

EVALUATION OF ANTIBACTERIAL EFFICACY OF FOUR DIFFERENT DENTAL VARNISHES- AN *IN VITRO* STUDY

**By
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In partial fulfillment of the requirement for the Degree of

MASTER OF DENTAL SURGERY

PEDIATRIC AND PREVENTIVE DENTISTRY

**SATHYABAMA DENTAL COLLEGE AND HOSPITAL
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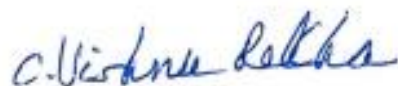
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Dental caries is a chronic infectious disease that affects the quality of life in children. It is the destruction of dental hard acellular tissue by acidic by-products from the bacterial fermentation of dietary carbohydrates especially sucrose. It progresses slowly in most of the people which results from an ecological imbalance in the equilibrium between tooth minerals and oral biofilms which is characterised by microbial activity, resulting in fluctuations in plaque pH due to bacterial acid production, buffering action from saliva and the surrounding tooth structure. The microbial community of caries is diverse and contains many facultatively and obligately-anaerobic bacteria.(1) Various preventive measures have been implemented in order to halt the causative microorganisms and thus halt the disease process.

The discovery of *S.mutans* from human caries lesion by **Clarke** in 1924 was overlooked for decades, and it wasn't until the 1960s that gnotobiotic animal studies became possible, allowing it to be demonstrated categorically that caries disease was a transmissible disease and fermentable carbohydrates in the diet played a critical role. Oral streptococci from humans could cause caries lesions in rodents fed with a high sugar diet. Also added on interventions, such as an antibacterial mouthwash, could prevent caries lesions.(2)

Streptococcus mutans and *Lactobacillus acidophilus* are the main bacterial strains involved in the decay process as causative and progressive factors, respectively. The antibacterial activities of varnishes

against *Lactobacillus acidophilus* have received little attention in most research. Because each microorganism's structural and metabolic features differ from those of other microbes, results from studies using *Streptococcus mutans* cannot be applied to *Lactobacillus acidophilus*.(3)

Recent studies have used molecular methods to identify the most common bacteria found in biofilm concealing lesions, avoiding the bias imposed by growing organisms on both selective and nonselective agar plates.(4)

A biofilm is a collection of organisms such as viruses, fungi, and bacteria that live at a density interface and are embedded in a self-secreted extracellular matrix. According to estimations, the mouth cavity contains almost 19,000 phylotypes. Dental caries occurs when the microbial homeostasis of dental plaque is disrupted, causing the mineral equilibrium between enamel and the biofilm fluid to break down. Biofilms are microbial communities that are incredibly complex (5).

Although untreated dental caries can progress, it can be prevented, reversed, or arrested in its early stages. This cycle can be influenced by a number of factors, including patient education, lowering the rate of free sugar intake, controlling plaque formation, increasing salivary buffering capacity, increasing salivary flow rate, and using additional caries preventive measures such as antibacterial agents and fluoride (6).

Many distinct compositional varnishes have emerged as a result of improvements in dental materials and technology. For more than three decades, varnish has been widely utilized as a caries prevention technique (7). In this context, the varnish layer assumes the role of a matrix, which enables the slow release of active substances, such as fluoride compounds, antimicrobial compounds or oxidative bleaching agents, on the tooth surface.

Schmidt, a German researcher, invented professionally applied fluoride varnishes in 1964, and employed them to prevent tooth cavities. There are already around thirty fluoride-containing varnishes in the market (8). Fluoride varnishes have shown great clinical efficacy in the reduction of dental caries, in addition to their ease of application and high patient acceptability. For decades, the explanation for fluoride's caries-preventive activity has been understood. Fluoride's benefits are proportional to the effective concentration and the frequency with which it is applied. When applied alone, fluoride varnish has been demonstrated to have a lower effect on *S. mutans* salivary levels, leading to the development of novel varnishes with additives. Fluor protector contains 0.9% difluorosilane in a polyurethane varnish base with ethyl acetate and isoamyl propionate solvents. The fluoride content is equivalent to 0.1%, or 1000 ppm in solution.(9)

To the conventional sodium fluoride varnish, a combination of calcium and phosphate coated with xylitol is an addition. The calcium and phosphate ions released by xylitol in saliva react with the fluoride ions in

saliva to generate protective fluorapatite on the teeth. Pulpdent embrace varnishes containing xylitol have been found to be promising solutions for increasing enamel remineralization. Because the five-carbon sugar xylitol cannot be fermented by *S.mutans*, it has favourable effects such as a lower incidence of caries and *S. mutans* levels in the oral flora (10).

Varnishes containing chlorhexidine, a well-known chemotherapeutic drug used against periodontal-pathogenic bacteria, have been used as a bacterial activity-reducing alternative. Their use is based on chlorhexidine's ability to reduce dental plaque production as well as its bactericidal and bacteriostatic effects. Microorganisms are selectively controlled by the Cervitec Plus varnish system, which contains an efficient mix of the active chemicals chlorhexidine and thymol. Cervitec plus varnish considerably inhibits the occurrence of caries in the tooth's occlusal fissures.(11)

Another preventive alternative tested in vitro, in situ and in vivo studies and used in recent years is the casein phosphopeptide-amorphous calcium phosphate nanocomplex. MI varnish with fluoride is used for prevention as well as arrest of dental caries progression. This milk product facilitates remineralization, inhibits demineralization, and prevents dental caries by forming a calcium phosphate ion reservoir for enamel to resist acid attack.(12)

Different methods to assess the antimicrobial efficacy of dental varnishes are susceptibility assays, such as agar diffusion techniques, minimum inhibitory concentration and minimum lethal concentration measurements, and time-kill curve analysis. The antibacterial activity with agar diffusion can be measured qualitatively. Because diffusion is dependent on the product's physical-chemical properties, the diameter of the inhibition zones cannot be used to compare different formulae, and the absence of an inhibition zone does not necessarily mean that the product is not active. Henceforth, minimum inhibitory concentration using broth dilution assay and in addition to characterizing the product's activity spectrum, the rate of kill must also be determined. (13). Furthermore preferences on selected dental varnish material are because the literature is evolved based on the inclusion of these new components which could improve the antibacterial activity of dental varnishes. Although the caries remineralizing or arresting effect was demonstrated in literature, until now, level of evidence regarding the comparative analysis and time kill curve analysis is low. This study was performed as an initiative to analyze different combinations of dental varnish material not only based on minimum inhibitory concentration but also including time kill curve assay. As a result, the ongoing dissertation work is intended to add more evidences and fill knowledge void in the literature.

The null hypothesis proposed in this study is that there is no difference among the antibacterial potential of the four different dental varnishes, Pulpdent Embrace varnish (Xylitol coated calcium phosphate varnish), MI varnish (CPP-ACP varnish), Chlorehexidine varnish (Cervitec plus varnish) and Fluoride varnish (Fluor protector varnish).

The aim of this study was to compare the antibacterial efficacy of the commercially available four different fluoride varnishes; Pulpdent embrace varnish (5% sodium fluoride with Xylitol coated calcium phosphate varnish), MI varnish (casein phosphopeptide-amorphous calcium phosphate varnish), Cervitec plus varnish (Chlorehexidine varnish) and Fluor protector varnish (fluoride varnish) in terms of their antibacterial effect against *S.mutans* and *L.acidophilus*.

OBJECTIVES

- ❖ To assess if there is any superiority in the antibacterial efficacy among Pulpdent embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish.
- ❖ To assess if there is any superiority in the required application time period among Pulpdent embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish.

The phrase "infectious disease" indicates that the illness is brought on by a specific microbe or chemical that has 'infected' a person. Caries was explained as an "infectious and transmissible" disease for nearly a half-century.

Rodrigues(1931), in the first half of the previous century focused on the role of Lactobacillus. He claimed that incidence of *L.acidophilus* in the salivary fluid of persons susceptible to dental caries can be influenced by various hygienic measures.

Loesche et al (1976) developed "Non-specific plaque hypothesis" based on the work of researchers like Black and Miller. The most widespread belief concerning dental infections at the end of the nineteenth century was that they were caused by a non-specific overgrowth of all bacteria in dental plaque.

Scheinin et al (1976) commenced the Turku Sugar Studies, adding a seminal study in dental caries research, involving the total replacement of almost all dietary sugars with xylitol. Since then, the majority of xylitol caries studies have been partial substitution or chewing gum/ lozenges supplementation studies.

Walter J. Loesche et al (1976) proposed the "Specific Plaque Hypothesis", claiming that dental caries was caused by an infection in the dental plaque caused by specific bacteria, the most important of which

were "*mutans streptococci*" (main species: *Streptococcus mutans* and *Streptococcus sobrinus*) and *lactobacilli*. The discovery of several species led to the hypothesis that oral disease could be caused by a variety of pathogens.

Balanyk and Sandham (1985) developed the first varnish, containing 10% chlorhexidine, as a way of increasing its substantiveness and the effective release of chlorhexidine into the oral cavity.

Pettersen et al (1991) reported application of cervitec during a 3-month interval is very effective even at low concentrations. Also no serious side effects have been observed when chlorhexidine was used in the form of a varnish, possibly because of the low concentration and the frequency of application compared to other chlorhexidine treatments.

Marsh and Martin (1992) stated that the relationship between *Streptococcus mutans* and dental caries is not robust. Caries can form even when relatively significant quantities of *Streptococcus mutans* are present on tooth surfaces.

Philip D. Marsh (1994) proposed a hypothesis that combined key concepts of the earlier hypotheses. In his "ecological plaque hypothesis" he stated that the disease is the result of an imbalance in the total microflora due to ecological stress, resulting in an enrichment of some "oral pathogens" or disease-related microorganisms.

Van Houte (1994) focussed on the role of *mutans streptococci*, and adds on a positive association with caries activity and rank very high among the oral microbiota with regard to their ability for acid production as well as growth and survival at acidic pH values.

Bratthall et al. (1995) applied cervitec three times over a period of 2 years, for children aged 7–8 and 12–13 years and found that fissure caries was significantly reduced in permanent molars.

Ogaard et al (1997) conducted a study on patients receiving treatment with fixed orthodontic appliances. Fluoride varnish (fluor protector) and an antimicrobial varnish (cervitec) were tested. Author concluded that cervitec varnish significantly reduces the number of *mutans streptococci* in plaque during treatment with fixed orthodontic appliances.

Reynolds et al (1997) at the University of Melbourne, Australia, patented CPP-ACP, a nano complex. It's made up of phosphopeptide casein protein (CPP), which comes from milk and is constructed by phosphorylated serine and glutamic amino acid residues.

Bradshaw and Marsh (1998) claimed that subjects, who frequently consume a considerable amount of fermentable carbohydrates, select for bacteria that ferment these carbohydrates and produce acids. This leads to more sugar fermentation and thus acid production, increasing the cariogenic bacteria even more.

Araujo et al (2002) investigated the effect of cervitec on *mutans streptococci* in the occlusal plaque of erupting first permanent molars and the impact on occlusal caries in these teeth. Cervitec reduced *mutans streptococci* in plaque on erupting permanent molars, potentially leading to a significant reduction in caries incidence.

Fejerskov (2003) established a link between the cause and dental caries. Oral bacteria that are necessary for the development and progression of dental caries are found in dental plaque biofilms. *Streptococcus mutans* is a key player in the etiology of dental caries and is widely regarded as the most common pathogen associated with the disease. Due to its high adherence to the tooth surface and the formation of acid from fermentable carbohydrates, which maintains the local pH low, it causes mineral loss. Biofilms are thought to be cariogenic because they account for *S. mutans* high adherence. The principal pathogenic bacteria associated with dental biofilms are *Streptococcus mutans* and *Streptococcus sobrinus* and *Lactobacillus*.

Fejerskov and Nyvad (2003) stated it is not merely semantics, but a paradigm shift, to consider dental caries as a 'complex disease caused by an imbalance in physiologic equilibrium between tooth mineral and biofilm fluid.

Larsen et al (2003) stated that despite its importance, fluoride alone is insufficient to prevent demineralization and potential mineral redeposition

into hydroxyapatite crystals. Maintaining the structural integrity of hydroxyapatite crystals also requires the presence of bioavailable calcium and phosphate in the plaque fluid.

Marsh (2003) expanded ecological plaque hypothesis by linking changes in microbial composition to changes in environmental factors such as the presence of nutrients and essential co-factors, pH, and redox potential.

Larsson et al (2004) evaluated efficacy of antibacterial varnish using caries diagnosing device DIAGNOdent. Cervitec proved to have potential and protective role on fissure caries.

Twetman et al (2004) concluded that clinical data on caries prevention effects are scarce, and that recent literature on the use of chlorhexidine varnishes for caries prevention in risk categories is inconclusive.

Cross et al (2005) claims that casein phosphopeptide containing the cluster sequence –Ser(P)- Ser(P)-Ser(P)-Glu-Glu- is the reason behind the stabilizing property of amorphous calcium phosphate in metastable solution. Casein phosphopeptide may bind to 25 calcium ions, 15 phosphate ions, and 5 fluoride ions per molecule, allowing calcium phosphate to be stabilized in solution. When the pH is neutral or alkaline, amorphous calcium phosphate is produced. Casein phosphopeptide can form colloidal complexes by releasing its weight in calcium and phosphate through its phosphorylated residue. Casein phosphopeptide controls the

growth of these amorphous calcium phosphate aggregates, preventing them from reaching the essential size for precipitation.

Plotzitz et al (2005) conducted a prospective clinical study to assess the effect of 3-monthly applications of the chlorhexidine-containing varnish cervitec on mutans streptococci colonisation of a child's oral cavity and caries prevalence. It was discovered that simply applying a chlorhexidine-containing varnish does not prevent early childhood caries, but it may influence the tendency of carious lesions to progress.

Beltran-Aguilar et al (2006) developed fluoride varnish with the goal of extending the duration of the tooth's contact time with the fluoride in the varnish. Fluoride uptake would be amplified and improved in this manner. Because the amount of fluoride placed in the mouth by fluoride varnish is approximately one-tenth that of other professionally applied products, fluoride varnish has a lower potential for harm than other forms of high concentration topical fluoride agents.

Andersson et al (2007) evaluated that white spot lesion regression can be obtained with the same casein phosphopeptide-amorphous calcium phosphate paste or tooth-paste containing sodium fluoride. Nonetheless, patients who used casein phosphopeptide-amorphous calcium phosphate-based paste had superior cosmetic results than those who used sodium fluoride based paste, presumably due to the presence of additional mineral.

Jeevarathan et al (2007) investigated the effect of fluor protector varnish on the concentration of *S.mutans* present in the plaque of children who did not have caries. The scientists showed that the high fluoride concentration in fluorprotector was linked to a decrease in bacterial count in their research. This could possibly be connected to the varnish's adhesion properties, such that *S. mutans* could not adhere to the enamel surface because of the varnish's covering layer.

Ribeiro et al (2007) highlighted on the number of studies investigated on this material at different concentrations (1%, 3%, 20%, 30%, 40%, and 50%) in order to assess its ability to lower *mutans streptococci* levels in the saliva and dental biofilm. Author claims that 1% chlorhexidine varnish one time a day for three days and application for three times with an interval of four days had no significant difference.

Autio Gold et al (2008) reviewed the literature on the effectiveness of different modes of chlorhexidine delivery for caries prevention and provide guidelines for chlorhexidine use in caries management. Author claimed that chlorhexidine varnishes for caries prevention should be researched further because of the equivocal literature and scant clinical data on gels and varnishes.

Morgan et al (2008) found that casein phosphopeptide-amorphous calcium phosphate in a sugar-free gum considerably reduced the

progression of caries in an ideally fluoridated environment in a randomised, controlled, double blind caries experiment.

Reynolds et al (2008) stated that for maximally effective remineralisation, calcium, phosphate and fluoride must all be available at sufficient levels in a bioavailable form in the oral environment. The goal of the research was to see if casein phosphopeptide-amorphous calcium phosphate could improve fluoride incorporation in plaque and promote enamel remineralization in situ. Author concluded that dentifrice with 2% casein phosphopeptide-amorphous calcium phosphate yielded remineralization levels comparable to those obtained with a dentifrice containing 2800 ppm fluoride. All other formulations were outperformed by a dentifrice containing 2% casein phosphopeptide-amorphous calcium phosphate plus 1100 ppm fluoride.

Fejerskov and Kidd (2009) stated that dental caries is characterised as the signs and symptoms of chemical breakdown of the tooth surface. Metabolic event in the biofilm overlaying the tooth structure causes this process. Dental caries can develop on any surface of a tooth where dental plaque is allowed to build over time and gradually mature into a biofilm.

Llena C et al (2009) in a review update claims that both biofilm and subsurface incipient caries lesions, as well as lower level demineralization of enamel or dentine surfaces previously treated with casein phosphopeptide-amorphous calcium phosphate based compounds have

been reported to have very high amounts of calcium and phosphate. Pastes containing casein phosphopeptide-amorphous calcium phosphate complexes can prevent tooth demineralization, promote enamel remineralization, and increase fluoride activity when applied to the surface of a tooth with early carious lesions. Clinically, pastes based on casein phosphopeptide-amorphous calcium phosphate compounds have been used to remineralize white spot lesions, and a similar effect to self-applied fluorides has been reported in preventing the formation of new caries lesions in xerostomia patients.

Poulsen et al (2009) performed a review which included six clinical trials on the effect of fluoride varnishes, which also confirmed the evidence that fluoride varnishes appear to have a caries-preventive effect on the young permanent dentition, while the effect on caries in the primary dentition remains to be limited.

Soderling et al (2009) based on the findings of studies on mother-child transmission of *mutans streptococci*; caregivers of young children under the age of two should consume xylitol on a regular basis. Consumption of xylitol on a regular basis could also be recommended for people who would benefit from a decrease in *mutans streptococci* counts as well as the amount and adhesivity of plaque.

Paula et al (2010) assessed the effect of combining 1% chlorhexidine varnish with xylitol chewing gum on *S. mutans* and biofilm levels in 6-8

year old children. Based on the outcome, cervitec was efficient and superior to single treatments in controlling biofilm and suppressing *S. mutans*.

Buzalaf et al (2011) claimed that fluoride inhibits the production of acids produced by oral bacteria such as *mutans streptococci*. This is a topical impact that leads to a reduction in bacterial colonization of the tooth and, as a result, a reduction in enamel demineralization. Fluoride ions inhibitory effect on oral bacteria has been linked to bacterial enzyme inhibition through in vitro investigations.

George Hajishengallis et al (2012) extended his concept to oral microbiology by proposing "The keystone-pathogen hypothesis." According to this hypothesis, certain low-abundance microbial infections might cause inflammatory disease by increasing the volume and modifying the composition of the normal microbiota.

Garcia Godoy et al (2013) conducted a study to compare the efficacy of xylitol based dentifrices with and without fluoride on root caries, and found no statistical difference in the mean lesion depths.

Marinho et al (2013) assessed the effectiveness of topically applied fluoride varnishes for preventing dental caries in children and adolescents. The author concluded that a substantial caries inhibiting effect of fluoride varnish in both permanent and primary teeth was reported.

Agouropoulos et al (2014) evaluated the effect of biannual fluoride varnish applications in preschool children as an added measure to a preventive programme consisting of school based oral health education and supervised tooth brushing with 1000 ppm fluoride toothpaste. The study used fluoride varnish (0.9% difluorosilane) biannually as test group and concluded that when 1000 ppm fluoride toothpaste is used daily in community-based caries prevention programmes for high-caries-risk preschool children, a fluoride varnish may offer little to caries prevention.

Carey et al (2014) mentioned about the controversy of fluoride varnish, since food and drug administration has not yet authorized fluoride for caries prevention. Because clinical efficacy caries investigations have been ambiguous or at best product based, the food and drug administration has been hesitant to approve fluoride varnish. Non-fluoride components in varnish products, such as varnish resin, flavouring, or other additives, may have anti-caries effectiveness or work in tandem with fluoride in the product. As a result, the food and drug administration lacks adequate information to describe the fluoride varnish efficacy requirements.

Grychtol et al (2014) investigated the influence of a preparation containing CPP/ACP. In contrast, author concluded under the conditions the tested preparation containing CPP/ACP has no significant impact on the initial bacterial colonization on enamel and dentin. Thus, it cannot be recommended for biofilm management.

Milgrom et al (2014) concluded that "occasional application of fluoride varnish following American Academy of Pediatrics guidance is safe for toddlers" in a study on the pharmacokinetics of fluoride in six toddlers aged 12 to 15 months after application of 5 % sodium fluoride varnish.

Llena et al (2015) evaluated the effects of novel casein phosphopeptide formulations casein phosphopeptide-amorphous calcium phosphate and casein phosphopeptide-amorphous calcium fluoride phosphate versus fluoride varnish on the remineralisation of enamel white spot lesions over a 12-week follow-up period. Outcome of the study revealed that casein phosphopeptide-amorphous calcium phosphate is not superior to fluoride varnish by any of the measures studied.

Marsh et al (2015) reveals dental caries is a polymicrobial biofilm-mediated disease triggered by environmentally induced dysbiosis in the resident oral microbiome. The significance to create ways to control the microbial composition of dental plaque for caries prevention has been highlighted by a greater understanding of oral microbial ecology, particularly the relevance of the equilibrium between cariogenic and commensal microflora.

Millburn et al (2015) concluded that Embrace varnish had the greatest initial fluoride release, exceeding ten times the release of a fluoride varnish (Vanish) in the first four hours; however, Embrace varnish had the highest rate of fluoride depletion and lowest substantivity of all varnishes tested.

Author claims that Embrace varnish is a fluoride varnish that combines xylitol-coated calcium and phosphate in a resin matrix to create a new generation of fluoride varnish. The fluoride release time of this varnish is increased as a result of this combination. When xylitol is dissolved in saliva, calcium and phosphate ions are released, which combine with fluoride ions to produce fluorapatite on the enamel surface.

Walsh et al (2015) in a review claims the effects of chlorhexidine-containing oral products (toothpastes, mouthrinses, varnishes, gels, gums and sprays) on the prevention of dental caries in children and adolescents. At three and six months, two of the trials revealed some evidence of *mutans streptococci* inhibiting effect. If the hypothesis of reduced numbers of *mutans streptococci* in the oral environment leading to lower caries increments is correct, then further trials should consider the use of chlorhexidine-containing products at no less than three or six month intervals.

Chokshi et al (2016) evaluated the remineralizing potential of fluor protector varnish, a casein phosphopeptide-amorphous calcium phosphate based paste, and another paste based on functionalized tri-calcium phosphate using laser confocal microscopy. The results showed that fluoride varnish was more effective than CPP-ACP based paste in decreasing the depth of incipient dental caries lesions.

Gao et al (2016) conducted a comprehensive study of fluoride treatment for preventing and treating dental caries in children that was professionally applied. The authors emphasized the efficiency of fluoride varnish for remineralization of early childhood caries in their meta-analysis, which found that the overall percentage of remineralization was 63.6 percent higher than no therapy.

Lenzi et al (2016) in her meta-analysis compared fluoride varnish to no treatment for reversing enamel carious lesions in children aged three to twelve. Despite the meta-analysis high level of heterogeneity, the authors found that fluoride varnish was an effective product that inhibits and can slow the advancement of enamel caries in both primary and permanent teeth.

Anderson et al (2017) found that biannual application of fluoride varnish had no effect on caries advancement on the buccal surfaces of primary incisors or the occlusal surfaces of primary molars.

Chukwuma et al (2017) explained that because of the permeability between xylitol and enamel, which enhances calcium absorption, xylitol also works as a calcium carrier for remineralization of teeth through complicated interactions. By facilitating the flow and accessibility of calcium ions, xylitol can aid in the remineralization of deeper layers of demineralized enamel.

Hajishengalis et al (2017) correlated the prime importance of *S.mutans*. Human dental caries has long been linked to *S. mutans*, a member of the *mutans streptococci* group. Extensive clinical, epidemiological and experimental animal researches have conclusively established that *mutans streptococci* is closely linked to the condition, particularly in early childhood caries, though not solely.

Mishra et al (2017) conducted a systematic review to look into the role of fluoride varnish in preventing early childhood caries. The most recent year of the search was 2015, and studies published in the previous 36 years were included. In total, seventeen studies were examined. Fluoride varnishes with concentrations of 1% and 5% were employed. When 1% varnish was used, the caries preventive fraction ranged from 6.4% to 30%, and when 5% fluoride varnish was employed, the caries preventive fraction ranged from 5% to 63%.

Shahmoradi et al (2017) reported that several proteins including statherins are responsible for stabilizing calcium and phosphate in saliva and preventing their spontaneous precipitation. Fluoride varnishes with added calcium and phosphate compositions have recently been introduced with the purpose of increasing the amount of calcium and phosphate in saliva, as well as the desire to improve demineralization prevention efficacy and possibly remineralization of the lesion. Fluoride varnishes with additional amorphous calcium phosphate (Enamel Pro varnish), tri-calcium phosphate (Clinpro White varnish), casein

phosphopeptide stabilized amorphous calcium phosphate (MI varnish), and Xylitol coated calcium and phosphate (Embrace varnish) are just a few examples.

Bowen et al (2018) mentioned biofilms form when microorganism clusters adhere to surfaces and secrete extracellular matrix, which aids in the attachment of additional microorganisms. Cariogenic biofilms initiated by *S. mutans* are acidic, hypoxic, but rich in carbohydrates, which creates an ideal microenvironment for opportunistic organisms like lactobacilli to grow and accelerate caries progression. Author also includes that the primary role of *S. mutans* as a pathogen may be due to its exceptional ability to alter the local physicochemical environment by utilising dietary sugar to assemble an insoluble polymeric matrix, thereby providing mechanical stability and protecting the acid milieu within which commensals may perish and more potent acidogenic/aciduric organisms thrive to become dominant, making them difficult to treat.

Jafari et al (2018) reported the use of xylitol-based varnishes and casein phosphopeptide-amorphous calcium phosphate varnishes resulted in a significant reduction in the amount of *streptococcus mutans* in the oral cavity.

Liptak et al (2018) investigated the effects of cervitec varnish on the level of *mutans streptococcus* and *lactobacillus* colonies. Based on findings, author concluded that in practice monthly use of an innovative varnish

containing 1% chlorhexidine and 1% thymol can be an effective professional method to prevent dental plaque-induced oral disease, and this can result in a significant improvement of oral health in orthodontic patients.

Philip et al (2019) investigated whether casein phosphopeptide-amorphous calcium phosphate could influence microbial counts, acidogenicity, and the relative abundance of specific caries and health associated bacterial species in polymicrobial biofilms. Casein phosphopeptide-amorphous calcium phosphate has virulence attenuating properties, which can impact a favorable microbial ecological alteration in the biofilm.

Troconis CC et al (2019) contributed in a review that the use of dental products containing casein phosphopeptide-amorphous calcium phosphate has a preventive potential for dental caries, but it is not greater than sodium fluoride. However, when casein phosphopeptide-amorphous calcium phosphate or casein phosphopeptide-amorphous calcium phosphate fluoride containing products are used, there is a greater likelihood of regression of enamel white spot lesions.

Urquhart et al (2019) stated that, topical fluoride treatment has become widely regarded as a critical factor in successful caries prevention without significant fluoride ingestion. Fluorides are also effective as therapeutic

agents for the prevention of caries lesions in non-invasive caries treatment.

Yadav S et al (2019) concluded that xylitol alters the growth and metabolism of several bacterial species, but *streptococcus mutans* appears to be the target organism. Furthermore, it is likely that the reduction of insoluble extracellular polysaccharides is important for the xylitol-induced decrease in counts as well as the transmission of *mutans streptococci*.

Feifei LD et al (2020) proved that biannual application of fluoride varnish compared with fissure sealant results in non-significant difference about caries prevention efficacy of first permanent molar at 2-3 years follow up.

Elhangour et al (2021) compared the remineralizing effect of embrace varnish with fluoride varnish on caries-like lesions in primary teeth regarding calcium and phosphate enamel content. Topical fluoride varnish containing xylitol-coated calcium phosphate (Embrace varnish) achieved significantly superior remineralization results on artificial enamel caries when compared to conventional sodium fluoride varnish.

Hradec Kralove et al (2021) reviewed the evidence behind the current fluoride caries prevention method as well as the risks associated with it, particularly in children. The author claims that, when fluoride is used in conjunction with other fluoride vehicles, the cumulative fluoride exposure

of children under the age of six must be considered. Reviews strongly support the recommended use of fluoride in pediatric population and adopt them by Pediatric and Preventive Dentistry community.

Jullien et al (2021) reported on the recommendations and supporting evidence on the effectiveness and potential harms of the different fluoride interventions in preventing dental caries in children under 5 years of age. Author claims that, professionally-applied topical fluorides such as gels and varnishes are effective in preventing dental caries, and are generally indicated for persons at high risk of dental caries and for patients with special needs, especially in communities with low exposure to fluoride.

Sharda et al (2021) favoured the use of casein phosphopeptide-amorphous calcium phosphate topical fluoride combination therapy while others reveal no significant difference in post-intervention *S.mutans* colony count. The results of the sub-group pooled analysis, suggest that combination treatment is more effective than fluoride alone in lowering salivary *S. mutans* load.

The present in vitro study was conducted in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital, Chennai.

Other facilities involved in the study

- Research Centre, Balaji Dental College and Hospital, Pallikaranai.

Materials used in the study

Experimental materials

- ❖ 5% sodium fluoride with xylitol coated calcium phosphate varnish (Pulpdent embrace varnish, Pulpdent Corporation)
- ❖ Casein phosphopeptide-amorphous calcium phosphate varnish (MI varnish, GC India)
- ❖ Chlorhexidine varnish (Cervitec Plus varnish, Ivoclar Vivadent)
- ❖ Fluoride varnish (Fluor protector varnish, Ivoclar Vivadent)

Materials & Equipment for evaluation of antibacterial effect

- Culture plate
- Cotton swab
- Micropipette
- Wire loop
- 96 well plate
- Blood agar
- Mutans sanguis agar
- Lactobacillus MRS agar

- Brain heart infusion broth
- Lactobacillus MRS broth
- *Streptococcus mutans* strain (MTCC- 497)
- *Lactobacillus acidophilus* strain (MTCC- 10307)
- Incubator

Streptococcus mutans and *Lactobacillus acidophilus* were procured from Council of scientific and Industrial Research Institute of Microbial Technology, Chandigarh in a lyophilized (Freeze dried) form.

METHODOLOGY

Culture preparation was done for *streptococcus mutans* and *Lactobacillus acidophilus*.

***Streptococcus mutans* culture preparation**

S.mutans strain was added to 0.4 ml of brain heart infusion broth. This suspension was added onto sheep's blood agar plate and streaked. The streaked plate was incubated at 37°C for 24 hours under anaerobic conditions. Growth of *S.mutans* culture was observed after 24 hours. *S.mutans* was sub cultured at weekly intervals to maintain their viability until subjected to the study.

***Lactobacillus acidophilus* culture preparation**

Lactobacillus acidophilus strain was added to 0.4 ml of lactobacillus MRS broth. This suspension was added onto lactobacillus MRS agar plate and streaked. The streaked plate was incubated at 37° C for 24 hours under

anaerobic conditions. Growth of *Lactobacillus acidophilus* culture was observed after 24 hours. *Lactobacillus acidophilus* was sub cultured at weekly intervals to maintain their viability until subjected to the study.

The antibacterial efficacy was analysed using minimum inhibitory concentration and time kill curve assay.

Minimum inhibitory concentration of *Streptococcus mutans*

Pure colonies of *streptococcus mutans* were added to 1ml of brain heart infusion broth. To this, sterile human serum was added. This prepared inoculum was incubated at 37°C for 24 hours. Inoculum's turbidity was as per McFarlands standards (approximately 1.5×10^8 colony forming units per ml). (McFarlands standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing prior to assay). This inoculum was introduced into 96-well titre plates for spot inoculation. Pulpdent embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish were added to this inoculum in the respective titre wells. 5 µl of broth dilution assay from each well was spot inoculated on mutans sanguis agar and incubated at 37°C for 24 hours.

Inhibition of growth of *streptococcus mutans* was observed and scored for each dental varnish. The lowest antibacterial concentration that completely inhibited visible growth of *streptococcus mutans* was recorded as the minimum inhibitory concentration.

Minimum inhibitory concentration of *Lactobacillus acidophilus*

Pure colonies of *Lactobacillus acidophilus* were added to 1ml of lactobacillus MRS broth. This prepared inoculum was incubated at 37°C for 24 hours. Inoculum's turbidity was as per McFarlands standards.

The inoculum was subjected to spot inoculation for broth dilution assay. This inoculum was introduced into 96-well titre plates. Pulpdent Embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish were added to this inoculum in the respective titre wells. 5 µl of broth dilution assay from each well was spot inoculated on mutans sanguis agar and incubated at 37°C for 24 hours.

Inhibition of growth of *Lactobacillus acidophilus* was observed and scored for each dental varnish. The lowest antibacterial concentration that completely inhibited visible growth of *Lactobacillus acidophilus* was recorded as the minimum inhibitory concentration.

Time kill curve assay against *Streptococcus mutans*

Time kill curve assay was analysed for all four dental varnishes against *S.mutans*. 50 freshly extracted premolars were autoclaved at 121°C under a pressure of 15 psi for 20 minutes. These autoclaved teeth were subjected to biofilm formation by vertically immersing them in test tubes containing 10 µl of culture suspension of *S.mutans*. *S.mutans* culture suspension was matched to McFarlands standards. These test tubes were incubated at 37°C for 48 hours. Two teeth samples were assessed for cell viability. Swabs were taken from these two teeth using applicator tip and was swirled in 1 ml of brain heart infusion broth. This

was transferred into agar plates and was observed for growth of *S.mutans*. This was used as the positive control.

The remaining 48 teeth were divided into four groups containing 12 teeth each.

Group A – Pulpdent embrace varnish

Group B – MI varnish

Group C – Cervitec plus varnish

Group D – Fluor protector varnish

Pulpdent Embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish were applied to the teeth of the respective group. Swabs were taken from the four groups after 2, 5, 10 and 15 minutes. These swabs were swirled into 10 µl of brain heart infusion broth and inoculated into sterile mutans sanguis agar plate and incubated at 37°C for 24 hours. Colony forming units were observed and counted using digital colony counter.

Time kill curve assay against *Lactobacillus acidophilus*

Time kill curve assay was analysed for all four dental varnishes against *L.acidophilus*. 50 freshly extracted premolars were autoclaved at 121°C under a pressure of 15 psi for 20 minutes. These autoclaved teeth were subjected to biofilm formation by vertically immersing them in test tubes containing 10 µl of culture suspension of *L.acidophilus*. *L.acidophilus* culture suspension was matched to McFarlands standard. These test tubes were incubated at 37°C for 48 hours. Two teeth samples were

assessed for cell viability. Swabs were taken from these two teeth using applicator tip and was swirled in 1 ml of lactobacillus MRS broth. This was transferred into agar plates and was observed for growth of *L.acidophilus*. This was used as the positive control.

The remaining 48 teeth were divided into four groups containing 12 teeth each.

Group A – Pulpdent embrace varnish

Group B – MI varnish

Group C – Cervitec plus varnish

Group D – Fluor Protector varnish

Pulpdent Embrace varnish, MI varnish, Cervitec plus varnish and Fluor protector varnish were applied to the teeth of the respective group. Swabs were taken from the 4 groups after 2, 5, 10 and 15 minutes. These swabs were swirled into 10 µl of lactobacillus MRS broth and inoculated into sterile MRS agar plate. Agar plate was incubated at 37°C for 24 hours. Colony forming units were observed and counted using digital colony counter.

**MINIMUM INHIBITORY CONCENTRATION OF FOUR DENTAL
VARNISHES AGAINST *S.MUTANS***

Streptococcus mutans strain revived on sheep blood agar



Overnight inoculation in 0.4 ml brain heart infusion broth



Incubated at 37°C for 1 Hour



BROTH MICRO-DILUTION ASSAY

1µl Pulpdent
Embrace™ varnish
with CXP prepared
in broth media

1µl MI Varnish with
RECALDENT™
varnish prepared in
broth media

1µl Cervitec plus
varnish prepared in
broth media

1µl Fluor protector
varnish prepared in
broth media



Placed in the first well of the 96 microtitre plate



Gradual increase of dilutions from second well to eleventh well



10 µl culture suspensions of *streptococcus mutans* added to all the wells



Incubated overnight at 37°C



5 µl of the culture from each well transferred onto mutans sanguis agar
plate



Incubated for 24 hours



Inhibition of the growth of *Streptococcus mutans* scored as the minimum
inhibitory concentration

**MINIMUM INHIBITORY CONCENTRATION OF FOUR DENTAL
VARNISHES AGAINST *L.ACIDOPHILUS***

Lactobacillus acidophilus strain revived on *Lactobacillus* MRS agar



Overnight inoculation in 0.4ml *Lactobacillus* MRS broth



Incubated at 37°C for 1 Hour



BROTH MICRO-DILUTION ASSAY

1µl Pulpdent
Embrace™ varnish
with CXP prepared
in broth media

1µl MI Varnish with
RECALDENT™
varnish prepared in
broth media

1µl Cervitec plus
varnish prepared in
broth media

1µl Fluor protector
varnish prepared in
broth media



Placed in the first well of the 96 microtitre plate



Gradual increase of dilutions from second well to eleventh well



10 µl culture suspension of *Lactobacillus acidophilus* added to all the wells



Incubated overnight at 37°C



5 µl of the culture from each well transferred onto *Lactobacillus* MRS

Agar plate

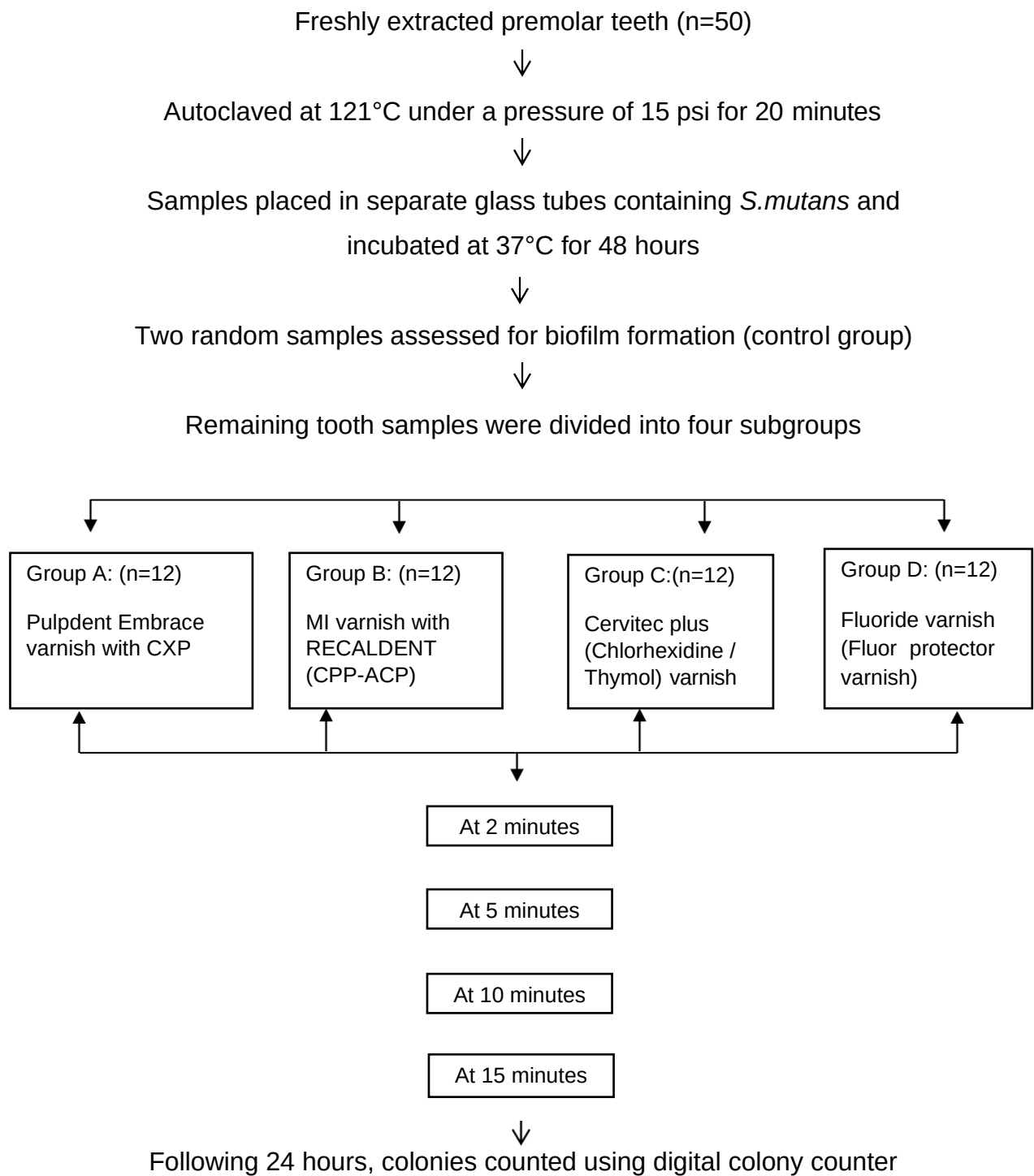


Incubated for 24 hours



Inhibition of the growth of *Lactobacillus acidophilus* scored as the
minimum inhibitory concentration

**TIME KILL CURVE ASSAY OF FOUR DENTAL VARNISHES
AGAINST S.MUTANS**



**TIME KILL CURVE ASSAY OF FOUR DENTAL VARNISHES
AGAINST *L.ACIDOPHILUS***

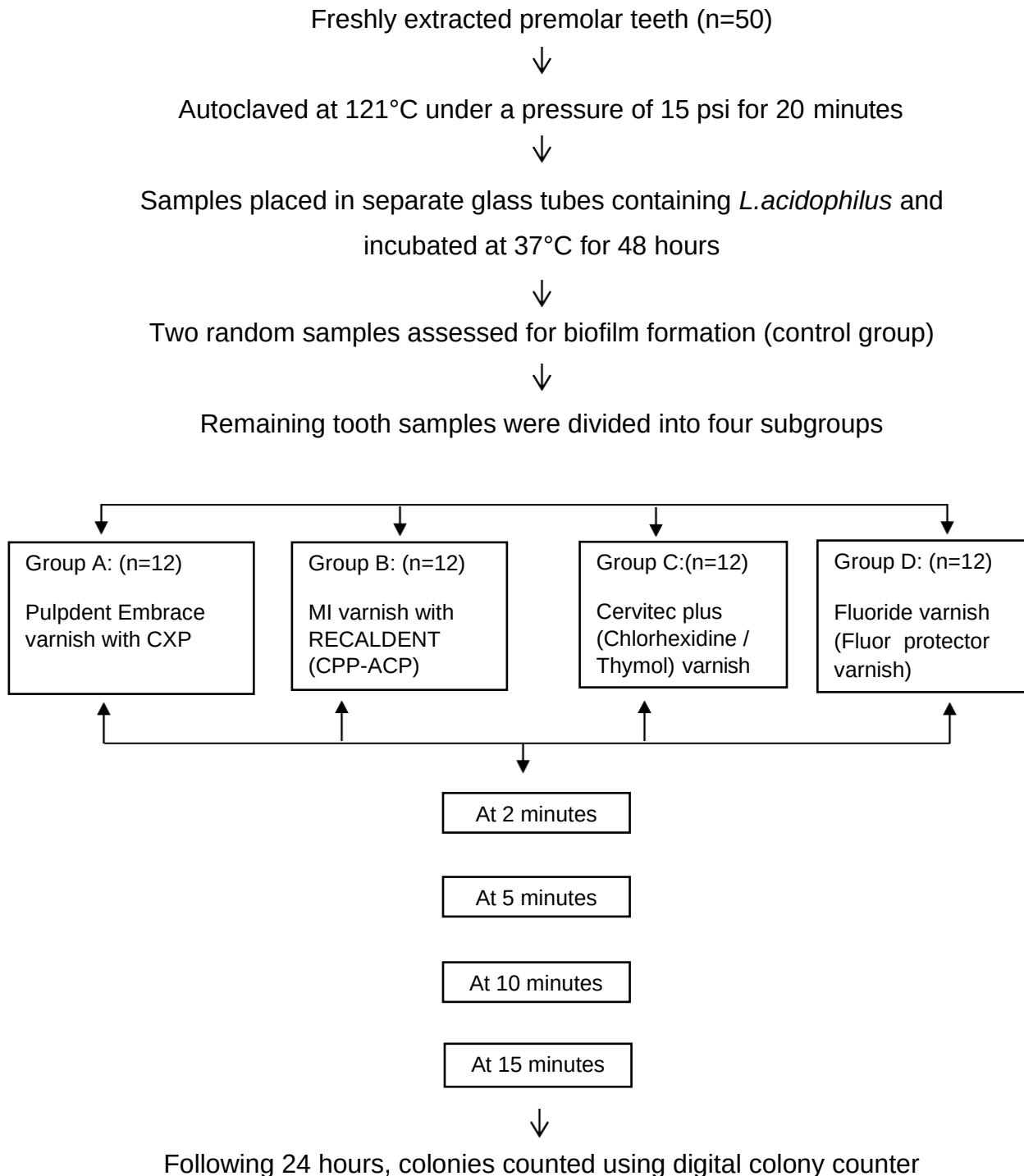




Figure 1: Pulpdent Embrace varnish



Figure 2: MI varnish



Figure 3: Cervitec plus varnish



Figure 4: Fluor protector varnish



Figure 5: Mutans sanguis Agar, Brain Heart Infusion Broth, Lactobacillus MRS Agar, and Lactobacillus MRS Broth



Figure 6 : Streptococcus mutans strain (MTC-497) & Lactobacillus acidophilus strain (MTCC -10307)



Figure 7: Incubator



Figure 8: Digital Colony Counter



Figure 9: Tooth samples



Figure 10 : Broth preparation

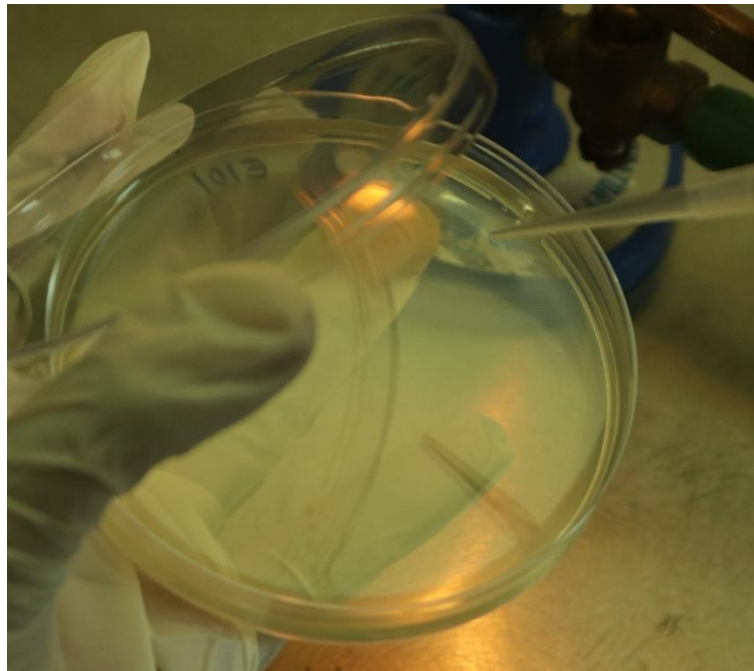


Figure 11: Spot Inoculation medium preparation

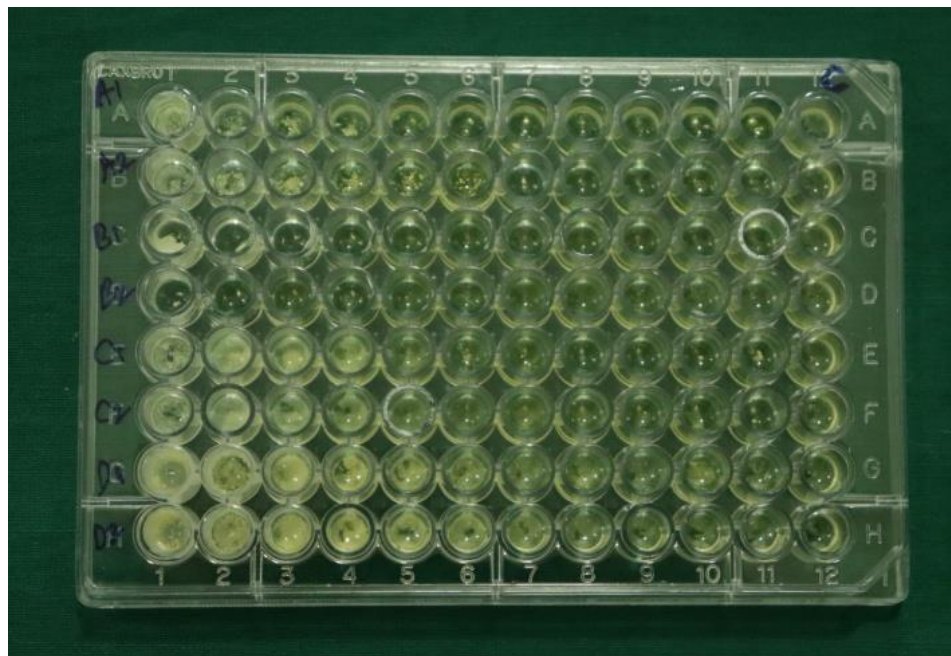


Figure 12: Broth Dilution Assay against *S.mutans* at doubling dilutions in a microtitre plate

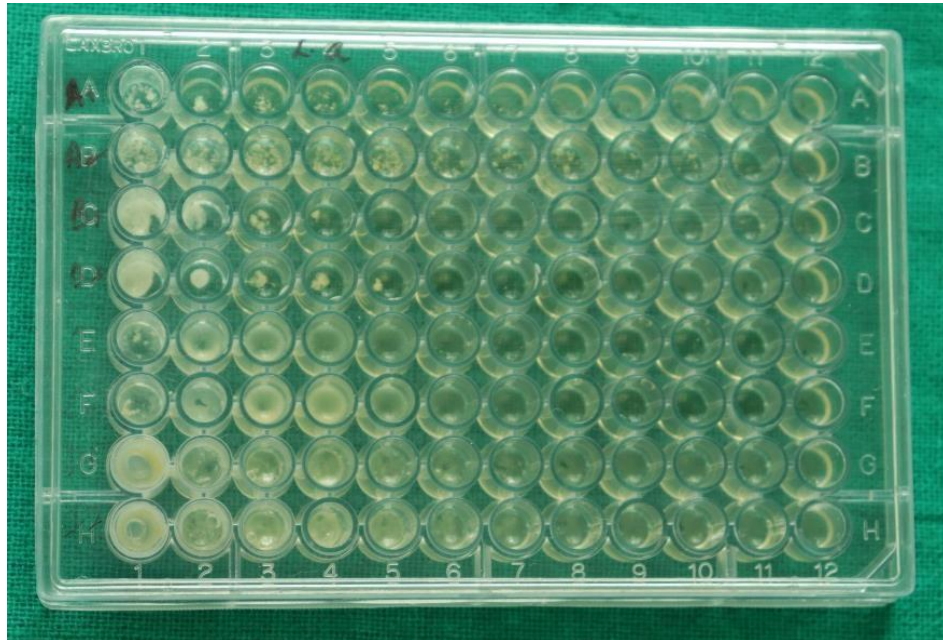


Figure 13: Broth Dilution Assay against *L.acidophilus* at doubling dilutions in a microtitre plate

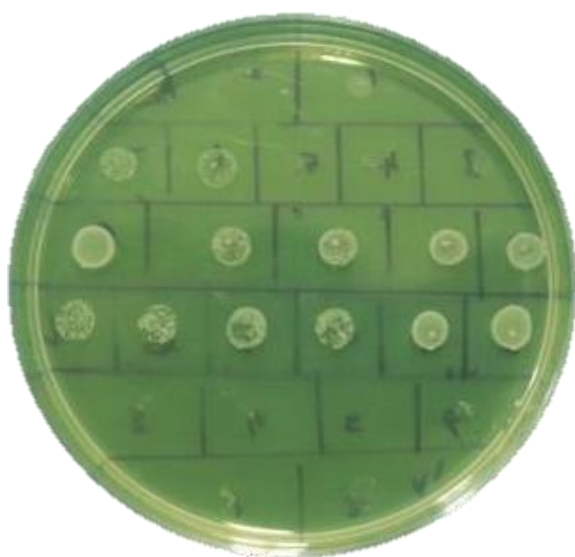


Figure 14: Inhibition of growth of *S.mutans* attained by Pulpdent Embrace varnish

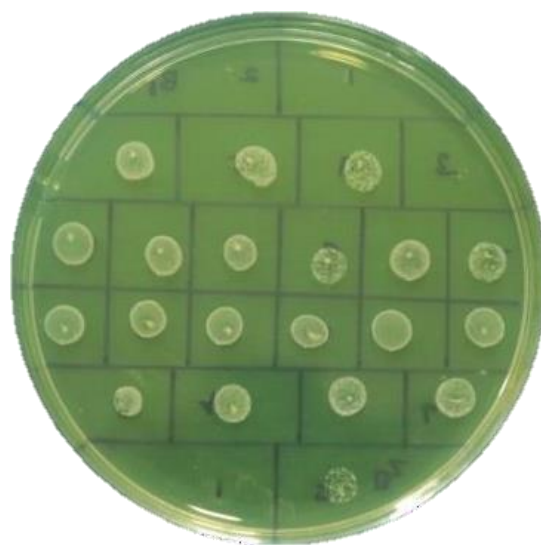


Figure 15: Inhibition of growth of *S.mutans* attained by MI varnish

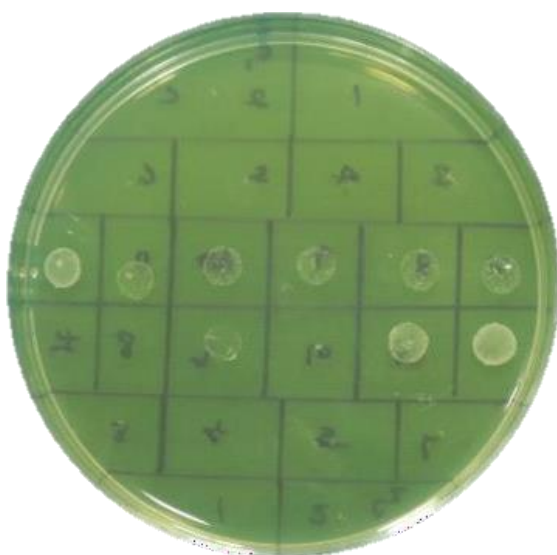


Figure 16: Inhibition of growth of *S.mutans* attained by Cervitec plus varnish

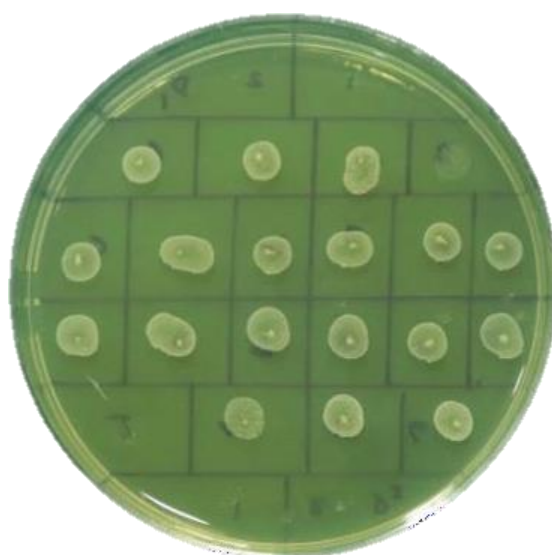


Figure 17: Inhibition of growth of *S.mutans* attained by Fluor protector varnish

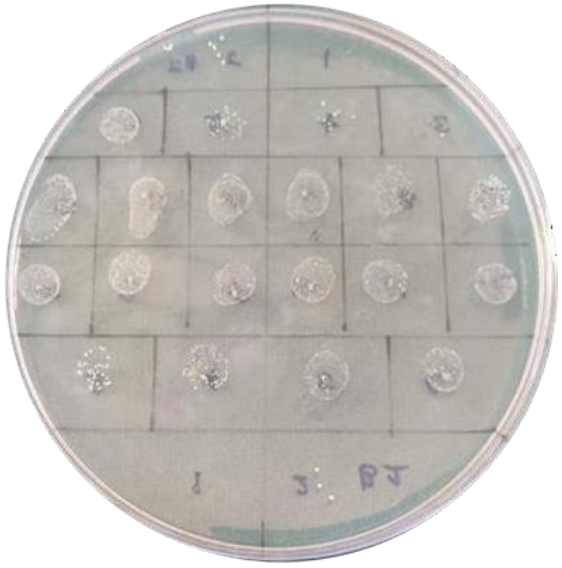


Figure 19: Inhibition of growth of *L.acidophilus* attained by MI varnish

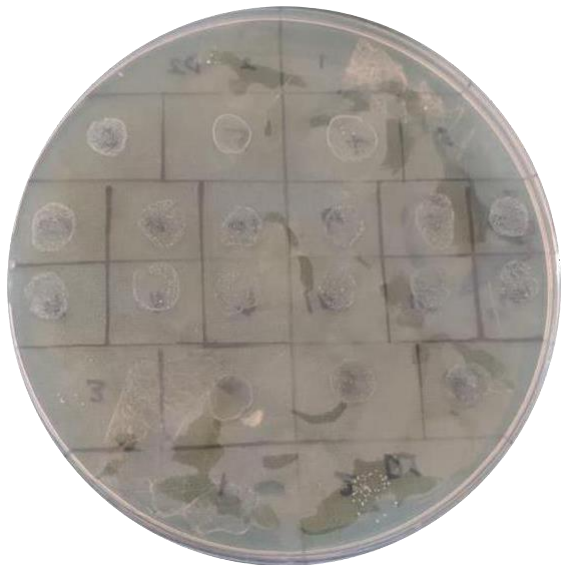


Figure 21: Inhibition of growth of *L.acidophilus* attained by Fluor protector varnish

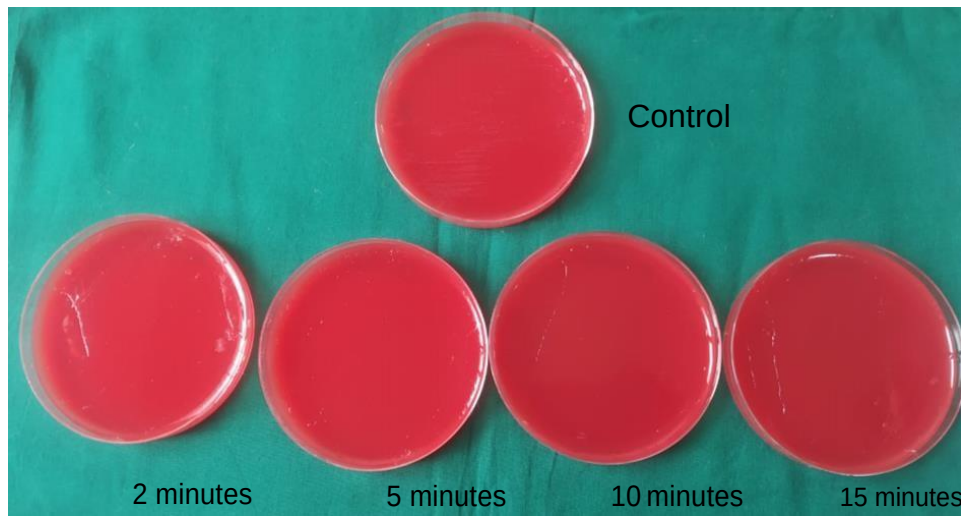


Figure 22: Activity against *S.mutans* for Pulpdent embrace varnish

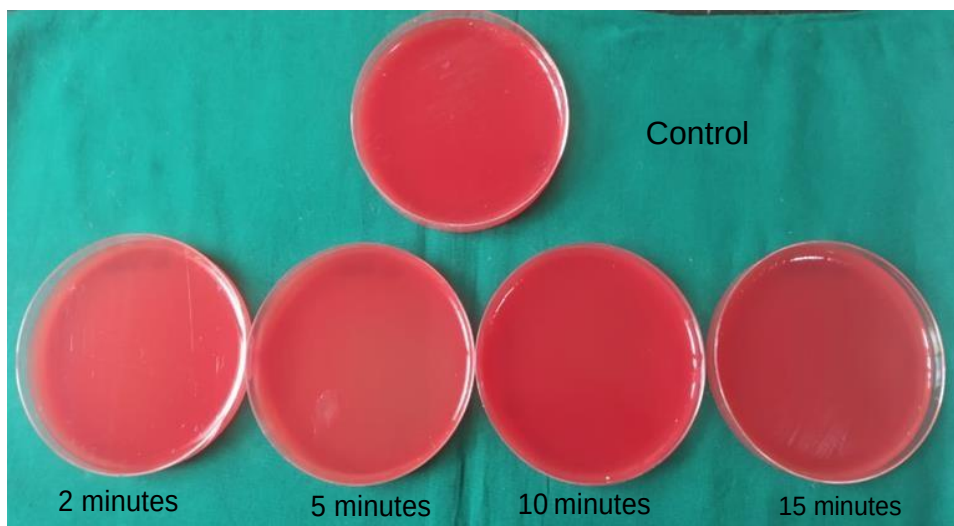


Figure 23: Activity against *S.mutans* for MI varnish

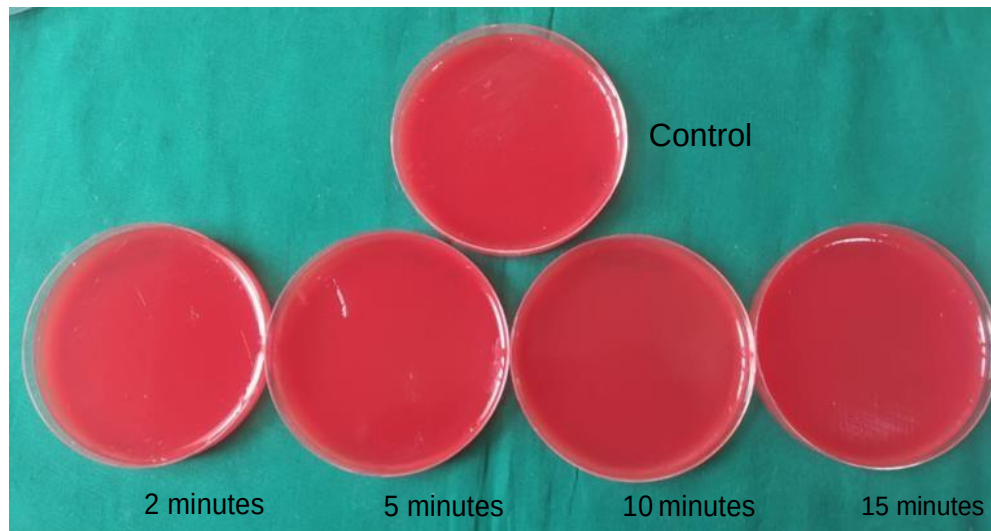


Figure 24: Activity against *S. mutans* for Cervitec plus varnish

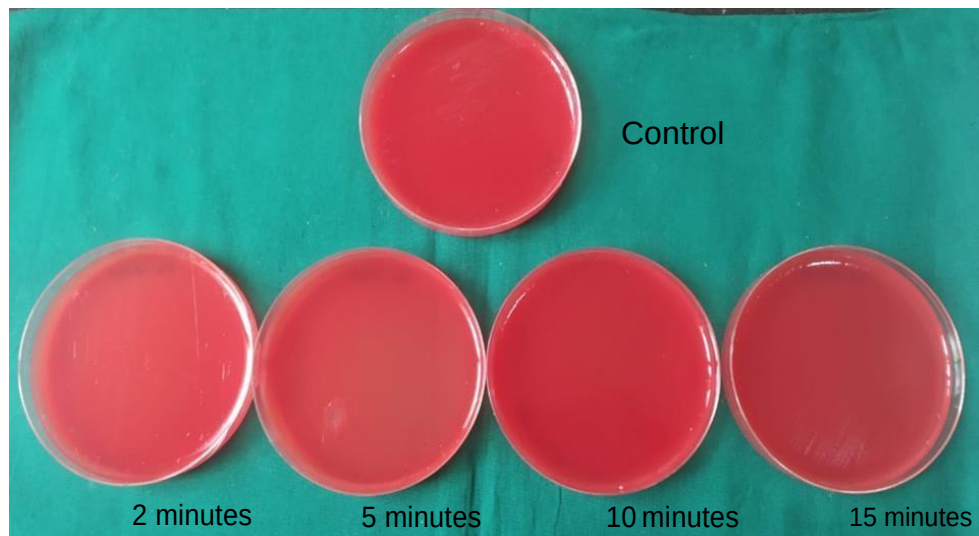


Figure 25: Activity against *S. mutans* for Fluor protector varnish

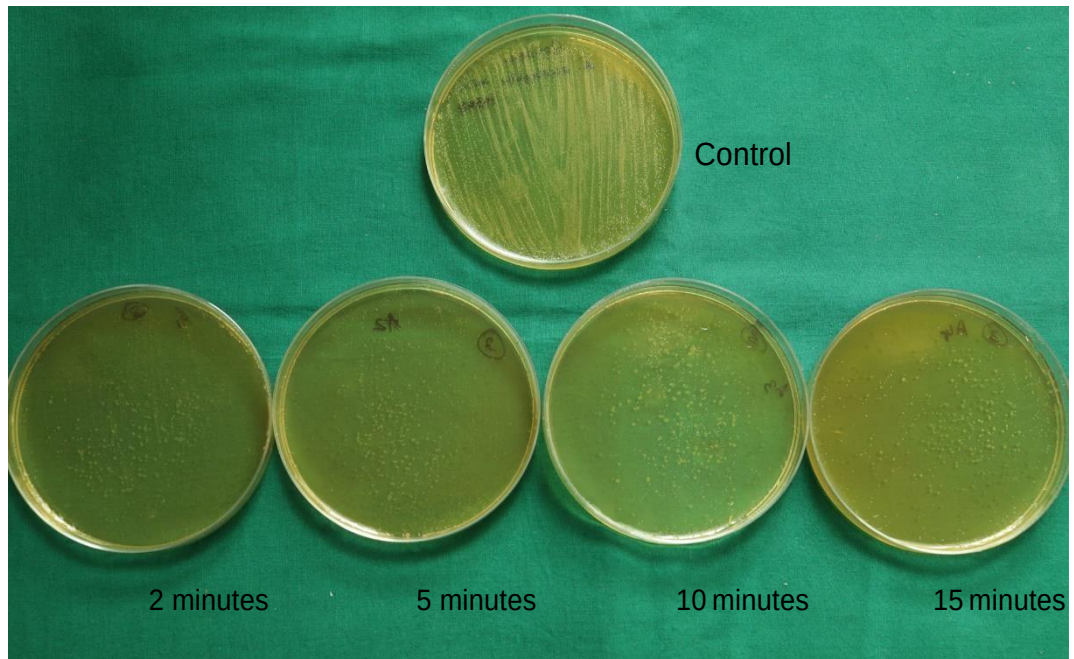


Figure 26: Activity against *L.acidophilus* for Pulpdent embrace varnish

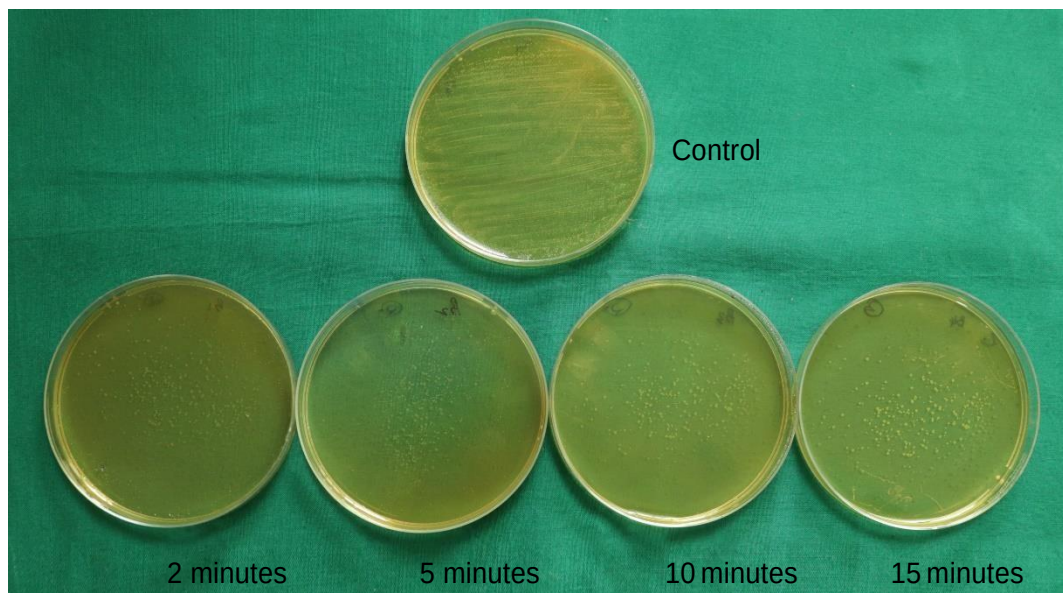


Figure 27: Activity against *L.acidophilus* for MI varnish

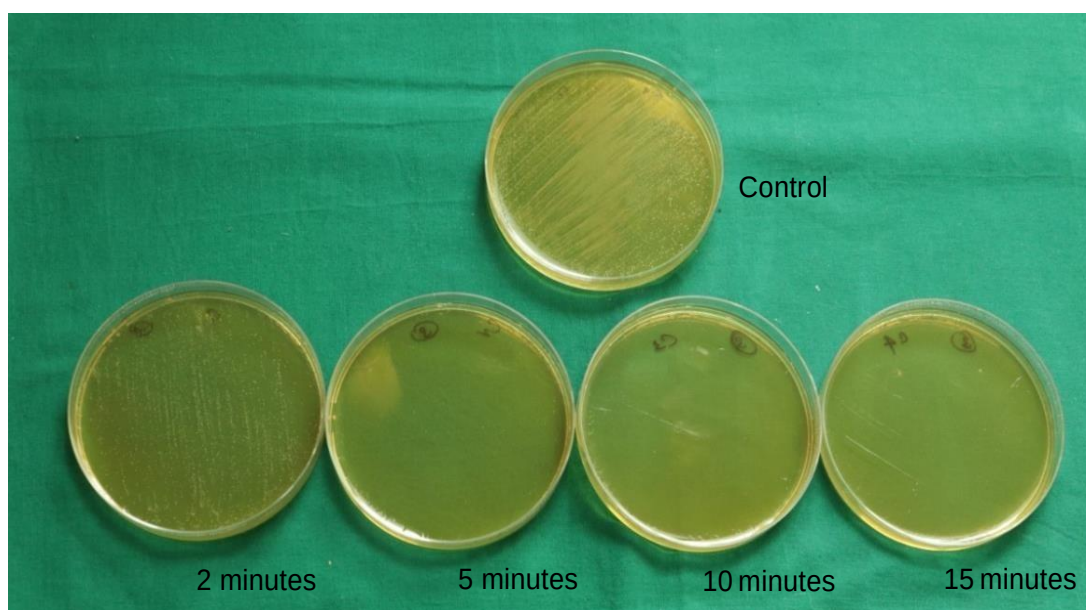


Figure 28: Activity against *L.acidophilus* for Cervitec plus varnish

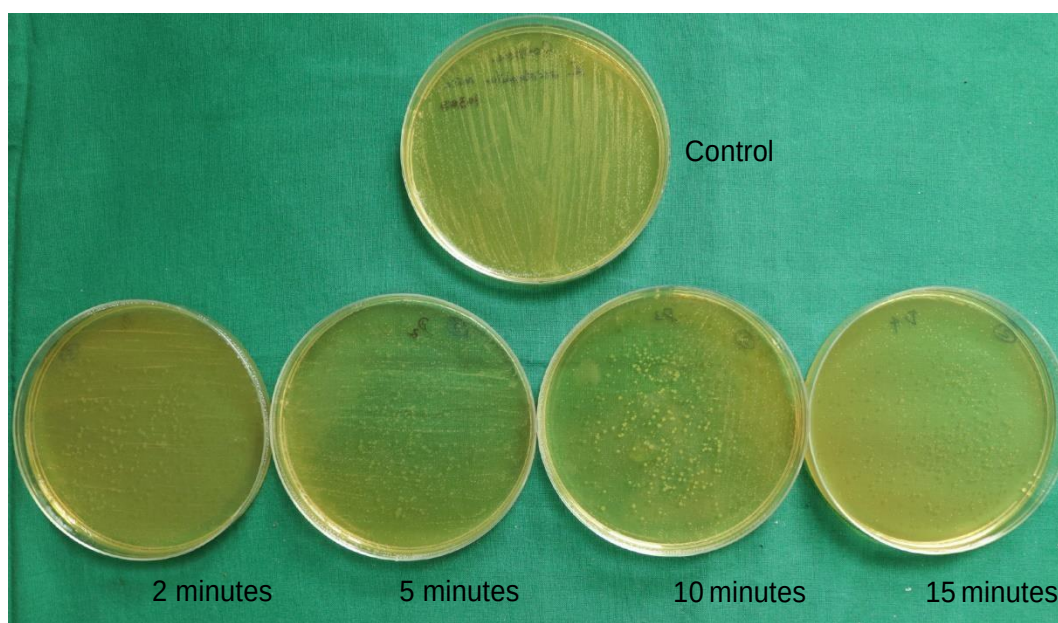


Figure 29: Activity against *L.acidophilus* for Fluor protector varnish

STATISTICAL ANALYSIS

- ❖ The mean difference values of the colony forming units/ml of the four groups were analysed using the **One-way ANOVA** test.
- ❖ Pairwise comparison was done using Post-Hoc test, since one way ANOVA showed statistically significant differences.

Formulae used for analysis:

One Way ANOVA

$$F = \frac{MST}{MSE}$$

Post hoc Tukey-b test

$$\bar{y}_i - \bar{y}_j \pm \frac{Q(1 - \alpha; r, n_T - r)}{\sqrt{2}} s \sqrt{\frac{1}{n_i} + \frac{1}{n_j}}$$

The present in vitro study was conducted to compare and evaluate the antibacterial effect of four different dental varnishes, on the basis of minimum inhibitory concentration and time kill curve analysis for 2, 5, 10 and 15 minutes.

Minimum Inhibitory Concentration

The Broth micro dilution assay was performed to check the efficacy of the varnishes that completely inhibited (~99%) the growth of the colonies on the agar plate. The assay was repeated twice for minimizing lab related errors.

GROUP	50 μL	25 μL	12.5 μL	6.25 μL	3.12 μL	1.56 μL	0.78 μL	0.3 μL	0.19 μL	0.09 μL	0.004 μL	CONTROL
Pulpdent Embrace varnish	N	N	N	N	N	N	N	Y	Y	Y	Y	Y
Pulpdent Embrace varnish	N	N	N	N	N	Y	Y	Y	Y	Y	Y	Y
MI varnish	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y
MI varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Cervitec Plus varnish	N	N	N	N	N	N	N	N	Y	Y	Y	Y
Cervitec Plus varnish	N	N	N	N	N	N	N	N	Y	Y	Y	Y
Fluor Protector varnish	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fluor Protector varnish	N	N	N	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y= Growth of *Streptococcus mutans* colonies

N= No growth of *Streptococcus mutans* colonies

Table 1: Minimum inhibitory concentration of four dental varnishes against *Streptococcus mutans*

Table 1 depicts the inhibition of *Streptococcus mutans* by pulpdent embrace varnish upto 0.78 μL. Highest potential of *S.mutans* inhibition was noted in cervitec plus varnish which showed inhibition at minimal of 0.3 μL concentration. Lowest potential was with MI varnish, which revealed no growth inhibition even at 12.5 μL. Similarly fluor protector varnish showed no growth of colonies till 12.5 μL concentration.

GROUP	50 μL	25 μL	12.5 μL	6.25 μL	3.12 μL	1.56 μL	0.78 μL	0.3 μL	0.19 μL	0.09 μL	0.004 μL	CONTROL
Pulpdent Embrace varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Pulpdent Embrace varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
MI varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
MI varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Cervitec Plus varnish	N	N	N	N	N	N	N	N	Y	Y	Y	Y
Cervitec Plus varnish	N	N	N	N	N	N	N	Y	Y	Y	Y	Y
Fluor Protector varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Fluor Protector varnish	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Y= Growth of *Lactobacillus acidophilus* colonies

N= No growth of *Lactobacillus acidophilus* colonies

Table 2: Minimum inhibitory concentration of four dental varnishes against *Lactobacillus acidophilus*

Table 2 depicts the inhibition of *Lactobacillus acidophilus* by cervitec plus varnish upto 0.3 μL concentration. On the contrary, pulpdent embrace, MI varnish and fluor protector varnish revealed no growth inhibition at 50 μL.

Time Kill Curve Assay against *S.mutans*

Time (minutes)	Control		Pulpdent Embrace varnish		MI varnish		Cervitec Plus varnish		Fluor Protector varnish		One way ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	p-Value
2	2736000.000 ^c	-	2533.33 ^a	2034.15	87333.67 ^b	6566.23	100.000 ^a	100.000	69066.67 ^b	20599.46	<.001
5	2736000.000 ^b	-	1466.67 ^a	1466.67	35100.33 ^a	32450.65	.000 ^a	.000	16000.00 ^a	16000.00	<.001
10	2736000.000 ^b	-	933.33 ^a	933.33	33967.00 ^a	33019.13	.000 ^a	.000	8000.00 ^a	8000.00	<.001
15	2736000.000 ^b	-	133.33 ^a	133.33	66.67 ^a	66.67	.000 ^a	.000	6333.33 ^a	6333.33	<.001

Note: Superscripts a, b, and c are the distinct subgroups formed in the Post-Hoc test

Table 3: Antibacterial efficacy of four different dental varnishes against *Streptococcus mutans* at different time periods.

Table 3 shows the comparison of four dental varnishes at four different time intervals. i.e, 2, 5, 10 and 15 minutes. All the four dental varnishes used in this study revealed statistically significant difference and proved a potential benefit compared to the control group (p value < 0.001). Pairwise comparison was done using Post-Hoc test.

Cervitec plus and pulpdent embrace varnish showed statistically significant inhibitory effect against *S.mutans* compared to MI varnish and Fluor protector varnish at 2 minutes. At 5 minutes, 10 minutes and 15 minutes time period all the four dental varnishes revealed no statistically significant inhibitory effect against *S.mutans*.

Mean plot against *S.mutans*

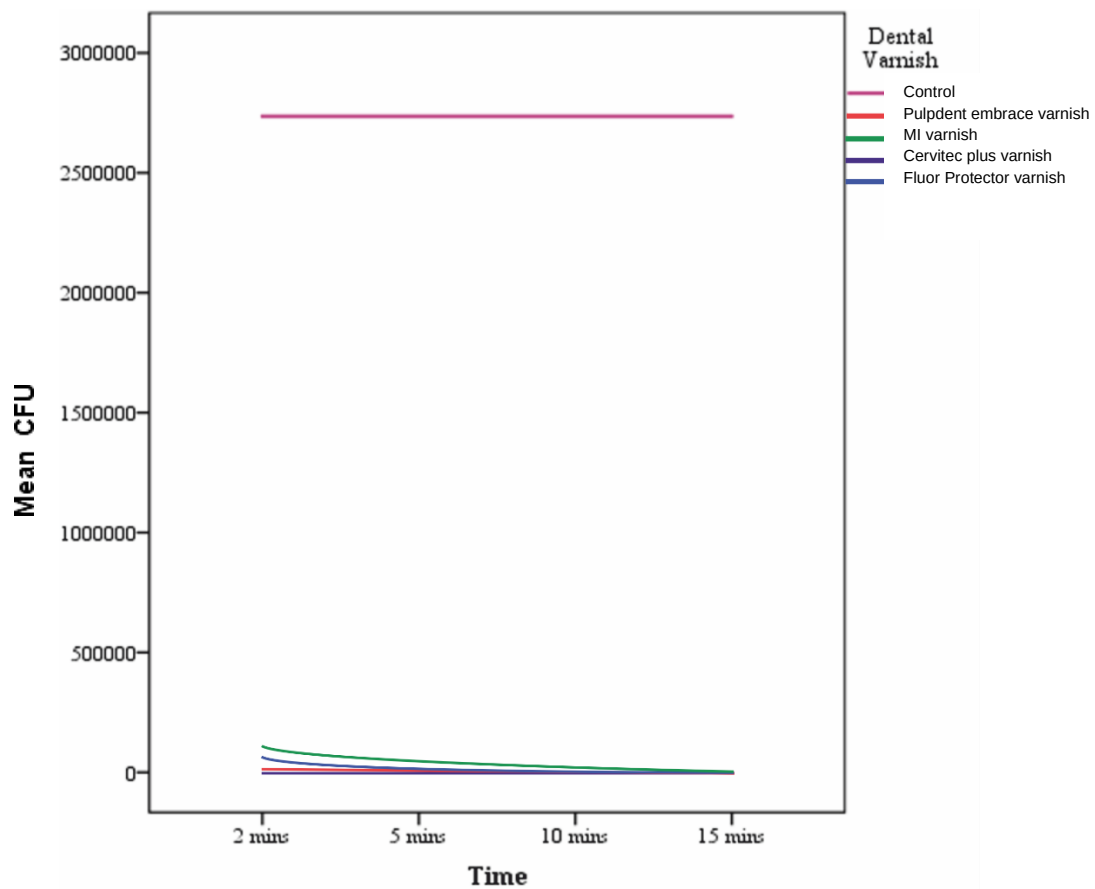


Figure 30: Mean plot revealing time kill curve analysis at different time periods against *S.mutans*

Time kill curve analysis plotted at four different timings did show a significant difference compared to the control group. Cervitec plus varnish showed a conclusive difference compared to the other dental varnishes. Pulpdent embrace varnish also showed higher activity in all the four time periods. Initially at 2 and 5 minutes, MI varnish and Fluor protector varnish had a slow inhibition effect compared to 10 and 15 minutes.

Time Kill curve assay against *Lactobacillus acidophilus*

Time (minutes)	Control		Pulpdent Embrace varnish		MI varnish		Cervitec Plus varnish		Fluor Protector varnish		One way ANOVA
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	P-Value
2	7420000.00 ^c	--	121866.67 ^a	7156.67	70266.67 ^a	15602.28	1233.33 ^a	433.33	659333.33 ^b	243146.96	<.001
5	7420000.00 ^b	--	82266.67 ^a	7156.66	20900.00 ^a	8261.36	.000 ^a	.000	395333.33 ^a	217275.04	<.001
10	7420000.00 ^b	--	48000.00 ^a	2837.84	1566.67 ^a	1516.94	.000 ^a	.000	203400.00 ^a	136600.49	<.001
15	7420000.00 ^b	--	23200.00 ^a	1665.33	66.67 ^a	66.67	.000 ^a	.000	60666.67 ^a	18414.97	<.001

Note: Superscripts a, b, and c are the distinct subgroups formed in the Post-Hoc test

Table 4: Antibacterial efficacy of four dental varnishes against *Lactobacillus acidophilus* at different time periods

Table 4 shows colony forming units of *L. acidophilus*, of control and dental varnishes at different time periods i.e, 2, 5, 10 and 15 minutes. All the four dental varnishes used in this study revealed statistically significant difference and proved a potential benefit compared to the control group (p value < 0.001). Cervitec plus, Pulpdent embrace and MI varnish comparatively showed statistically significant inhibitory effect against *L.acidophilus* compared to fluor protector varnish at 2 minutes. For 5 and 10 minutes time period, all the four dental varnishes showed no significant difference between the groups against *L.acidophilus*. At 15 minutes time period, Cervitec plus, Pulpdent embrace and MI varnish were more effective when compared to fluor protector varnish.

Mean plot against *L.acidophilus*

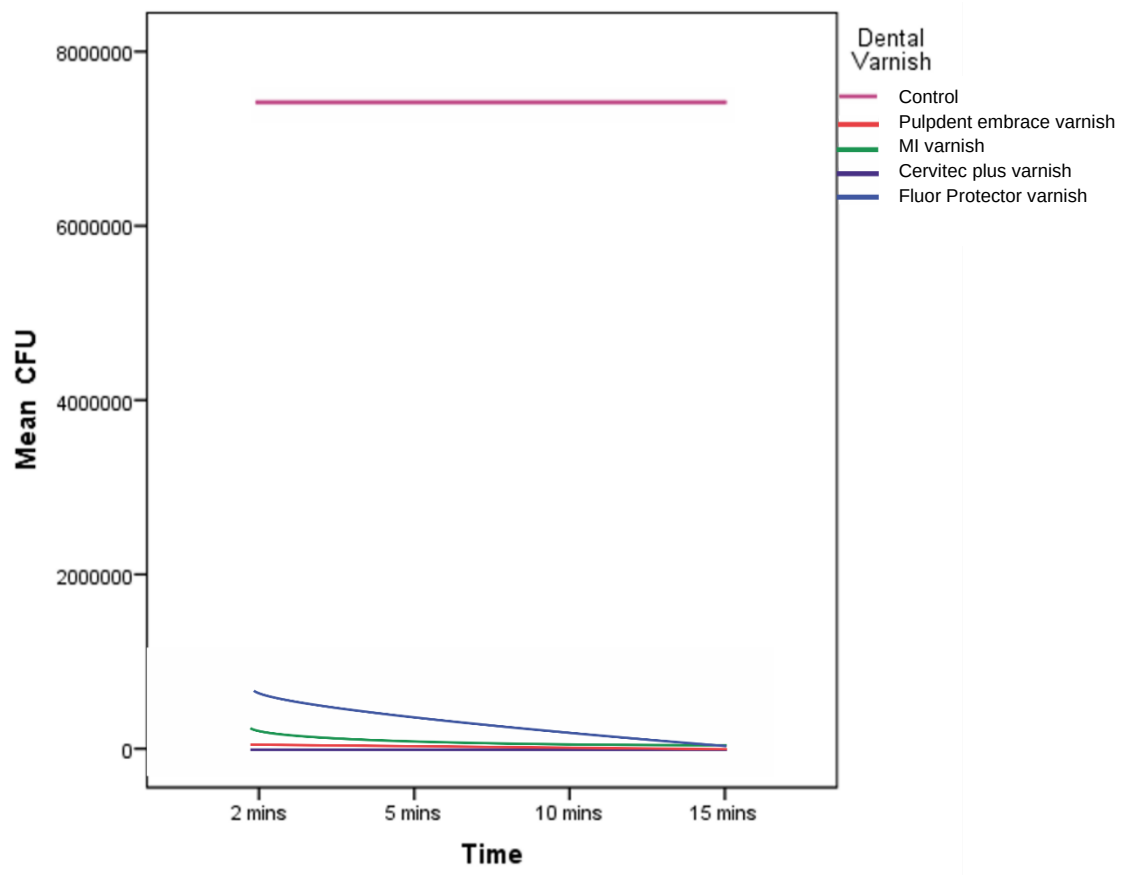


Figure 31: Mean plot revealing time kill curve analysis at different time periods against *L.acidophilus*.

Time kill curve analysis plotted at four different timings did show a significant difference compared to the control group. Cervitec plus, Pulpdent embrace and MI varnish were more effective at all the time periods when compared to fluor protector varnish. Fluor protector varnish showed a slower reduction at 2, 5 and 10 minutes compared to other dental varnishes used in this study.

The global public health issue of childhood dental caries, as well as the population need, has become evident and urgent. Dental caries is one of the most common disorders afflicting people all over the world. To address this issue, several oral health promotion projects have been developed around the world, with a plethora of clinical and cost-effectiveness studies. Antibacterial agents come in a variety of forms on the global market, including dentifrices, mouthwashes, sprays, and gels. Antibacterial compounds, such as varnishes, have been added into sustained-release systems in recent years for improved advantages.

The presence of specific bacteria, particularly *Streptococcus mutans* and other non-streptococcus species such as *Lactobacillus acidophilus*, which produce acid and bring the plaque to the critical pH, was the primary etiologic factor for dental caries. *Streptococcus mutans* and *Lactobacillus acidophilus* species are dominant microorganisms in advanced caries lesions, and these two are regarded as key microorganisms in the pathology of dental caries. *S.mutans* is the organism responsible for the onset of dental caries, whereas *L. acidophilus* is responsible for the progression of dental caries (Jafari et al). This explains why this study included research on the antibacterial efficacy against *Streptococcus mutans* and *Lactobacillus acidophilus*.

Varnishes are easy to apply, safe, well-accepted by patient, not sensitive to moisture and can harden even under saliva. Focus towards

varnish material and its usage is gaining attention from clinicians as well as patients, this is the major reason for selection criteria of this present study. Development in the field of preventive dental materials has introduced many new materials with different formulations. These dental varnish materials have justified the addition of ingredients by overcoming some of the pre-existed limitations. Evidences comparing different formulations especially focusing on the minimum inhibitory concentration and time kill curve assay is lacking in literature. Thus in this study, dental varnish materials with different composition were selected, compared and studied. Each material used in this present study was selected based on the individual material property and variation in its composition, Pulpdent embrace varnish (5% sodium fluoride with xylitol coated calcium phosphate varnish), MI varnish (casein phosphopeptide-amorphous calcium phosphate varnish), Cervitec plus varnish (chlorhexidine varnish) and Fluor protector varnish (fluoride varnish).

In order to prove a material on antibacterial property, some standard tests are being done. Minimum inhibitory concentration and Time kill curve assay were the methods used to study the antibacterial efficacy in this study. The broth micro dilution assay is an effective alternative to the disc diffusion method for determining the antibacterial efficacy of materials that are thixotropic and exhibit insufficient dissolution onto filter paper discs. Along with this the time kill curve assay helps with the time period required for the material to exhibit its maximum and minimum potential.

In this study cervitec plus has proved its potential even in its lowest concentration. The antibacterial effect of each varnish was optimal against both *S.mutans* and *L.acidophilus*. The highest antibacterial intensity was observed in cervitec plus varnish (containing chlorehexidine and thymol), followed by pulpdent embrace varnish (containing xylitol). This is in accordance with Liptak et al 2018, who investigated the effects of cervitec plus on the level of *mutans streptococcus* and *lactobacillus* colonies and showed significant improvement in oral health. Chlorhexidine is known to be an excellent antimicrobial agent, particularly in reducing the levels of *S. mutans*, which is very sensitive to this substance. Various forms of chlorehexidine as mouthrinses and chewing gums have proved its potential against *S.mutans* and *L.acidophilus* which is in accordance with Evans et al (2015) and Paula et al (2010). Minimum inhibitory concentration interprets the potential of antibacterial agent used even at its lowest potential. Among the four dental varnishes studied cervitec varnish showed inhibition of growth of microorganism at its lowest concentration 0.3 µL. The result is in accordance with other studies done by Ogaard (2001), Larsson et al (2004) and cochrane review by Walsh et al (2015).

Results of this present study is in contrast with Plotzitz et al (2005), who claimed mere application of a chlorhexidine-containing varnish does not prevent early childhood caries, but may influence the progression tendency of carious lesions. It is also in contrast with Twetman et al's (2004) study who claimed that the evidence for an anticaries effect of chlorehexidine containing varnishes was rated as

inconclusive for caries active school children and adolescents with daily exposure to fluorides.

The minimum inhibitory concentration against *S.mutans* for pulpdent embrace varnish was 0.78 µL and against *L.acidophilus* was 25 µL. This difference could be due to the difference in the nature of microorganism tested. Pulpdent embrace varnish lacked to prove its potential significantly against *L.acidophilus* compared to *S.mutans*. This is in accordance to an in vitro investigation done by Erdem (2012) and he found that fluor protector + cervitec group had the strongest antibacterial effect, which is significant for delaying bacterial colonisation and biofilm formation. In case of xylitol, according to the literature, xylitol consumption results in favourable outcomes such as a lower incidence of caries and a significant reduction of *Streptococcus mutans* levels in the oral environment due to *Streptococcus mutans* inability to ferment this five-carbon sugar xylitol (Janneson et al 2002).

In the present study, the application of fluoride varnish was not statistically significant from the result obtained with the chlorhexidine varnish. This is in accordance with chiba et al (2019) and Baygin et al (2014). In addition to this, usage of fluoride varnishes have a limited effect on the total salivary levels of *Streptococcus mutans* species (Bapat et al, Yadav et al, and Jafari et al) .Hence recommendations to use in combination with other active agent such as xylitol was done to improve the overall benefit of the varnish.

Shen et al has shown that, in addition to dosage, differences in carriers and the intensity of uniformity affect the maintenance and release behaviour of various fluoride varnishes. They claimed that the fluoride content of different varnishes could vary even between doses taken from the same tube, and that the uneven appearance of the varnish coming out of the tube indicates heterogeneity in the ingredients, resulting in a range of fluoride content. This influences the strength of the antibacterial effect.

Inconclusive result in case of fluor protector could be due to the combinational property of the material remineralising property and antibacterial property. This is explained by Seppa et al (1983) following the application of fluor protector and duraphat monitored parotid saliva peak level. Author highlights on presence of fluoride content and the antibacterial efficacy of the material determine the potential of this material.

MI varnish did not prove a statistically significant difference in the present study. Casein phosphopeptide-amorphous calcium phosphate has shown promising anticariogenic effects among non-fluoride agents for caries prevention. With respect to post-intervention *S.mutans* colony count, few trials significantly favour the use of casein phosphopeptide-amorphous calcium phosphate–topical fluoride combined therapy (Sharda et al 2021). Thus justifies the outcome of present study against both *S.mutans* and *L.acidophilus*. On contrary the fluoride varnish containing CPP–ACP showed the greatest reduction in *S. mutans* count according to

Yadav et al (2019) but this could be due to prior oral prophylactic measures done and does not justify this present study due to limitations mentioned by the author.

Time kill curve assay helps with the time period at which the material is highly potential. This study compared four different dental varnishes against two pathogens, at various time periods. Against both microorganisms cervitec plus showed highest potential even at its lowest concentration. Cervitec plus revealed a sustainable action at all the observed time periods (2, 5, 10 and 15 minutes). Compared to the other dental varnishes used, Cervitec plus varnish required 2 minutes time to exhibit its full potential against *S.mutans*. Pulpdent embrace and MI varnish in 2 minutes exhibited good activity compared to fluor protector varnish. From time kill curve analysis of each material, the time taken by the material to express its maximum potential and substantivity can be interpreted. Though there was no statistically significant difference in 5, 10 and 15 minutes, each material exhibited statistically significant difference compared to the control. Thus analyzing the data, 2 minutes time period showed good antibacterial effect for all the four materials against both microorganisms.

This present study is an addition to literature and an evidence proving cholrehexidine containing dental varnish material to be an effective one against both *S.mutans* and *L.acidophilus*. Pulpdent embrace varnish with its addition of xylitol is a potent dental varnish material, as it showed

inhibition of *S.mutans* and *L.acidophilus*. MI varnish exhibited influence over *S.mutans* and was not significant against *L.acidophilus*. Fluor protector varnish showed no significant effect against both *S.mutans* and *L.acidophilus*.

The conclusions drawn from this in vitro study were as follows,

- Cervitec plus varnish exhibited better antibacterial efficacy when compared to pulpdent embrace varnish, MI varnish and fluor protector varnish against both *Streptococcus mutans* and *Lactobacillus acidophilus*. Pulpdent embrace varnish showed better inhibition compared to MI varnish and fluor protector varnish. Fluor protector varnish showed the least activity against both *S.mutans* & *L.acidophilus*.
- Even at the minimal of 0.3 µL cervitec plus varnish performs its best on controlling the growth of microorganisms, *S.mutans* and *L.acidophilus*. Cervitec plus showed highest potential even at its lowest concentration.
- Cervitec plus varnish revealed a sustainable action at all the observed time periods (2, 5, 10 and 15 minutes) compared to pulpdent embrace varnish, MI varnish, fluor protector varnish. Time kill curve analysis revealed similar results against both *S.mutans* and *L.acidophilus*.

Chlorehexidine based dental varnish material has its major antibacterial efficacy compared to other materials. On both minimum inhibitory concentration and time kill curve assay, cervitec plus varnish worked better when compared to pulpdent embrace, MI varnish and Fluor protector against *Streptococcus mutans* and *Lactobacillus acidophilus*.

The limitations of this study includes not considering the interaction with other pathogenic bacteria in the oral environment. This study focused solely on *Streptococcus mutans* and *Lactobacillus acidophilus*. Time kill curve analysis could have been performed for longer time periods also. Depth penetration of each dental varnish could have been analysed to improve this study. In addition, intended concentrations of varnishes were selected for measuring their antibacterial effects and other environmental factors associated were not included in this study. Simulating the oral cavity biofilm study models could have been used to analyse the antibiofilm property of these dental varnishes.

Inorder to overcome these limitations evaluation of antibacterial efficacy for a longer time period should be studied. Furthermore investigation should be done using different biofilm models to substantiate the results against various oral pathogens simulating the salivary medium. In addition to these properties depth of penetration is an important feature for any dental varnish, evidences based on depth of penetration of each dental varnish could have been added.

The present in vitro study was carried out in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital. The study intended to compare and evaluate the antibacterial efficacy of four different dental varnishes and also to evaluate the potential of each material based on time kill curve analysis. Study included commercially available dental varnishes with varied active ingredients in addition, namely

- ❖ Pulpdent Embrace™ varnish
- ❖ MI varnish
- ❖ Cervitec plus varnish
- ❖ Fluor protector varnish

Minimum inhibitory concentration

Broth micro-dilution assay was performed. Overnight culture of *Streptococcus mutans* was inoculated in 0.4 ml of brain heart infusion broth and incubated at 37°C for 1 hour. Serial dilutions of all the dental varnishes were prepared and *Streptococcus mutans* suspension was added to all the wells including the culture control. The plates were incubated overnight at 37°C. The minimum inhibitory concentration was recorded as the lowest concentration of the test solution which did not permit the growth of *Streptococcus mutans*. Similarly *L.acidophilus* was prepared on Lactobacillus MRS broth and respective Lactobacillus MRS

agar medium. The minimum inhibitory concentration was recorded for *L.acidophilus*.

Time Kill curve Analysis

The method used to determine the bactericidal potential of antimicrobial agents helps us to analyse the time period required for the full potential of the material. Activity of each dental varnish was studied at 2, 5, 10 and 15 minutes. 10 µl of brain heart infusion broth and lactobacillus MRS broth was inoculated into sterile mutans sanguis agar plate and sterile MRS agar respectively. The plate was then incubated at 37°C for 24 hours. After 24 hours, the colonies were counted using digital colony counter to determine the colony forming units/ml.

Statistical analysis

The values obtained were subjected to statistical analysis using the ANOVA test for intergroup comparison. Pairwise comparison was done using Post Hoc test to check the superiority of the efficacy of one material over the other.

Results

Cervitec plus varnish is superior based on antibacterial efficacy in both minimum inhibitory concentration and time kill curve assay, when compared to Pulpdent embrace, MI varnish and Fluor protector varnish against *Streptococcus mutans* and *Lactobacillus acidophilus*. Cervitec plus revealed a sustainable action at all the observed time periods (2, 5, 10 and

15 minutes). Even at the minimal of 0.3 μ L, cervitec plus varnish performs its best on controlling the growth of microorganisms.

Conclusion

Based on the results of this study, it has been concluded that cervitec plus varnish exhibited better antibacterial efficacy. Minimum inhibitory concentration of cervitec plus was higher and showed inhibition of growth of microorganism. In the observed time period, cervitec plus significantly inhibited growth of bacteria when compared to the other dental varnishes used. Time kill curve analysis against both *S.mutans* and *L.acidophilus* was effective with cervitec plus varnish. Based on the results, cervitec plus varnish helps with prevention of dental caries and can be used in primary prevention.

1. Marsh PD, Martin MV. Oral microbiology. 5th edition. Edinburgh (UK): Churchill Livingstone; 2009
2. Lemos, J. A., Palmer, S. R., Zeng, L., Wen, Z. T., Kajfasz, J. K., Freires, I. A., Abranches, J., & Brady, L. J. (2019). The Biology of *Streptococcus mutans*. *Microbiology spectrum*, 7(1), 10.1128/microbiolspec.GPP3-0051-2018.
<https://doi.org/10.1128/microbiolspec.GPP3-0051-2018>
3. Soleimani B, Goli H, Naranjian M, Mousavi S J, Nahvi A. Comparison of Antimicrobial Activity of Fluoride Varnishes Against *Streptococcus mutans* and *Lactobacillus acidophilus*: An In Vitro Study. *Iran J Pediatr*. 2021;31(3):e111422. doi: 10.5812/ijp.111422.
4. Becker MR, Paster BJ, Leys EJ, et al. Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* 2002;40(3):1001–9
5. Khalid GS, Hamrah MH, Ghafary ES, Hosseini S, Almasi F. Antibacterial and Antimicrobial Effects of Xanthorrhizol in the Prevention of Dental Caries: A Systematic Review. *Drug Des Devel Ther*. 2021;15:1149-1156. Published 2021 Mar 11. doi:10.2147/DDDT.S290021
6. Baik A, Alamoudi N, El-Housseiny A, Altuwirqi A. Fluoride Varnishes for Preventing Occlusal Dental Caries: A Review. *Dentistry Journal*. 2021;9(6).

7. Kohn, W.G.; Maas, W.R.; Malvitz, D.M.; Presson, S.M.; Shaddix, K.K. Recommendations for Using Fluoride to Prevent and Control Dental Caries in the United States. 2021.
8. Mahdi Shahmoradi, Neil Hunter, Michael Swain, "Efficacy of Fluoride Varnishes with Added Calcium Phosphate in the Protection of the Structural and Mechanical Properties of Enamel", *BioMed Research International*, vol. 2017, Article ID 7834905, 7 pages, 2017. <https://doi.org/10.1155/2017/7834905>
9. Nahum, S., Rogelio José, S., Edith, L., Hugo, T., Ulises, V., Alejandra, M. Fluoride Release and Recharge in Conventional Varnishes, Compared to a Giomer and a Resin Modified Glass Ionomer. *Journal of Dental Materials and Techniques*, 2021; 10(2): 62-70. doi: 10.22038/jdmt.2021.44814.1331.
10. Cardoso, C. A., Cassiano, L. P., Costa, E. N., Souza-E-Silva, C. M., Magalhães, A. C., Grizzo, L. T., Caldana, M. L., Bastos, J. R., & Buzalaf, M. A. (2016). Effect of xylitol varnishes on remineralization of artificial enamel caries lesions in situ. *Journal of dentistry*, 50, 74–78. <https://doi.org/10.1016/j.jdent.2016.03.011>
11. Weiger, R., Friedrich, C., Netuschil, L., & Schlagenhauf, U. (1994). Effect of chlorhexidine-containing varnish (Cervitec) on microbial vitality and accumulation of supragingival dental plaque in humans. *Caries research*, 28(4), 267–271. <https://doi.org/10.1159/000261984>.
12. Kooshki, F., Pajoohan, S., & Kamareh, S. (2019). Effects of treatment with three types of varnish remineralizing agents on the microhardness of demineralized enamel surface. *Journal of clinical*

- and experimental dentistry*, 11(7), e630–e635. <https://doi.org/10.4317/jced.55611>
13. Bugno, Adriana & Nicoletti, M. & Almodóvar, Adriana & Caldas Pereira, Tatiana & Auricchio, Mariângela. (2007). Antimicrobial efficacy of Curcuma zedoaria extract as assessed by linear regression compared with commercial mouthrinses. *Brazilian Journal of Microbiology - BRAZ J MICROBIOL.* 38. 10.1590/S1517-83822007000300011.
 14. Rodriques FE: Quantitative incidence of *Lactobacillus acidophilus* in the oral cavity as a presumptive index of susceptibility to dental caries. *J Am Dent Assoc* 1931;18:2118–2135.
 15. Loesche W. J. (1976). Chemotherapy of dental plaque infections. *Oral Sci. Rev.* 9, 65–107
 16. Scheinin A, Mäkinen KK, Ylitalo K. Turku sugar studies. V. Final report on the effect of sucrose, fructose and xylitol diets on the caries incidence in man. *Acta Odontol Scand.* 1976;34(4):179-216. doi: 10.3109/00016357608997711. PMID: 795260.
 17. Balanyk TE, Sandham HJ. Development of sustained-release antimicrobial dental varnishes effective against *Streptococcus mutans* in vitro. *Journal of dental research.* 1985 Dec;64(12):1356-60.
 18. Petersson LG, Maki Y, Twetman S, Edwardsson S. *Mutans streptococci* in saliva and interdental spaces after topical applications of an antibacterial varnish in schoolchildren. *Oral microbiology and immunology.* 1991 Oct;6(5):284-7.

19. Marsh PD, Martin M: Oral Microbiology, ed 3. London, Chapman & Hall, 1992.
20. Marsh P. D. (1994). Microbial ecology of dental plaque and its significance in health and disease. *Adv. Dent. Res.* 8, 263–271
21. Van Houte, J. (1994) 'Microbiological Predictors of Caries Risk', *Advances in Dental Research*, 7(2), pp. 87–96. doi: 10.1177/08959374930070022001.
22. Bratthall D, Serinirach R, Rapisuwon S, Kuratana M, Luangjarmekorn V, Luksila K, Chaipanich P: A study into the prevention of fissure caries using an antimicrobial varnish. *Int Dent J* 1995;45:245–254
23. Ogaard B, Larsson E, Glans R, Henriksson T, Birkhed D. Antimicrobial effect of a chlorhexidine-thymol varnish (Cervitec) in orthodontic patients. A prospective, randomized clinical trial. *Journal of orofacial orthopedics= Fortschritte der Kieferorthopadie: Organ/official journal Deutsche Gesellschaft fur Kieferorthopadie.* 1997 Jan 1;58(4):206-13.
24. Reynolds EC (1997). Remineralization of enamel subsurface lesions by casein phosphopeptide-stabilized calcium phosphate solutions. *J Dent Res* 76:1587-1595.
25. Bradshaw DJ, Marsh PD. Analysis of pH-driven disruption of oral microbial communities in vitro. *Caries Res.* 1998;32(6):456-62. doi: 10.1159/000016487. PMID: 9745120.
26. Araujo AM, Naspitz GM, Chelotti A, Cai S. Effect of Cervitec® on mutans streptococci in plaque and on caries formation on occlusal

- fissures of erupting permanent molars. Caries research. 2002;36(5):373-6.
27. Fejerskov O. Changing paradigms in concepts on dental caries: consequences for oral health care. Caries Res. 2003 Jun;38(3):182–91.
28. Larsen MJ, Pearce EI. Saturation of human saliva with respect to calcium salts. Archives of oral biology. 2003 Apr 1;48(4):317-22.
29. Marsh P. D. (2003). Are dental diseases examples of ecological catastrophes? *Microbiology* 149, 279–294 10.1099/mic.0.26082-0
30. Sköld-Larsson K, Fornell AC, Lussi A, Twetman S. Effect of topical applications of a chlorhexidine/thymol-containing varnish on fissure caries assessed by laser fluorescence. Acta Odontologica Scandinavica. 2004 Jan 1;62(6):339-42.
31. Twetman S. Antimicrobials in future caries control?. Caries research. 2004;38(3):223-9.
32. Cross KJ, Huq NL, Palamara JE, Perich JW, Reynolds EC (2005). Physicochemical characterization of casein phosphopeptide amorphous calcium phosphate nanocomplexes. J Biol Chem 280:15362-15369
33. Plotzitz B, Kneist S, Berger J, Hetzer G. Efficacy of chlorhexidine varnish applications in the prevention of early childhood caries. European journal of paediatric dentistry. 2005 Mar;6(3):149.
34. Beltran-Aguilar ED, Goldstein JW, Lockwood SA. Fluoride varnishes: a review of their clinical use, cariostatic mechanism, efficacy and safety. JADA. 2000; 131(5):589–596. [PubMed: 10832252]

35. Andersson A, Sköld-Larsson K, Haligren A, Petersson LG, Twetman S, Hallgren A. Effect of a dental cream containing amorphous cream phosphate complexes on white spot lesion regression assessed by laser fluorescence. *Oral health & preventive dentistry*. 2007 Jun 1;5(3).
36. Jeevarathan, J.; Deepti, A.; Muthu, M.S.; Rathna Prabhu, V.; Chamundeeswari, G.S. Effect of fluoride varnish on *Streptococcus mutans* counts in plaque of caries-free children using Dentocult SM strip mutans test: A randomized controlled triple blind study. *J. Indian Soc. Pedod. Prev. Dent*. 2007, 25, 157–163
37. Ribeiro LG, Hashizume LN, Maltz M. The effect of different formulations of chlorhexidine in reducing levels of mutans streptococci in the oral cavity: a systematic review of the literature. 2007. In: *Database of Abstracts of Reviews of Effects (DARE): Quality-assessed Reviews [Internet]*. York (UK): Centre for Reviews and Dissemination (UK); 1995-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK74500>.
38. Autio-Gold J. The role of chlorhexidine in caries prevention. *Operative dentistry*. 2008 Nov;33(6):710-6.
39. Morgan MV, Adams GG, Bailey DL, Tsao CE, Fischman SL, Reynolds EC. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. *Caries Research*. 2008;42(3):171-84.

40. Reynolds EC, Cai F, Cochrane NJ, Shen P, Walker GD, Morgan MV, Reynolds C. Fluoride and casein phosphopeptide-amorphous calcium phosphate. *Journal of Dental Research*. 2008 Apr;87(4):344-8.
41. Fejerskov O, Kidd E. *Dental Caries: The Disease and Its Clinical Management*. John Wiley & Sons; 2009. 641 p.
42. Llena C, Forner L, Baca P. Anticariogenicity of Casein Phosphopeptide-amorphous Calcium Phosphate: A Review of the Literature. *J Contemp Dent Pract* 2009 May; (10)3:001-009.
43. Poulsen S. Fluoride-containing gels, mouth rinses and varnishes: an update of evidence of efficacy. *European Archives of Paediatric Dentistry*. 2009 Sep 1;10(3):157-61.
44. Soderling EM. Xylitol, mutans streptococci, and dental plaque. *Adv Dent Res*. 2009;21(1):74-8. doi: 10.1177/0895937409335642. PMID: 19717413.
45. Paula VA, Modesto A, Santos KR, Gleiser R. Antimicrobial effects of the combination of chlorhexidine and xylitol. *British dental journal*. 2010 Dec;209(12):E19-.
46. Buzalaf, M.A.R.; Pessan, J.P.; Honório, H.M.; Ten Cate, J.M. Mechanisms of action of fluoride for caries control. In *Fluoride and the Oral Environment*; Karger Publishers: Berlin, Germany, 2011; pp. 97–114
47. Hajishengallis G., Darveau R. P., Curtis M. A. (2012). The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10, 717–725 10.1038/nrmicro2873

48. García-Godoy FR, Kao LM, Flaitz CM, Hicks J. Fluoride dentifrice containing xylitol: in vitro root caries formation. *Am J Dent*. 2013 Feb 1;26:56-60.
49. Marinho V, Worthington H, Walsh T, Clarkson J. Fluoride varnishes for preventing dental caries in children and adolescents (Review). *Cochrane Database Syst Rev*. 2013;Issue 7:Art. No.: CD002279
50. Agouropoulos A, Twetman S, Pandis N, Kavvadia K, Papagiannoulis L. Caries-preventive effectiveness of fluoride varnish as adjunct to oral health promotion and supervised tooth brushing in preschool children: a double-blind randomized controlled trial. *Journal of dentistry*. 2014 Oct 1;42(10):1277-83.
51. Carey CM. Focus on fluorides: update on the use of fluoride for the prevention of dental caries. *Journal of Evidence Based Dental Practice*. 2014 Jun 1;14:95-102.
52. Grychtol S, Basche S, Hannig M, Hannig C. Effect of CPP/ACP on initial bioadhesion to enamel and dentin in situ. *The Scientific World Journal*. 2014 Jan 1;2014.
53. Milgrom P, Taves DM, Kim AS, Watson GE, Horst JA. Pharmacokinetics of fluoride in toddlers after application of 5% sodium fluoride dental varnish. *Pediatrics*. 2014 Sep 1;134(3):e870-4.
54. Llena C, Leyda AM, Forner L. CPP-ACP and CPP-ACFP versus fluoride varnish in remineralisation of early caries lesions. A prospective study. *Eur J Paediatr Dent*. 2015 Sep 1;16(3):181-6.

55. Marsh PD. The commensal microbiota and the development of human disease - an introduction. *J Oral Microbiol.* 2015 Sep;7(1):29128
56. Milburn JL, Henrichs LE, Banfield RL, Stansell MJ, Vandewalle KS. Substantive fluoride release from a new fluoride varnish containing CXP. *Dentistry.* 2015;5(12):1-6.
57. Walsh T, Oliveira-Neto JM, Moore D. Chlorhexidine treatment for the prevention of dental caries in children and adolescents. *Cochrane Database of Systematic Reviews.* 2015(4).
58. Chokshi K, Chokshi A, Konde S, Shetty SR, Chandra KN, Jana S, Mhambrey S, Thakur S. An in vitro comparative evaluation of three remineralizing agents using confocal microscopy. *Journal of clinical and diagnostic research: JCDR.* 2016 Jun;10(6):ZC39.
59. Gao SS, Zhang S, Mei ML, Lo EC, Chu CH. Caries remineralisation and arresting effect in children by professionally applied fluoride treatment—a systematic review. *BMC oral health.* 2016 Dec;16(1):1-9.
60. Lenzi TL, Montagner AF, Soares FZ, de Oliveira Rocha R. Are topical fluorides effective for treating incipient carious lesions?: A systematic review and meta-analysis. *The Journal of the American Dental Association.* 2016 Feb 1;147(2):84-91.
61. Anderson M, Dahllöf G, Soares FC, Grindefjord M. Impact of biannual treatment with fluoride varnish on tooth-surface-level caries progression in children aged 1–3 years. *Journal of dentistry.* 2017 Oct 1;65:83-8.

62. Chukwuma CI, Islam MS. Xylitol: One Name, Numerous Benefits 19. Sweeteners. 2017;409.
63. Hajishengallis G, Reis ES, Mastellos DC, Ricklin D, Lambris JD. Novel mechanisms and functions of complement. Nat Immunol. 2017 Nov 16;18(12):1288-1298. doi: 10.1038/ni.3858. PMID: 29144501; PMCID: PMC5706779.
64. Mishra, P.; Fareed, N.; Battur, H.; Khanagar, S.; Bhat, M.A.; Palaniswamy, J. Role of fluoride varnish in preventing early childhood caries: A systematic review. Dent. Res. J. 2017, 14, 169–176.
65. Shahmoradi M, Hunter N, Swain M. Efficacy of fluoride varnishes with added calcium phosphate in the protection of the structural and mechanical properties of enamel. BioMed research international. 2017 Dec 7;2017.
66. Bowen WH, Burne RA, Wu H, Koo H. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. Trends Microbiol. 2018;26:229–42
67. Jafari K, Hekmatfar S, Fereydunzadeh M. In vitro comparison of antimicrobial activity of conventional fluoride varnishes containing xylitol and casein phosphopeptide-amorphous calcium phosphate. Journal of International Society of Preventive & Community Dentistry. 2018 Jul;8(4):309.
68. Lipták L, Szabó K, Nagy G, Márton S, Madléna M. Microbiological changes and caries-preventive effect of an innovative varnish containing chlorhexidine in orthodontic patients. Caries research. 2018;52(4):272-8.

69. Philip N, Leishman SJ, Bandara HM, Walsh LJ. Casein phosphopeptide-amorphous calcium phosphate attenuates virulence and modulates microbial ecology of saliva-derived polymicrobial biofilms. *Caries research*. 2019;53(6):643-9.
70. Madrid-Troconis CC, Perez-Puello SD. Casein phosphopeptide-amorphous calcium phosphate nanocomplex (CPP-ACP) in dentistry: state of the art. *Revista Facultad de Odontología Universidad de Antioquia*. 2019 Jun;30(2):248-62.
71. Urquhart O, Tampi MP, Pilcher L, Slayton RL, Araujo MWB, Fontana M, et al. Nonrestorative Treatments for Caries: Systematic Review and Network Meta-analysis. *J Dent Res* 2019; 98(1): 14–26.
72. Yadav S, Sachdev V, Malik M, Chopra R. Effect of three different compositions of topical fluoride varnishes with and without prior oral prophylaxis on *Streptococcus mutans* count in biofilm samples of children aged 2–8 years: A randomized controlled trial. *Journal of Indian Society of Pedodontics and Preventive Dentistry*. 2019 Jul 1;37(3):286.
73. Li F, Jiang P, Yu F, Li C, Wu S, Zou J, Xu X, Ye L, Zhou X, Zheng L. Comparison between fissure sealant and fluoride varnish on caries prevention for first permanent molars: a systematic review and meta-analysis. *Scientific reports*. 2020 Feb 13;10(1):1-9.
74. Elhangour SZ, Bakry NS, Soliman R, Nagui DA. Effect of fluoride varnish containing xylitol-coated calcium and phosphate on the remineralization of caries like lesions in primary teeth. *Alexandria Dental Journal*. 2021 Dec 1;46(3.3):181-7.

75. Ivančaková RK, Radochová V, Kováčsová F, Merglová V. Exogenous Intake of Fluorides in Caries Prevention: Benefits and Risks. ACTA MEDICA. 2021 Aug 2;64(2):71-6
76. Jullien S. Prophylaxis of caries with fluoride for children under five years. BMC pediatrics. 2021 Sep;21(1):1-1.
77. Sharda S, Gupta A, Goyal A, Gauba K. Remineralization potential and caries preventive efficacy of CPP-ACP/Xylitol/Ozone/Bioactive glass and topical fluoride combined therapy versus fluoride mono-therapy—a systematic review and meta-analysis. Acta Odontologica Scandinavica. 2021 Aug 18;79(6):402-17.
78. Evans A, Leishman SJ, Walsh LJ, Seow WK. Inhibitory effects of antiseptic mouthrinses on *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus*. Aust Dent J. 2015 Jun;60(2):247-54; quiz 270. doi: 10.1111/adj.12312. Epub 2015 May 19. PMID: 25989101.
79. Paula, V., Modesto, A., Santos, K. *et al.* Antimicrobial effects of the combination of chlorhexidine and xylitol. *Br Dent J* 209, E19 (2010). <https://doi.org/10.1038/sj.bdj.2010.887>
80. Pinar Erdem A, Sepet E, Kulekci G, Trosola SC, Guven Y. Effects of two fluoride varnishes and one fluoride/chlorhexidine varnish on *Streptococcus mutans* and *Streptococcus sobrinus* biofilm formation in vitro. Int J Med Sci. 2012;9(2):129-36. doi: 10.7150/ijms.3637. Epub 2012 Jan 7. PMID: 22253559; PMCID: PMC3258554.
81. Jannesson, L., Renvert, S., Kjellsdotter, P., Gaffar, A., Nabi, N., & Birkhed, D. (2002). Effect of a triclosan-containing toothpaste

- supplemented with 10% xylitol on mutans streptococci in saliva and dental plaque. A 6-month clinical study. *Caries research*, 36(1), 36–39. <https://doi.org/10.1159/000057588>.
82. Chiba, Fernando & Garbin, Cléa & Saliba, Tania & Garbin, Artênio & Moimaz, Suzely. (2019). Effect of Chlorhexidine and Fluoride Varnishes on Streptococcus mutans Count and Dental Plaque Index. *Journal of Health Sciences*. 21. 371. 10.17921/2447-8938.2019v21n4p371-5.
83. Baygin O, Tuzuner T, Kusgoz A, Senel AC, Tanriver M, Arslan I. Antibacterial effects of fluoride varnish compared with chlorhexidine plus fluoride in disabled children. *Oral Health Prev Dent* 2014;12(4):373-82. doi: 10.3290/j.ohpd.a32129.
84. Bapat SA, Shashikiran N, Gugawad S, Gaonkar N, Taur S, Hadakar S, Chaudhari P. In-vitro Comparison of Anti-microbial Efficacy and Enamel Microhardness after Application of Two Types of Fluoride Varnishes. *J Clin of Diagn Res*. 2020; 14(4):ZC05ZC08. <https://www.doi.org/10.7860/JCDR/2020/43917/13627>.
85. Shen, C., & Autio-Gold, J. (2002). Assessing fluoride concentration uniformity and fluoride release from three varnishes. *Journal of the American Dental Association* (1939), 133(2), 176–182. <https://doi.org/10.14219/jada.archive.2002.0141>.
86. Seppä, L.; Hanhijärvi, H. Fluoride concentration in whole and parotid saliva after application of fluoride varnishes. *Caries Res*. 1983, 17, 476–480.

Master Chart 1

Time kill curve assay against *S.mutans* at 2 minutes, 5 minutes, 10 minutes and 15 minutes

DENTAL VARNISH	CONTROL	2 min	5 min	10 mins	15 mins
PulpdentEmbrace varnish	2736000	6600	4400	2800	400
MI varnish	2736000	100001	100001	100001	200
Cervitec plus varnish	2736000	0	0	0	0
Fluor protector varnish	2736000	29200	0	0	0
DENTAL VARNISH	CONTROL	2 mins	5 mins	10 mins	15 mins
PulpdentEmbrace varnish	2736000	400	0	0	0
MI varnish	2736000	78000	2400	1600	0
Cervitec plus varnish	2736000	0	0	0	0
Fluor protector varnish	2736000	80000	0	0	0
DENTAL VARNISH	CONTROL	2mins	5 mins	10 mins	15 mins
PulpdentEmbrace varnish	2736000	600	0	0	0
MI varnish	2736000	84000	2900	300	0
Cervitec plus varnish	2736000	300	0	0	0
Fluor protector varnish	2736000	98000	48000	24000	19000

Master chart 2

Time kill curve assay against *L.acidophilus* at 2 minutes, 5 minutes, 10 minutes and 15 minutes

DENTAL VARNISH	CONTROL	2 min	5 min	10 mins	15 mins
Pulpdent Embrace varnish	7420000	107600	68800	51600	20800
MI varnish	7420000	47200	5900	4600	200
Cervitec plus varnish	7420000	800	0	0	0
Fluor protector varnish	7420000	840000	214000	12200	24000
DENTAL VARNISH	CONTROL	2 mins	5 mins	10 mins	15 mins
Pulpdent Embrace varnish	7420000	128000	84800	50000	22400
MI varnish	7420000	63600	34400	100	0
Cervitec plus varnish	7420000	2100	0	0	0
Fluor protector varnish	7420000	178000	144000	130000	76000
DENTAL VARNISH	CONTROL	2mins	5 mins	10 mins	15 mins
Pulpdent Embrace varnish	7420000	130000	93200	42400	26400
MI varnish	7420000	100000	22400	0	0
Cervitec plus varnish	7420000	800	0	0	0
Fluor protector varnish	7420000	960000	828000	468000	82000

Master chart 3

Post Hoc Tests – Tukey HSD – Homegeneous Subsets

Dental Varnish	(2 minutes) Subset for alpha = 0.05		
	1	2	3
Cervitec plus varnish	100.00		
Pulpdent Embrace varnish	2533.33		
Fluor Protector varnish		69066.67	
MI varnish		87333.67	
Control			2736000.00
Sig.	1.000	.681	1.000

Table 1: Pairwise comparison at 2 minutes against *S.mutans*

Dental Varnish	(5 minutes) Subset for alpha = 0.05	
	1	2
Cervitec plus varnish	.00	
Pulpdent Embrace varnish	1466.67	
Fluor Protector varnish	16000.00	
MI varnish	35100.33	
Control		2736000.00
Sig.	.566	1.000

Table 2: Pairwise comparison at 5 minutes against *S.mutans*

Dental Varnish	(10 minutes) Subset for alpha = 0.05	
	1	2
Cervitec plus varnish	.00	
Pulpdent Embrace varnish	933.33	
Fluor Protector varnish	8000.00	
MI varnish	33967.00	
Control		2736000.00
Sig.	.540	1.000

Table 3: Pairwise comparison at 10 minutes against *S.mutans*

Dental Varnish	(15 minutes) Subset for alpha = 0.05	
	1	2
Cervitec plus varnish	.00	
MI varnish	66.67	
Pulpdent Embrace varnish	133.33	
Fluor Protector varnish	6333.33	
Control		2736000.00
Sig.	.540	1.000

Table 4: Pairwise comparison at 15 minutes against *S.mutans*

Master chart 4

Post Hoc Tests – Tukey HSD – Homegeneous Subsets

Dental Varnish	(2 Minutes) Subset for alpha = 0.05		
	1	2	3
Cervitec plus varnish	1233.33		
MI varnish	70266.67		
Pulpdent Embrace varnish	121866.67		
Fluor Protector varnish		659333.33	
Control			7420000.00
Sig.	.930	1.000	1.000

Table 5: Pairwise comparison at 2 minutes against *L.acidophilus*

Dental Varnish	(5 minutes) Subset for alpha = 0.05	
	1	2
Cervitec plus varnish	.00	
MI varnish	20900.00	
Pulpdent Embrace varnish	82266.67	
Fluor Protector varnish	395333.33	
Control		7420000.00
Sig.	.095	1.000

Table 6: Pairwise comparison at 5 minutes against *L.acidophilus*

Dental Varnish	(10 Minutes) Subset for alpha = 0.05	
	1	2
Cervitec plus varnish	.00	
MI varnish	1566.67	
Pulpdent Embrace varnish	48000.00	
Fluor Protector varnish	203400.00	
Control		7420000.00
Sig.	.205	1.000

Table 7: Pairwise comparison at 10 minutes against *L.acidophilus*

Dental Varnish	(15 minutes) Subset for alpha = 0.05		
	1	2	3
Cervitec plus varnish	.00		
MI varnish	66.67		
Pulpdent Embrace varnish	23200.00		
Fluor Protector varnish		60666.67	
Control			7420000.00
Sig.	.339	.057	1.000

Table 8: Pairwise comparison at 15 minutes against *L.acidophilus*



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15th September 2020

Ref: 164/IRB-IBSEC/SIST Dated 15th September 2020

To,

Dr. A. Wilson Aruni, PhD, FAMPV
Pro Vice chancellor,
Chair Person, Institutional
Biosafety & Ethical Committee
(IBEC), SIST

Dr. Angelin Aneesha. R
II MDS
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Sathyabama Dental College & Hospital
Chennai -119

Dr. B. Thayumanavan, MDS
Dean, Sathyabama Dental College
Secretary & Convener
Institutional Review Board &
Institutional Biosafety & Ethical
Committee (IBEC), SIST

This is to inform you that the Institutional Bio-Safety & Ethical Committee has approved your study proposal entitled “**Evaluation of Antibacterial efficacy of four different dental varnishes –An *in vitro* study**”, based on the presentation of the proposal made and recommendations of the Institutional Bio-Safety & Ethical Committee/IRB held on 10th August 2020.

The validity of the Ethical clearance is for one year and you are expected to submit six-month progress and the final report of the study to IHBSEC/IRB

Dr. Thayumanavan
Secretary & Convener
IBEC, SIST

Dr. A. Wilson Aruni
Deputy Chair Person
IBEC, SIST



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04.01.2022

To whomsoever it may concern

This is to inform that Dr. Angelin Aneesha. R, post graduate student, Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital has done her research on “Evaluation of Antibacterial efficacy of four different dental varnishes – an *in-vitro* study “ using our research facility under my guidance and expertise from 19 – 04 – 2021 to 29 – 12– 2021. She had made use of equipments present at Research laboratory for oral and systemic health, Sree Balaji Dental College and Hospital.

Regards

Dr.K. MAHALAKSHMI, Ph.D.,
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