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TITLE: A Comparative Evaluation Of The Antimicrobial Activity Of Calcium Hydroxide Paste, Combination Of Calcium Hydroxide With 2% Chlorhexidine And Synbiotic Paste As An Intracanal Medicament Against Enterococcus Faecalis: An In Vitro Microbiological Study

RUNNING TITLE: Evaluation Of Antimicrobial Efficacy Of Calcium Hydroxide, Calcium Hydroxide And 2% Chlorhexidine And Synbiotic Paste Against E. Faecalis.

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ABSTRACT

Background:

Amongst all available intracanal medicaments, calcium hydroxide has been considered as a ideal agent for elimination of root canal pathogens. Due to the immense resilience portrayed by *E. faecalis* due to its ability to penetrate deep into dentin and survive extreme conditions, it is only prudent to consider the use of newer strategies as against conventional strategies to better aid in eradication of the *E. faecalis* bacteria from root canals and hence a synbiotic product may be considered as a novel alternative for the eradication of *E.faecalis.*

Methodology

96 single rooted premolars were inoculated with *E.faecalis* (ATCC 29212) invitro and were the subjected to testing by inoculation into Mueller hinton agar to confirm bacterial growth and for colony counting. This was then followed by random division of samples into three groups. Group I-Calcium hydroxide, Group II-Calcium

hydroxide and 2%Chlorhexidine and Group III-Synbiotic paste. The medicaments were then placed into the canals of the inoculated samples and placed in an incubator for 7 days. Then the samples were reinoculated into Mueller Hinton agar and colonies were counted and collected in a data collection table.

Results

Mean Colony forming units was 114.06 x 10^3±9.31 premedication and 82.37x10^3±7.29 post medication for calcium hydroxide. This difference in colony forming units was statistically highly significant. For Calcium hydroxide + Chlorhexidine mean colony forming units was reduced to 70.34x10^3±6.35 post medication from 113.46x10^3±11.25 premedication respectively. For synbiotic the colony forming units was 118.21x10^3±8.30 premedication and 86.15x10^3±5.78 post medication. This difference was statistically high significant

Conclusion

This study revealed that calcium hydroxide, its combination with chlorhexidine and synbiotic paste are all effective as intracanal medicaments against *E.faecalis*. The use of synbiotic paste as an intracanal medicament, may be advocated as an effective intracanal medicament but further studies need to be conducted in order to better understand it efficacy

Keywords- calcium hydroxide, chlorhexidine, synbiotic.

1. Introduction

1.1 Background

Dental caries is a biofilm-mediated, sugar-driven, multifactorial, dynamic disease that results in the phasic demineralization and remineralization of dental hard tissues. [(1)] The evaluation of the prevalence of this condition, across different types of dentitions, portrays that the highest overall prevalence was noted in the mixed dentition (58%) category, followed by the primary (54%) type. This indicates that there is a progressive rise in the prevalence of caries in the transition phase that exists between the primary and mixed dentition. [(2)] Since, it possesses the potential to inflict significant damage to the tooth, it is imperative to intervene therapeutically; as a deep carious lesion may inadvertently result in tissue necrosis, thus allowing the microbes to infiltrate the root canal system and subsequently, invade the peri-radicular tissues. Such entrenchment of microbes is responsible for eliciting a reactionary response, which manifests frequently by the development of a periapical granuloma. Thus, in order to achieve healing, it is only prudent to focus on pathogen removal, which can be acquired by the proper biomechanical preparation and disinfection of root canals. [(1)]

There are several etiologic agents that may be held responsible for causing endodontic infections, however, the most nefarious of them all is *E. faecalis*. [(3)]

E. faecalis is a facultative anaerobe and gram-positive bacterium that is associated with persistent endodontic infections. Organisms such as enterococci and streptococci are capable of surviving adverse conditions of root canals. This ability to survive adverse conditions is because of its unique capability to invade the depths of dentinal tubules and survive extreme periods of low pH and limited nutrition. Thus, the placement of intracanal medicaments between appointments, is advocated, as it helps in eradication of the surviving microorganisms and promotes the successful outcome of the treatment. [(3)]

Amongst all available intracanal medicaments, calcium hydroxide has been considered as a gold standard agent for elimination of root canal pathogens. Consequently, it has been used extensively in root canal treatment that is performed in multiple visits. [(3)]

During the past decade, however, Chlorhexidine has received increased attention as an irrigant and medicament. Studies have shown that the combining chlorhexidine with calcium hydroxide has a synergistic extension of its antibacterial efficacy. [(4)]

The immense resilience portrayed by *E. faecalis* is a result of its ability to penetrate deep into dentin and survive extreme conditions. Thus, it is only prudent to consider the use of newer strategies as against conventional strategies to better aid in eradication of the E. faecalis bacteria from root canals. [(5)]

Amongst newer strategies that maybe considered to eradicate root canal pathogens, a concept of competitive inhibition maybe utilised. Competitive inhibition refers to deteriorating the action of a microorganism by introducing another microorganism which requires similar nutrients.

Thus, one can assume that the inoculation of microorganisms in the root canal, can be followed by a cascade of events, where in the newly introduced microorganism competes with the pre-existing root canal pathogens for a common source of nutrition. [(5)] This common source of nutrition may be derived from probiotics,

prebiotics and synbiotics.

WHO defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [4]. WHO defines prebiotics as a non-viable food component that confer health benefit(s) by modulation of microbiome of the host. [5]

A synbiotic product is a combination of probiotic compounds and prebiotic compounds which beneficially affects the host in improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria. [5]

Since, the term "synbiotics" is directed towards the synergistic effect, this term is strictly reserved for combination in which the fibrous prebiotic component selectively enhances the probiotic organisms. Synbiotics were developed to overcome possible survival difficulties for probiotics. [4]

It is postulated that synbiotics carry the potential to competitively inhibit pre-existing pathogenic microflora and thus may eradicate the need for antimicrobial therapy. [4][5]

Thus, the study was conducted for the purpose of performing a comparative assessment of the antimicrobial effect of synbiotic paste as an intracanal medicament with calcium hydroxide and calcium hydroxide with 2% chlorhexidine against *E. faecalis.*

1.2 Aim:

The study aimed to evaluate the antimicrobial efficacy of calcium hydroxide paste, calcium hydroxide with 2% chlorhexidine and synbiotic paste as an intracanal medicament against E. faecalis

1.3 Objectives:

1. To evaluate the antimicrobial efficacy of calcium hydroxide paste against *E.faecalis.*
2. To evaluate antimicrobial efficacy of combination of calcium hydroxide paste with 2% chlorhexidine against *E. faecalis*.
3. To evaluate antimicrobial efficacy of synbiotic paste as intracanal medicament against *E. faecalis.*
4. To compare the antimicrobial efficacy of calcium hydroxide paste, calcium hydroxide with 2% chlorhexidine and synbiotic paste against *E. faecalis.*

2. Materials and methods

The study included samples which were primarily single rooted premolars and teeth with closed apex. Samples which were Carious, with developmental defects or with calcifications in canals were excluded.

96 single-rooted premolars were selected based on the previously mentioned inclusion and exclusion criterias. The samples were decoronated to a standardized length of 11 mm when measured from the apex. The working length were set to 10mm, 1mm short of anatomic apex. The biomechanical preparation of root canals were done using protaper rotary files in crown down technique for apical size standardization. After that copious intracanal irrigation was done with 5.25% sodium hypochlorite and with 0.9% normal saline solution to remove smear layer. The root canals were dried with paper points and the apex were sealed with light cure composite. The external surfaces were coated with nail polish. To make identification and handling easier, the teeth were mounted vertically in acrylic resin.

After setting of the acrylic resin, samples were placed in an autoclave sachet and autoclaved at 121℃ for 20 minutes. *E.faecalis* (ATCC 29212) was inoculated in brain heart infusion (BHI) broth for 24 hours. The turbidity were verified using McFarland turbidity scale and was adjusted to 0.5, corresponding to $1.5x10^8$ organisms per millilitre. The sterilized root canals were filled with 50 microliters of *E. Faecalis* suspension, using sterile micropipettes. All specimens were incubated at 37℃ for 24 hours. Each root canal was then inoculated with the 24 hour old cultured broths of bacterial solution, until the canal orifice using a sterile endodontic needle in a microbiological safety cabinet. After inoculation, the samples were incubated at 37°C for 21 days. Every 3rd day, the canals were re-inoculated with fresh bacterial samples.

Bacterial samples were collected using sterile paper points, transferred into Eppendorf tubes (containing 2 mL normal saline), vortexed for 1 minute and then inoculated in Mueller Hinton agar at 37℃ for 24 hours, to evaluate the growth of E. faecalis in the canals. The canal contents was then aspirated and rinsed with 5 mL saline and patted dry with sterile paper points.

The specimens were then randomly divided into 3 groups for intracanal medicaments. The three groups were:

Group 1: calcium hydroxide paste Group 2: calcium hydroxide with 2% chlorhexidine solution Group 3: synbiotic paste

Preparation of Intracanal medicaments:

For the calcium hydroxide paste group, the Calcium hydroxide paste (RC Cal) were placed into the root canals.For the calcium hydroxide with chlorhexidine group – 150 mg of Calcium hydroxide powder was mixed with 0.2 mL of 2% Chlorhexidine liquid. For the Synbiotic paste- one capsule was broken and the powder content of the capsule was mixed with 0.2mL of glycerine

Placement of intracanal medicament: 0.2 mL of prepared medicaments were placed in the root canals and completely filled.

The canals were sealed with temporary restoration and incubated at 37°C for 7 days. After 7 days of incubation, the temporary restoration were removed from each of the canals entrance. The bacterial samples from the canals were collected using sterile paper points, transferred into Eppendorf tubes (containing 2 mL normal saline) , vortexed for 1 minute and then inoculated in Mueller Hinton agar and incubated at 37°C for 24 h for obtaining results in CFU. All the results were collected in the data collection table and appropriate statistical analysis were performed.

3.Results:

Table 3.1 and graph 3.1 show that the mean colony forming units prior to medicament placement are - calcium hydroxide was 114.06x(10^3) ±9.31,calcium hydroxide +2% chlorhexidine was 113.46x(10^3)±11.25 and synbiotic was 118.21x(10^3)±8.30 respectively.

Table 3.2 and graph 3.2 show the mean colony forming units post medicament placement in all three groups and show reduction in comparison to premedication placement in all groups.

Table 3.3 and graph 3.3 show that the Mean Colony forming units was 114.06 x 10^3±9.31 premedication and 82.37x10^3±7.29 post medication for calcium hydroxide. This difference in colony forming units was statistically highly significant. ($p<0.001$) For Calcium hydroxide + Chlorhexidine mean colony forming units was reduced to 70.34x10^3±6.35 post medication from 113.46x10^3±11.25 premedication respectively. ($p<0.001$) For synbiotic the colony forming units was 118.21x10^3±8.30 premedication and 86.15x10^3±5.78 postmedication. This difference was statistically high significant. ($p<0.001$

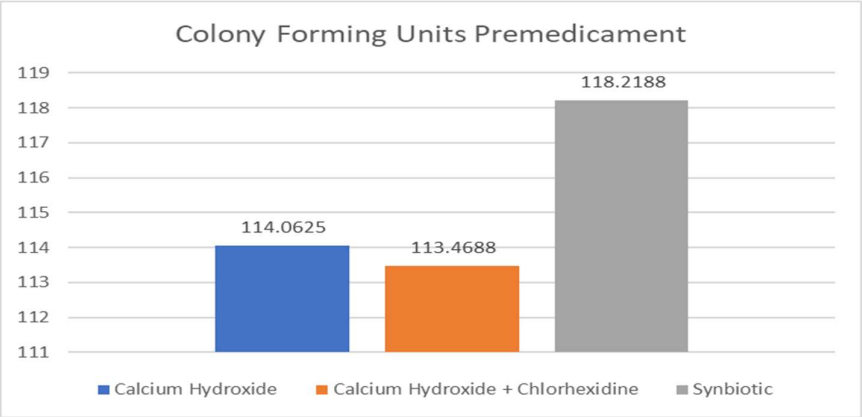
	N	Mean (10^3)	Std. Deviation (10^3)	Minimum	Maximum
Calcium Hydroxide	32	114.0625	9.31505	93.00	129.00
Calcium Hydroxide + Chlorhexidine	32	113.4688	11.25076	89.00	130.00
Synbiotic	32	118.2188	8.30365	100.00	130.00

Table 3.1 : Mean colony forming units pre medication between calcium hydroxide, calcium hydroxide +chlorhexidine and symbiotic

	N	Mean (10^3)	Std. Deviation (10^3)	Minimum	Maximum
Calcium Hydroxide	32	82.3750	7.29450	68.00	99.00
Calcium Hydroxide + Chlorhexidine	32	70.3438	6.35310	60.00	83.00
Synbiotic	32	86.1563	5.78156	70.00	96.00

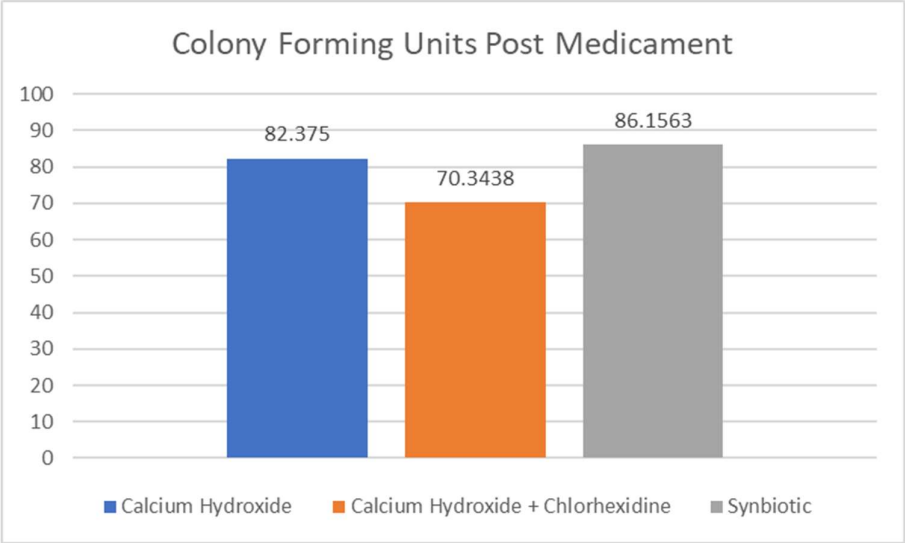
Table 3.2 : Mean colony forming units post medication between calcium hydroxide, calcium hydroxide +chlorhexidine and symbiotic

TABLE 3.3: Comparison between before and after medication of calcium hydroxide, calcium hydroxide +chlorhexidine and synbiotic

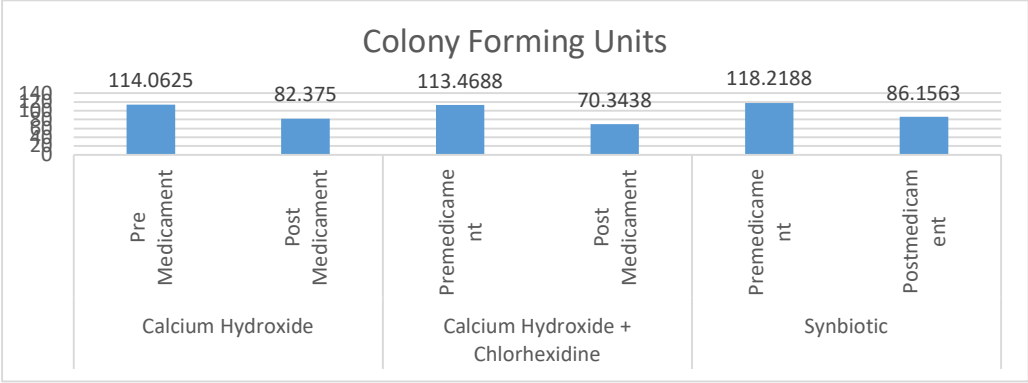


		Mean ($x10^3$)	Std. Deviation ($x10^3$)	t value ($x10^3$)	p value	95% Confidence Interval	
Calcium Hydroxide	Pre Medicament	114.0625	9.31505	29.242	<0.001*	29.47	33.89
	Post Medicament	82.3750	7.29450				
Calcium Hydroxide + Chlorhexidine	Premedicament	113.4688	11.25076	26.579	<0.001*	39.81	46.43
	Post Medicament	70.3438	6.35310				
Synbiotic	Premedicament	118.2188	8.30365	22.239	<0.001*	29.12	35.00
	Postmedicament	86.1563	5.78156				

GRAPH 3.1: MEAN COLONY FORMING UNITS PREMEDICATION IN CALCIUM HYDROXIDE, CALCIUM HYDROXIDE +CHLORHEXIDINE AND SYNBIOTIC GROUP



GRAPH 3.2: MEAN COLONY FORMING UNITS POSTMEDICATION IN CALCIUM HYDROXIDE, CALCIUM HYDROXIDE +CHLORHEXIDINE AND SYNBIOTIC GROUP



GRAPH 3.3: COMPARISON BETWEEN BEFORE AND AFTER MEDICATION OF CALCIUM HYDROXIDE, CALCIUM HYDROXIDE +CHLORHEXIDINE AND SYNBIOTIC

4. Discussion

The morphology, structure, functioning and pathology of the pulp and periradicular tissues of teeth forms the core domain in the subject of paediatric endodontics. Literature suggests that, bacteria may be identified as the root cause of endodontic infections. [13] These endodontic infections, affect the pulp tissue, subsequently leading to necrosis. The necrosed pulpal tissue has been associated with a plethora of microorganisms. Amongst the wide variety of organisms that coherently form the large community, ensconced in the root canals of infected teeth, it has been identified that anaerobic bacteria form a large majority in this community [14]

Commonly isolated anaerobic bacteria among these anaerobic microorganisms is the *E.faecalis.* E.faecalis has been held responsible for the persistence of endodontic infections. These bacteria stain gram positive and are highly resistant to the medicaments It also shows the potential to survive solitarily, without the support of other bacteria. In addition, E. Faecalis has the ability to produce a biofilm, which basically corresponds to the microbial community attached to the surface. This biofilm aids as a layer of protection against the host defence mechanisms. [15] [16]

Amongst various strategies that may be employed in the eradication of *E.faecalis*, the most commonly used method includes the utilisation of calcium hydroxide as an intracanal medicament. [8]

The study performed by Riaz et al [8] stated that calcium hydroxide has always been considered as a gold standard intracanal medicament against a wide array of root canal pathogens particularly *E,faecalis.* The author also mentions that the combination of calcium hydroxide with chlorhexidine, despite its documented efficacy and results are not being employed in routine. Thus the authors conducted the study with the objective to compare calcium hydroxide and combination of calcium hydroxide with 2% chlorhexidine when used as an interappointment dressing /intracanal medicament for 14 days. The comparison of these agents was based on the resolution of periapical radiolucency. The study was performed on 60 patients of either gender was included in the study. The age range was 15–60 years with the radiographic evidence of radiolucency of 2–4 mm in size. Both maxillary and mandibular teeth till first molar were selected. Teeth with previous endodontic treatment mentally handicapped and terminally ill patients were excluded. Selected patients were placed randomly into Group 1 (calcium hydroxide alone) and in Group 2 (calcium hydroxide in combination with 2% chlorhexidine gel). Obturation and permanent filling was done at 14th day. The patient was recalled after 90 days of obturation and a radiograph was taken. Final outcome was measured at the end of 90th day after obturation. The results suggested that calcium hydroxide with 2% chlorhexidine showed better resolution of lesion, than the group without chlorhexidine. Thus, the study concluded that calcium hydroxide in combination with 2% chlorhexidine as intracanal medicament results in better resolution of periapical radiolucency. (8) The resolution of periapical radiolucency may be associated with the increased antimicrobial effect of the combination of calcium hydroxide with chlorhexidine. The current study also shows that the combination of calcium hydroxide with chlorhexidine has better antimicrobial activity as compared to calcium hydroxide alone.

Moreover, the results of the current study is also in agreement with the study by Martinho et al. (26) which concluded that all the 7- and 14-day intracanal medications were effective in reducing bacteria and endotoxin as well as in lowering the levels of inflammatory cytokines, with chlorhexidine showing limited effectiveness against endotoxin. The addition of chlorhexidine to calcium hydroxide lowers its contact angle and improves the wettability of the medication on the root canal (27)

This could also be because of synergistic effect of calcium hydroxide and chlorhexidine on liposaccharides/ endotoxin produced by gram negative bacteria. Hence, both the intracanal medications complement their actions. The current study, however is not in agreement with the study by Schaffer and bossmann et al[27] whose study stated that chlorhexidine alone was a more effective antimicrobial than its combination with calcium hydroxide. Another study by Delgado., et al. [28] concluded that there was no difference between the antimicrobial activity of chlorhexidine with or without calcium hydroxide. Gomes., et al. [32] evaluated that 2% chlorhexidine gel was not effective in eliminating endotoxin from the primary infected root canals.

Studies by Alarbeed et al[29] concluded that calcium hydroxide was effective medication in reducing the postoperative pain also. Despite good antibacterial properties shown by previous studies, later studies by Ostravik and Heling et al [30] [31] have demonstrated the inability of calcium hydroxide to eliminate bacteria commonly found in the root canals and those penetrated in dentinal tubules . The limited action of calcium hydroxide could be because of the buffer effect that dentin exerts over calcium hydroxide, reducing its antimicrobial action. [30] [31]

Several unique methods of defeating E. faecalis were attempted using an array of irrigants. Ingenious treatment strategies were critically assessed, such as the use of passion fruit juice as an endodontic irrigant, [17] as well as the use of lasers and phytomedicine;[18,19]

Countless clinical trials have been executed in recent times in order to identify an ideal intracanal medication by utilising a number of combinations such as camphorated paramonochorophenol, chlorhexidine, iodine potassium iodine, erythromycin and clindamycin or tetracycline with calcium hydroxide. [10] The augmentation of chlorhexidine to calcium hydroxide has been backed by multiple studies. [11] However , further neoteric methods need to be reached in order to eliminate *E.faecalis.*

In an attempt to develop newer strategies, it is possible to consider the use of probiotic organisms for the eradication of *E.faecalis.* Probiotic strains have been utilised previously, in an attempt to of treat oral health problems. Specifically, probiotics have been proven to be efficacious in the management of periodontal problems, breath malodour, and prevention of caries. [2, 8]. However, no studies have been conducted so far to evaluate the combination of these probiotic strains with prebiotics (synbiotics) in endodontic therapy.

Lactobacillus and Bifidobacterium genera are the most commonly employed probiotic strains and coincidentally, they are commensals of the oral cavity, and have also been isolated in carious lesions. [9, 10] These microorganisms were among the pioneer species to be introduced in research. [11] It has been postulated that ATCC 53103 and Lactobacillus rhamnosus GG, possess the potential to reduce caries risk by releasing a growth inhibitory substance against the microorganism streptococcus sobrinus. [10]

The use of Probiotic organisms with associated prebiotics in the eradication of endodontic infection has not yet been evaluated extensively. Synbiotics possess greater prospective value against the use of antibacterial treatment in the future. In this study, an ingenious approach has been developed in an attempt to increase the success of endodontic therapy. This novel strategy involves utilising probiotic organisms, supplemented with additional fibre to eliminate pathogenic organisms, by competitive inhibition of pathogenic organisms. [20]

The current study performed an evaluation to compare the antimicrobial activity of calcium hydroxide paste, combination of calcium hydroxide with 2% chlorhexidine, synbiotic paste as medicament for root canals against *E.faecalis*.

The use of probiotics as an intracanal medicament was first considered by Bohora et al in 2019, where in a pilot study was executed in two phases to identify if the probiotics possessed the potential to inhibit endodontic pathogens. The study concluded that probiotic supernatants effectively inhibit *E.faecalis*.(4)

Shabaan et al conducted an in vitro study to assess the antibacterial activity of multi-strain probiotics supernatants as an intracanal medication on **E. faecalis** biofilm. Calcium hydroxide and Probiotics supernatants groups showed a significant ($p < 0.05$) percentage of overall bacterial reduction. The study concluded that Probiotics supernatants has a potential antibacterial effect on **E. faecalis** growth closely similar to the routinely used Calcium hydroxide**.**

Recently, however, multiple systematic reviews, meta-analyses, and expert opinions expressed criticism on their claimed effects and safety. The shift in the scientific world towards a better understanding of the human microbiome, before consumption of the probiotic organisms, is highly recommended.[20]

The limitations of the study are that because of the financial limitations, commercial synbiotics were utilized instead of extracting probiotic organisms and utilising them. The effective CFU count needed to eliminate or outcompete the pathogenic organisms is unknown.

Ideally, it is imperative to achieve sterility of the endodontic system for endodontic success; however, practicality suggests that it is nearly impossible to achieve sterility of root canals under regular conditions.[20]

Synbiotics used against *E.faecalis*, which was evaluated in vitro in this present study, yielded the result that its use as an intracanal medicament, caused statistically significant reduction in E. faecalis and is comparable to the gold standard intracanal medicament, calcium hydroxide and hence it can be considered as an acceptable method against pathogenic microflora. This study demonstrated that synbiotics can be researched further as a valuable aid in endodontic therapy.

The merits of the study are that there have been no studies involving the use of synbiotics as an intracanal medicament against *E.faecalis* and thus may have a pivotal role in pedodontics, as it may aid in eradication of *E.faecalis* from root canals of teeth in children. The use of synbiotic capsules to create a paste, that may be used as a medicament; may be considered as a useful, cost-effective replacement for the use of antibiotics to manage endodontic infections.

5.Conclusion

This study revealed that calcium hydroxide, its combination with chlorhexidine and synbiotic paste are all effective as intracanal medicaments against *E.faecalis*. However, the study evidently suggests that the combination of chlorhexidine and calcium hydroxide is superior in eradication of *E.faecalis*. The use of synbiotic paste as an intracanal medicament, may be advocated as an effective intracanal medicament but further studies need to be conducted in order to better understand it efficacy.

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