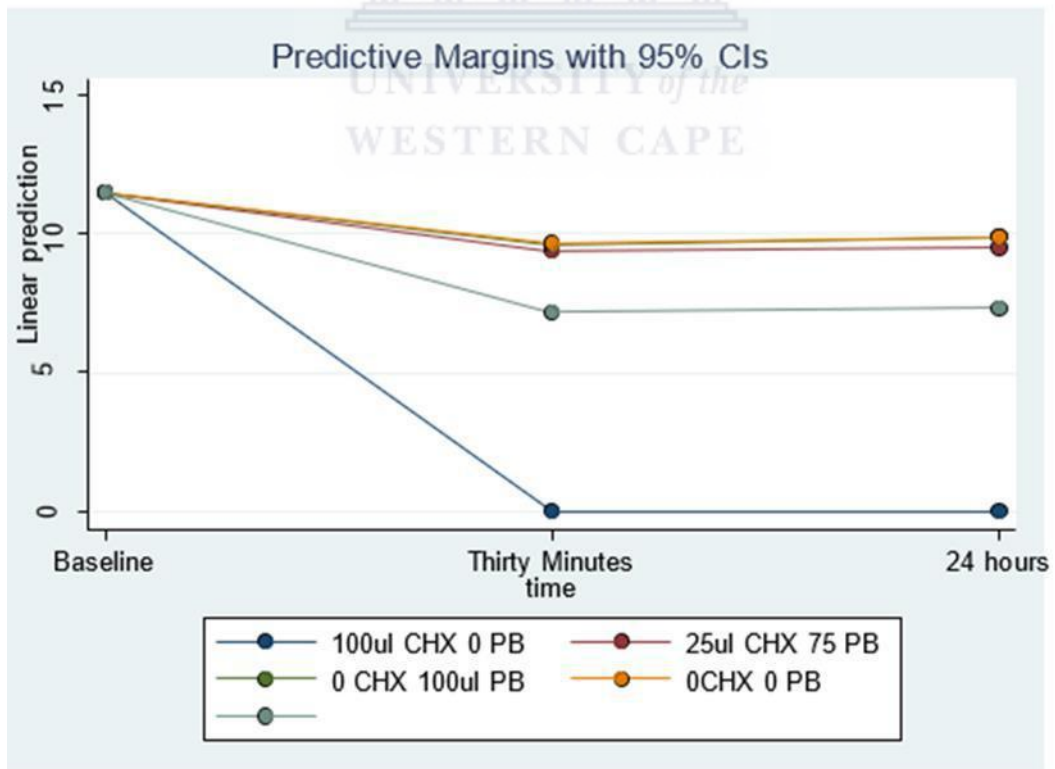


Figure 8. Predictive margin of time effect on log (CFU/mL).

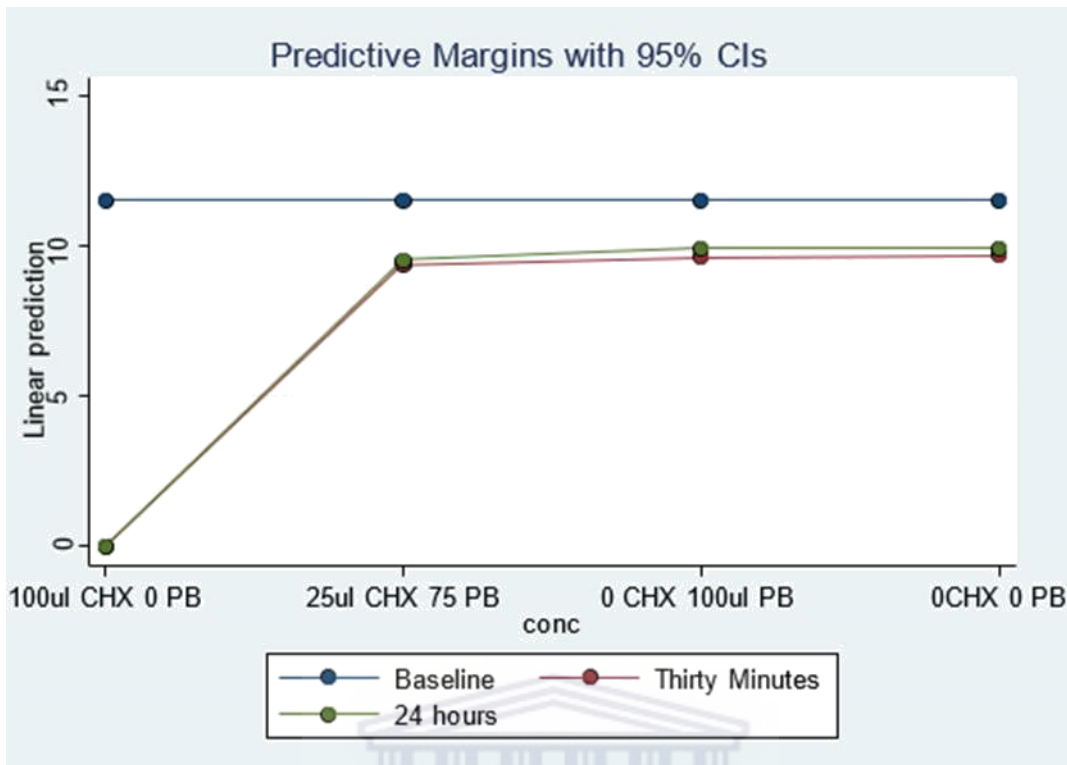


Figure 9. Predictive margin of combination treatment effect on log (CFU/mL).

A significant interaction between two predictor variables tell us that the relationship between one predictor and the response variable depends on the level of the other predictor. Here it means the relationship between Log CFUs and treatment varied with time.

At baseline, there was no statistically significant difference between the four treatments, $p > 0.05$.

Chapter six

Discussion

This study evaluated the in-vitro effect of combining CHX and PB (*Lactic acid bacterium, lactobacilli reuteri prodentis* strains) at different concentrations (xCHX_yPB) on *C. albicans*. Both the 25 CHX 75 PB combination and the 0 CHX 100 PB resulted in a decrease in Log CFU/mL, albeit not significantly different (mean difference of 0,384) (Table 7). However, the effect on the 0 CHX 100 PB combination was almost similar to the control (no treatments). *C. albicans* was completely eradicated by the 100 CHX combination.

Exploring probiotics as an adjuvant or alternative therapeutic approach in the management of fungal infections is reasonable, as not only do probiotics have antimicrobial properties, but are good for the overall host health (Matsubara *et al.*, 2016). Various combinations of microorganisms are used in the probiotics, but Lactobacilli has a long history of use in probiotic formulations.

The CFU at 25 CHX 75 PB was removed from the baseline reading at both 30 minutes and 24 hours with no significant differences between the two times (Figure 6). Similarly, an in-vitro study by Jorgensen *et al.*, (2017) investigated the antifungal potential of 2 strains of *Lactobacillus reuteri* probiotic bacterium (Lactic acid bacterium, *Lactobacilli reuteri prodentis*), against six oral *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, and *C. parapsilosis*). The study supported the fact that these 2 strains exhibited antifungal properties against 5 out of 6 species investigated, including (*C. albicans*) (Bonifait *et al.*, 2009).

Rossoni, *et al.*, (2018) evaluated the antimicrobial activity of 30 different lactobacilli strains of the *Lactobacillus* group against *C. albicans* biofilms. It is known that the effects of probiotics cannot be generalized as the exact mechanism of action is species and strain specific. Rossoni, *et al.*, (2018) concurred with our findings supporting the strain specific antifungal property of probiotics (lactobacilli strains). The authors reported that most *Lactobacillus* isolates exhibited antimicrobial activity against *C. albicans*. The fact that

the percentage reductions varied among the 30 *Lactobacillus* strains that were evaluated, supports the strain specific antifungal effect of *Lactobacillus*. Possible mechanisms by which this occurs are either by the production of acids or exometabolites by *Lactobacillus* strains at various rates, thereby inhibiting *C. albicans* growth.

Future studies should incorporate different combination of live micro-organisms within the probiotics e.g. (Lactobacilli, Bifidobacterium, Streptococcus) to broaden the antifungal destructive capacity as each species and strain would target specific microbial cell targets.

Numerous randomized control trails (RTC's) have revealed different outcomes with various probiotics strains. Petti *et al.*, (2001) found a reduction in salivary *Candida* after consumption of yogurt containing *Lactobacillus bulgaricus* (*Lactobacillus* group) and *S. thermophilus* (*Streptococcus* group). Haukioja (2010) showed that probiotics (*L. rhamnosus* GG and two different *L. reuteri* strains) (same group different strains) diminish the number of *Candida* yeast count. Harini, Anegundi (2010) showed that probiotics (strain not mentioned) are capable of maintaining equilibrium in the oral environment, which emphasizes the strain specific properties of probiotics. Which is suggested to be related to the mechanism of action probiotics by either secreting antimicrobial elements (organic acid or hydrogen peroxide) or competing for the attachment in different oral sites (Bonifait *et al.*, 2009).

The results obtained for this combination 25 CHX 75 PB could be related clinically that after complete eradication of the pathogens by CHX (treating agent), the oral environment restores its microflora by persister cells. These cells are generated by *C. albicans* biofilm upon initial attachment to the tooth surface. These small subpopulation of cells (persister cells) are capable of resisting the antimicrobial effect of CHX, thereby encouraging the formation of new population of persisters cells resulting in the recovery of the normal microflora (LaFleur *et al.*, 2010). A study revealed that chlorhexidine gluconate, beside suppressing the fungal growth during the PAFE period (suppression of growth that continues following brief exposure of an organism to an antimicrobial agent), it further modifies the pathogenic attributes of the yeast cells such as its ability to encourage germ tubes formation (initial stage of yeast) (Ellepola, Samaranayake 2000). The effect of re-establishing *Candida* carriage requires further investigation. Earlier studies revealed that lactic acid bacteria (strain used in our PB) is capable of producing various antimicrobial components such as organic acid and hydrogen peroxide (H₂O₂), that are capable of reducing the pathogens. Furthermore, PB would adhere to dental tissues as part of the biofilm and participate with the growth of other

organisms in the oral environment (Meurman, Stamatova 2007). The oral environment is not similar in all individuals and may have a genetic predisposition to colonization.

For the 0 CHX 100 PB, there was significant difference in growth between the two times (Table 6). The highest ratio of CFU were observed in this combination and was quite similar to the control (0 CHX 0 PB). This could be explained by the fact that probiotics act by maintaining the normal flora of individuals (Harini, Anegundi 2010). Another explanation which goes in line with our result was reported in a recent study, Matsubara *et al.*, (2016) evaluated the effect of probiotics on various stages of biofilm development. After incubation for 90 minutes, 24 hours and 48 hours, the authors found that during the first 90 minutes probiotics were unable to prevent *C. albicans* growth and an inhibitory effect was only seen with mature formation of probiotics growth. This may explain the fact that the 0 CHX 100 and 0 CHX and 0 PB were quite similar in CFU/mL (Figure 6). In other words, our PB were possibly not mature enough to exerts their inhibitory effects on *C. albicans*. This could be related clinically to a study that revealed that the ingestion of probiotic commercial lactobacilli (over a period of 2-3 weeks (mature probiotics) induced a significant increase in salivary *LGG* counts in two different groups (Aminabadi *et al.*, 2011). However, this alteration was not stable following cessation of the probiotic and throughout the course of the study in the second group ,which might be interpreted as a probiotic failure in bacterial competition (immature probiotics) (Aminabadi *et al.*, 2011).

When combining these treatments simultaneously, the ratio of CHX is required to be lower than that of PB otherwise CHX will modalities a fungicidal (killing) effect. Preferably, probiotics should be administered a period after CHX , to ensure that CHX has exerted its antimicrobial effects and to allow the probiotics to work at their full strength. .

The antimicrobial fungicidal effects of CHX at full strength is well established and is found to be a result of increased cell permeability with subsequent cell lysis (Varoni *et al.*, 2012, Machado *et al.*, 2010). The results from the present study for the 100 CHX combination concurred with other studies that reported that the antimicrobial effect of CHX, which are directed at non-specific reduction on both harmless (normal microflora) and harmful oral microorganisms (Varoni *et al.*, 2012).

Since our study was an in-vitro study ,the in-vivo adherence ability was not created and thus no persister cells have been seen after the exposure to 100 CHX combination. As have been established in literature persister cells play a role in re-establishing the oral environment after exposure to -cidal



agents (LaFleur *et al.*, 2010). If the 4 out of 30 plates we had during this study were persister is another research question that warrants further investigation.

Based on our results for (100 CHX), we can conclude that CHX should be initially administered (as a managing agent) followed by the administration of PB for restoration of the normal oral flora. Aminabadi *et al.*, (2011) found that CHX suppresses the oral microorganisms and thereby provides a unique opportunity for easy colonization of PB, meaning that CHX results in reduction of the pathogens while PB (beneficial live bacteria) will promote growth and stable colonization of the normal oral flora. Therefore, the benefit of combining CHX and PB will be as follows: after CHX completely eradicates the oral environment, leaving only the persister cells, PB will encourage these cells to grow and enhance the establishment of a normal oral flora. A possible protocol for eradicating *C.albicans* could be as follows: the regular use of CHX until clinical resolution is established followed by PB to restore the commensal flora.

Comparing all the combinations at the baseline reading, there was no statistically significant difference between the four combinations as the same microorganism was set for the four combinations (0.5 McFarland) (Figure 6). After 30 minutes of the administration (100 CHX with 0 PB), the log CFU/mL dropped to zero and the difference was statistically significant from the baseline reading and as well as at 24 hours as presented in Figure 6. This was consistent with the results obtained by Varoni *et al.*, 2012.

The smallest difference of colony counts of *C.albicans* was between 0 CHX 0 PB and 0 CHX with 100 PB, this difference was not statistically significant (Table 7). Since there was no CHX in these combinations, there was no eradication or reduction in colony counts of *C.albicans*. Instance the probiotics maintained the level of the organism and no further increase in the number of colony counts of *C.albicans* were noted. This supports the notion that PB was able to maintain the balance of the normal oral flora (Harini, Anegundi 2010). Alternatively, PB may not have been mature enough to exert its inhibitory effect (Matsubara *et al.*, 2016).

Regarding the 24 hours, the two smallest differences were equal. These differences were between 25 CHX with 75 PB and 0 CHX with 100 PB and 25 CHX with 75 PB and 0 CHX 0 PB (the control). All of these combinations had a higher probiotic ratio and suggests that probiotics are effective in diminishing the number of *candida* yeast count (Haukioja, 2010) as well as maintaining equilibrium at the same time (Harini, Anegundi 2010).

The predictive margins (Figure 9) indicates that the at (concentration) value ≥ 25 CHX 75 PB growth level would be maintained past 24 hours. If we wanted to maintain the growth levels



of the yeast below that indicated by 25 CHX 75 PB (most effective combination) we would have to increase CHX and decrease PB.

It was noticed that all combinations with a higher ratio of CHX showed no CFU (50 CHX 50 PB and 75 CHX 25 PB) and for this reason they were discontinued from the experiment. These observations were similar to those reported in the literature, the higher the dosage the more destructive effect CHX exerts on the organisms (Machado *et al.*, 2010).



Chapter seven

Conclusion and Recommendations

Conclusion

Chlorhexidine (CHX) and probiotics (PB) could be used at different combination concentration, provided that the chlorhexidine ratio is lower than the probiotics. Depending on the combination used, the number of colonies were slightly different. Chlorhexidine per se (100 CHX) destroyed all the candidal colonies. Conversely, the use of PB without CHX resulted in the highest number of *C.albicans* colonies. Combination 25 CHX 75 PB showed optimal reduction in CFU/mL. These findings suggest that chlorhexidine destroyed the organism (-cidal effect), while PB reduces the number of *C.albicans* colonies. These findings have been previously reported in the literature. Our treatment goal when treating *Candida* infection is to partially eradicate the organism but not completely, because if we completely eradicate the organism it will cause an imbalance in the normal flora of the oral cavity. Therefore, these two effects (-cidal and -static effects) are necessary when managing *Candida* infection. The suggested protocol for managing *Candida* infection should therefore be as followed; if complete eradication occurred it should be followed or treated at the same time with probiotics to maintain the normal flora of the oral cavity. Should these combinations be used simultaneously or subsequently is another question that require further research.

Study Limitations

One limitation of our study (in-vitro) was that some of the antimicrobial mechanisms such as adherence which is responsible for the competitive inhibition could not be optimally exerted.



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Recommendations

Further research is required to investigate the effect of the various combinations of PB (strains) and CHX on *C. albicans*. The effects of delivering the probiotics after administration of chlorhexidine should be investigated. Adherence is one of the keys killing mechanism of probiotics. The study methodology should incorporate the execution of this property so evaluate the true potential of probiotics in in vitro studies.



Bibliography

Akpan, A. and Morgan, R., 2002. Oral candidiasis. *Postgraduate medical journal*, 78(922), pp.455-459.

Alvarez-Olmos, M.I., Oberhelman, R.A., 2001. Probiotic agents and infectious diseases: a modern perspective on a traditional therapy. *Clinical infectious diseases*, 32(11), pp.1567-1576.

Aminabadi, N.A., Erfanparast, L., Ebrahimi, A. and Oskouei, S.G., 2011. Effect of chlorhexidine pretreatment on the stability of salivary lactobacilli probiotic in six-to twelve-year-old children: a randomized controlled trial. *Caries research*, 45(2), pp.148-154.

Arendrup, M.C., 2010. Epidemiology of invasive candidiasis. *Current opinion in critical care*, 16(5), pp.445-452.

Balagopal, S. and Arjunker, R., 2013. Chlorhexidine: the gold standard antiplaque agent. *Journal of Pharmaceutical sciences and Research*, 5(12), p.270.

Bernardi, F., Pincelli, M.R., Carloni, S., Gatto, M.R. and Montebugnoli, L., 2004. Chlorhexidine with an Anti-Discoloration System. A comparative study. *International journal of dental hygiene*, 2(3), pp.122-126.

Bonifait, L., Chandad, F. and Grenier, D., 2009. Probiotics for oral health: myth or reality? *Journal of the Canadian Dental Association*, 75(8).

Byadarahally Raju, S. and Rajappa, S., 2011. Isolation and identification of *Candida* from the oral cavity. *ISRN dentistry*, 2011.

Caglar, E., Kargul, B., Tanboga, I., 2005. Bacteriotherapy and probiotics' role on oral health. *Oral diseases*, 11(3), pp.131-137.

Chew, S.Y., Cheah, Y.K., Seow, H.F., Sandai, D. and Than, L.T.L., 2015. In vitro modulation of probiotic bacteria on the biofilm of *Candida glabrata*. *Anaerobe*, 34, pp.132-138.

Cildir, Sule Kavaloglu, Derya Germec, Nuket Sandalli, Fulya Isik Ozdemir, Tulin Arun, Svante Twetman, and Esber Caglar. "Reduction of salivary mutans streptococci in

orthodontic patients during daily consumption of yoghurt containing probiotic bacteria." *The European Journal of Orthodontics* 31, no. 4 (2009): 407-411.

Coleman, J.J., Okoli, I., Tegos, G.P., Holson, E.B., Wagner, F.F., Hamblin, M.R., Mylonakis, E., 2010. Characterization of plant-derived saponin natural products against *Candida albicans*. *ACS chemical biology*, 5(3), pp.321-332.

Coman, M.M., Verdenelli, M.C., Cecchini, C., Silvi, S., Orpianesi, C., Boyko, N. and Cresci, A., 2014. In vitro evaluation of antimicrobial activity of *Lactobacillus rhamnosus* IMC 501®, *Lactobacillus paracasei* IMC 502® and SYN BIO® against pathogens. *Journal of applied microbiology*, 117(2), pp.518-527.

Ellepola, A.N.B. and Samaranayake, L.P., 2000. The effect of brief exposure to sub-therapeutic concentrations of chlorhexidine gluconate on the germ tube formation of oral *Candida albicans* and its relationship to post-antifungal effect. *Oral diseases*, 6(3), pp.166-171.

Ellepola, A.N.B. and Samaranayake, L.P., 2001. Adjunctive use of chlorhexidine in oral candidoses: a review. *Oral diseases*, 7(1), pp.11-17.

Ericson, J.E., Benjamin Jr, D.K., 2014. Fluconazole prophylaxis for prevention of invasive candidiasis in infants. *Current opinion in pediatrics*, 26(2), p.151.

Farah, C.S., Lynch, N. and McCullough, M.J., 2010. Oral fungal infections: an update for the general practitioner. *Australian dental journal*, 55, pp.48-54.

Flichy-Fernández, A.J., Alegre-Domingo, T., Peñarrocha-Oltra, D. and Peñarrocha-Diago, M., 2010. Probiotic treatment in the oral cavity: An update. *Med Oral Patol Oral Cir Bucal*, 15(5), pp.e677-80.

Fourie, J., Khammissa, R.A.G., Ballyram, R., Wood, N.H., Lemmer, J. and Feller, L., 2016. Oral candidosis: an update on diagnosis, aetiopathogenesis and management. *South African Dental Journal*, 71(7), pp.314-318.

Gerbaldo, G.A., Barberis, C., Pascual, L., Dalcero, A., Barberis, L., 2012. Antifungal activity of two *Lactobacillus* strains with potential probiotic properties. *FEMS microbiology letters*, 332(1), pp.27-33.

Gill, H., Prasad, J., 2008. Probiotics, immunomodulation, and health benefits. In *Bioactive components of milk* (pp. 423-454). Springer, New York, NY.

Grudianov, A.I., Dmitrieva, N.A. and Fomenko, E.V., 2002. Use of probiotics Bifidumbacterin and Acilact in tablets in therapy of periodontal inflammations. *Stomatologiya*, 81(1), pp.39-43.

Harini, P.M. and Anegundi, R.T., 2010. Efficacy of a probiotic and chlorhexidine mouth rinses: A short-term clinical study. *Journal of Indian society of pedodontics and preventive dentistry*, 28(3), p.179.

Hatakka, K., Ahola, A., Yli-Knuutila, H., Richardson, M., Poussa, T., Suomalainen, T., Meurman, J.H. and Korpela, R., 2005. Cheese with probiotic bacteria reduces prevalence of oral *Candida* in the elderly. *Am J Clin Nutr*.

Hatakka, K., Ahola, A.J., Yli-Knuutila, H., Richardson, M., Poussa, T., Meurman, J.H. and Korpela, R., 2007. Probiotics reduce the prevalence of oral *Candida* in the elderly—a randomized controlled trial. *Journal of dental research*, 86(2), pp.125-130.

Hatakka, K., Savilahti, E., Pönkä, A., Meurman, J.H., Poussa, T., Näse, L., Saxelin, M. and Korpela, R., 2001. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. *Bmj*, 322(7298), p.1327.

Haukioja, A., 2010. Probiotics and oral health. *European journal of dentistry*, 4(3), p.348.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S. and Calder, P.C., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology*, 11(8), p.506.

Huovinen, P., 2001. Bacteriotherapy: the time has come: Bacterial interference is an increasingly attractive approach to prevention and therapy. *BMJ: British Medical Journal*, 323(7309), p.353.

Iqbal, M.Z., Qadir, M.I., Hussain, T., Janbaz, K.H., Khan, Y.H. and Ahmad, B., 2014. Probiotics and their beneficial effects against various diseases. *Pakistan journal of pharmaceutical sciences*, 27(2).

Ishikawa, K.H., Mayer, M., Miyazima, T.Y., Matsubara, V.H., Silva, E.G., Paula, C.R., Campos, T.T. and Nakamae, A.E., 2015. A multispecies probiotic reduces oral *Candida* colonization in denture wearers. *Journal of Prosthodontics*, 24(3), pp.194-199.

Jorgensen, M.R., Kragelund, C., Jensen, P.O., Keller, M.K. and Twetman, S., 2017. Probiotic *Lactobacillus reuteri* has antifungal effects on oral *Candida* species in vitro. *Journal of oral microbiology*, 9(1), p.1274582.

Kang MS, Kim BG, Chung J, Lee HC, Oh JS. Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds. *J Clin Periodontol*. 2006;33:226-32.

Kraft-Bodi, E., Jorgensen, M.R., Keller, M.K., Kragelund, C., Twetman, S., 2015. Effect of probiotic bacteria on oral *Candida* in frail elderly. *Journal of dental research*, 94(9_suppl), pp.181S-186S.

Kwon, J.H. and Farrell, R.J., 2003. Probiotics and inflammatory bowel disease. *BioDrugs*, 17(3), pp.179-186.

LaFleur, M.D., Qi, Q. and Lewis, K., 2010. Patients with long-term oral carriage harbor high-persisters mutants of *Candida albicans*. *Antimicrobial agents and chemotherapy*, 54(1), pp.39-44.

Li, D., Li, Q., Liu, C., Lin, M., Li, X., Xiao, X., Zhu, Z., Gong, Q. and Zhou, H., 2014. Efficacy and safety of probiotics in the treatment of *Candida*-associated stomatitis. *Mycoses*, 57(3), pp.141-146.

Machado, F.C., Portela, M.B., Cunha, A.C., Souza, I.P.R., Soares, R.M. and Castro, G.F., 2010. Antifungal activity of chlorhexidine on *Candida* spp. biofilm. *Rev Odontol UNESP*, 39(5), pp.271-5.

Markowiak, P. and Śliżewska, K., 2017. Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients*, 9(9), p.1021.

Matsubara, V.H., Bandara, H.M.H.N., Mayer, M.P., Samaranayake, L.P., 2016. Probiotics as antifungals in mucosal candidiasis. *Clinical Infectious Diseases*, 62(9), pp.1143-1153.

Matsubara, V.H., Wang, Y., Bandara, H.M.H.N., Mayer, M.P.A. and Samaranayake, L.P., 2016. Probiotic lactobacilli inhibit early stages of *Candida albicans* biofilm development by reducing their growth, cell adhesion, and filamentation. *Applied microbiology and biotechnology*, 100(14), pp.6415-6426.

Mayer, F.L., Wilson, D. and Hube, B., 2013. *Candida albicans* pathogenicity mechanisms. *Virulence*, 4(2), pp.119-128.

- Mendonça, F.H.B.P., Santos, S.S.F.D., Faria, I.D.S.D., Gonçalves e Silva, C.R., Jorge, A.O.C. and Leão, M.V.P., 2012. Effects of probiotic bacteria on Candida presence and IgA anti-Candida in the oral cavity of elderly. *Brazilian dental journal*, 23(5), pp.534-538.
- Meurman, J.H. and Stamatova, I., 2007. Probiotics: contributions to oral health. *Oral diseases*, 13(5), pp.443-451.
- Millsop, J.W. and Fazel, N., 2016. Oral candidiasis. *Clinics in dermatology*, 34(4), pp.487-494.
- Mundula, T., Ricci, F., Barbetta, B., Baccini, M. and Amedei, A., 2019. Effect of Probiotics on Oral Candidiasis: A Systematic Review and Meta-Analysis. *Nutrients*, 11(10), p.2449.
- Nagaraj, T., Ravi, B., Sankara, S.N. and Madhu, K., 2012. Probiotics and oral health. *Journal of Indian Academy of Oral Medicine and Radiology*, 24(2), p.146.
- Nagashima, A.I., Pansiera, P.E., Baracat, M.M. and Gómez, R.J.H.C., 2013. Development of effervescent products, in powder and tablet form, supplemented with probiotics *Lactobacillus acidophilus* and *Saccharomyces boulardii*. *Food Science and Technology*, 33(4), pp.605-611.
- Nagoba, B. (2018) 'Properties of *Candida albicans* Biofilms: An Update', *American Journal of Clinical Microbiology and Antimicrobials*, 1(4), p. 1017.
- Nobile, C.J. and Johnson, A.D., 2015. *Candida albicans* biofilms and human disease. *Annual review of microbiology*, 69, pp.71-92.
- Petti, S., Tarsitani, G. and D'Arca, A.S., 2001. A randomized clinical trial of the effect of yoghurt on the human salivary microflora. *Archives of oral biology*, 46(8), pp.705-712.
- Pradeep, K., Kuttappa, M.A. and Prasana, K.R., 2014. Probiotics and oral health: an update: clinical review. *South African Dental Journal*, 69(1), pp.20-24.
- Sajjan, P., Laxminarayan, N., Kar, P.P. and Sajjanar, M., 2016. Chlorhexidine as an antimicrobial agent in dentistry—a review. *Oral Health Dent Manag*, 15(2), pp.93-100.
- Sardi, J.C.O., Scorzoni, L., Bernardi, T., Fusco-Almeida, A.M., Giannini, M.M., 2013. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of medical microbiology*, 62(1), pp.10-24.

Scheibler, E., da Silva, R.M., Leite, C.E., Campos, M.M., Figueiredo, M.A., Salum, F.G. and Cherubini, K., 2018. Stability and efficacy of combined nystatin and chlorhexidine against suspensions and biofilms of *Candida albicans*. *Archives of oral biology*, 89, pp.70-76.

Seil, J.T., Webster, T.J., 2012. Antimicrobial applications of nanotechnology: methods and literature. *International journal of nanomedicine*, 7, p.2767.

Seneviratne, C.J., Jin, L., Samaranyake, L.P., 2008. Biofilm lifestyle of *Candida*: a mini review. *Oral diseases*, 14(7), pp.582-590.

Sherry, L., Jose, A., Murray, C., Williams, C., Jones, B., Millington, O., Bagg, J., Ramage, G., 2012. Carbohydrate derived fulvic acid: an in vitro investigation of a novel membrane active antiseptic agent against *Candida albicans* biofilms. *Frontiers in microbiology*, 3, p.116.

Strus, M., Kucharska, A., Kukla, G., Brzychczy-Włoch, M., Maresz, K. and Heczko, P.B., 2005. The in vitro activity of vaginal *Lactobacillus* with probiotic properties against *Candida*. *Infectious diseases in obstetrics and gynecology*, 13(2), pp.69-75.

Sutton, S., 2011. Accuracy of plate counts. *Journal of validation technology*, 17(3), pp.42-46.

Ujaoney, S., Chandra, J., Faddoul, F., Chane, M., Wang, J., Taifour, L., Mamtani, M.R., Thakre, T.P., Kulkarni, H., Mukherjee, P. and Ghannoum, M.A., 2014. In vitro effect of over-the-counter probiotics on the ability of *Candida albicans* to form biofilm on denture strips. *American Dental Hygienists' Association*, 88(3), pp.183-189.

Varoni, E., Tarce, M., Lodi, G. and Carrassi, A., 2012. Chlorhexidine (CHX) in dentistry: state of the art. *Minerva Stomatol*, 61(9), pp.399-419.

Vecchiarelli A, Pericolini E, Gabrielli E, Pietrella D. New approaches in the development of a vaccine for mucosal candidiasis: progress and challenges. *Front Microbiol* 2012; 3:1-7.

Verdenelli, M.C., Coman, M.M., Cecchini, C., Silvi, S., Orpianesi, C. and Cresci, A., 2014. Evaluation of antipathogenic activity and adherence properties of human *Lactobacillus* strains for vaginal formulations. *Journal of applied microbiology*, 116(5), pp.1297-1307.

Vilela, S.F., Barbosa, J.O., Rossoni, R.D., Santos, J.D., Prata, M.C., Anbinder, A.L., Jorge, A.O. and Junqueira, J.C., 2015. *Lactobacillus acidophilus* ATCC 4356 inhibits biofilm formation by *C. albicans* and attenuates the experimental candidiasis in *Galleria mellonella*. *Virulence*, 6(1), pp.29-39.

Vishnu, H.P., 2012. Probiotics and oral health. In Oral health care-pediatric, research, epidemiology and clinical practices. InTech.

Wolf, B.W., Garleb, K.A., Ataya, D.G. and Casas, I.A., 1995. Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. *Microbial ecology in health and disease*, 8(2), pp.41-50.



Appendix

	A	B	C	D	E	F
1	treatmen x2		x4	x5	control	
2	obs1					
3	obs2					
4	obs3					
5	obs4					
6	obs5					
7	obs6					
8	obs7					
9	obs8					
10	obs9					
11	obs10					
12	obs11					
13	obs12					
14	obs13					
15	obs14					
16	obs15					
17	obs16					
18	obs17					
19	obs18					
20	obs19					
21	obs20					
22	obs21					
23	obs22					
24	obs23					
25	obs24					
26	obs25					
27	obs26					
28	obs27					
29	obs28					
30	obs29					
31	obs30					

Sample of data collection sheet.

Note: four of these spread sheets were used during this study. Two for selective media (30 minutes and 24 hours incubation) and two for non-selective media (30 minutes and 24 hours incubation).



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