Comparative Antimicrobial Activity and Durability of Different Glass Ionomer Restorative Materials with and without Chlorohexidine

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Abstract: *Objectives*: To evaluate the *in vitro* antibacterial effect of three different restorative materials (Glass Ionomer Cements (GIC)) containing chlorohexidine on *Streptococcus mutans*, and *lactobacillus acidophilus.*

Materials and Methods: Three commercially available glass ionomer cements, i.e., Fuji 1X (GIC1), Ketac molar (GIC2) and Riva (GIC3) were evaluated each alone and in combination with chlorohexidine diacetate or chlorohexidine digluconate. GICs were manipulated in accordance with manufacturer's guidelines and embedded in wells made-up in plates of trypticase soy agar seeded with *Streptococcus mutans* and Lactobacillus MRS agar seeded with *Lactobacillus acidophilus*. The antibacterial activity was evaluated by using a caliper to measure the diameter of growth inhibition zones. The study was performed in triplicate and Duncan post-Hoc Multiple comparisons at p≤ 0.05 is used for means comparison.

Results: the three Glass ionomers with chlorohexidine diacetate powder (1%) showed the highest activity and prolonged effect on the tested strains compared to glass ionomers free from chlorohexidine and the other glass ionomers with chlorohexidine digluconate liquid. Also, it was found that Fuji IX glass ionomer showed higher and prolonged effect in comparison to Ketac-Molar and Riva glass ionomers. Glass ionomers in combination with chlorohexidine diacetate showed higher efficacy against *streptococcus mutans* than for lactobacillus acidophilus.

Conclusion: All three GIC's under evaluation, promoted growth inhibition of the cariogenic bacteria assayed. Fuji IX glass ionomer with chlorohexidine diacetate showed the highest efficacy and durability against the tested strains.

Keywords: Caries, *Streptococcus mutans, lactobacillus acidophilus,* Glass ionomers, chlorohexidine diacetate, chlorohexidine digluconate.

INTRODUCTION

Caries disease still remains a major public health problem despite the widespread use of fluoride and the decline in caries prevalence observed in the majority of highly industrialized countries [1]. In an attempt to obtain restorative materials that could prevent marginal gaps colonization, materials capable of releasing fluoride and providing antimicrobial activity have been developed, such as, Glass Ionomer cements (GIC), "compomers" and fluoridated composite resins [2, 3].

GIC is a promising restorative material due to its physical and chemical properties, such properties include its adhesion to dental structures, biocompatibility and fluoride release/uptake, which contributes to GICs preventive characters [4]. GICs materials are inexpensive compared with resin composites and less demanding with respect to the clinical application. The high viscosity GICs have better mechanical properties than traditional GICs that were developed by increasing the powder/liquid ratio for atraumatic restorative treatment (ART) [5]. It is found that therapeutic benefits may be gained by combining antibacterial agents with glass ionomer materials. Recently, researchers modified filling materials such as composite resins, acrylic resins, and GICs by adding chlorohexidine and quaternary ammonium compounds [6]. However, the incorporation of antibacterial agents in restorative materials frequently results in changes in the physical properties [7, 8] and it is critical that the type of restorative material shows strong enough physical properties to resist occlusal load. Therefore, antibacterial GICs, for use in the ART approach, require an optimum amount of antibacterial agents, which should not jeopardize the basic properties of the parent materials [9-12]. It was shown that the incorporation of CHX dihydrochloride and CHX diacetate into GICs can increase the antimicrobial effect without seriously compromising the physical properties of the original material [13]. Chlorohexidines as one of cationic disinfectants have received attention for their antibacterial properties. It has been proven to be the most effective and safe agent among several different antimicrobial agents in plaque reduction [14-16]. Its antibacterial effect is significantly longer than other agents due to long retention in oral structures from which it is slowly released [17, 18].

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MATERIALS AND METHODS

Two antibacterial compounds, chlorohexidine diacetate (CHX1) and Chlorohexidine digluconate (CHX2) (Sigma-Aldrich, Switzerland) and three types of glass ionomer restorative materials which are Fuji IX (GI1) (GC Corporation, Tokyo, Japan), Ketak Molar (GI2) (3 MESPE AG, Germany) and Riva self cure (GI3) (SDI Limitation, Austeralia) were used.

Chlorhexidine diacetate which is commercially available as a solid substance (powder) was added to glass ionomer powder in order to obtain 1% concentration of CHX in GI formulation. For Fuji IX 15g of powder was mixed with 0.151g of CHX powder. For Ketac molar, 12.5g of powder was mixed with 0.126g of CHX powder and for Riva, 15g of powder was mixed with 0.151g of CHX powder. The same procedure was used with Chlorhexidine digluconate solution which is available as an aqueous solution to obtain 1% concentration. For Fuji IX, 6.4ml of liquid was mixed with 0.0696 ml (69 µl) of CHX liquid, for Ketac Molar 8.5ml of liquid was mixed with 0.0858ml (85µl) of CHX liquid and for Riva 6.0ml of liquid was mixed with 0.060ml (60µl) of CHX liquid. The original ratio of powder/liquid for GI1 was 3.6g: 1g, 4.5g: 1g for GI2 and 3.3g: 1g for GI3 (1spoon of powder and 1 drop of liquid) and was used as a reference. Fifty disks of each type of glass ionomer materials (3 X 50) = 150 specimens in total, each specimen was prepared in a split Teflon ring with a central hole having dimensions (10mm in diameter X 2mm in thickness).

Agar Diffusion Testing

The antibacterial activity was evaluated against *Streptococcus mutans* ATCC® 25175™ and *Lactobacillus acidophilus* ATCC® 314™ (Microbiologics®, Lyophilized microorganisms, USA) using the agar diffusion test. These microorganisms were chosen because *Streptococcus mutans* is the main bacteria responsible for caries formation and *Lactobacillus acidophilus* is the principle bacteria related to caries progression [19, 20].

Each bacterial strain from stock cultures were cultivated overnight in specific culture media: Trypticase-soy agar for *Strep. mutans* (Becton Dickinson Microbiology systems, Cockeysville, MD21030, USA) and Lactobacillus MRS agar for *L. acidophilus* (Himedia laboratories PV, 23 Vadhani India, Est., LBS Marg., Mumbai, India) after incubation for 24h for *Strep. mutans* and 48h for *L. acidophilus* in incubator (Gallenkamp cooled incubator, IR211GA model, Pinal way, Loughborough, England) at 37˚c ± 1˚c, Two or three discrete representative overnight colonies of each tested strain were inoculated into 2 ml sterile saline and diluted to obtain a turbidity equal to 107 CFU/ml equivalent to 0.5 McFarland turbidity standard solution [(About 9.95 ml of solution A (1 % (V/V) of sulfuric acid) was mixed with 0.05 ml of solution B (1.175 % (W/V) aqueous solution of barium chloride dehydrate) slowly and with constant agitation in a clear glass test tube. The tube was sealed and stored in the dark at room temperature)] [21]. Petri dishes (15 cm diameter) containing 30 ml agar to a thickness of 2 mm were seeded by 0.5 ml of microbial suspension using Automatic micropipette (Huawei Adjustable micropipette (H) series, Zhejiang, China Mainland).

For each Petri dish, nine standardized wells with a diameter of 10mm were punched into the agar with the blunted end of a sterile Pasteur pipette. For each Petri dish 9 specimens (10mm in diameter x 2mm in thickness) were inserted in the wells onto agar with sterile forceps.

For monitoring the immediate antibacterial effect of the tested groups (day 0), the plates were incubated in incubator at 37˚c ± 1˚c for 48h. Then the diameters of the circular inhibition zones produced around the specimens (specimens + inhibition zones) were measured in millimeters with a digital caliper (Owner's manual, IOS-USA) at three different points, and the mean was recorded as the (day 0) value.

The specimens were then left in the same plates for five more days in the incubator (total of 7 days) and transferred to freshly inoculated plates and incubated at 37˚C for 24h for *Strep. mutans* and for 48h for *L. acidophilus* to obtain the inhibition zones for day 7. On that day, the respective culture media with fresh agar for the microorganisms were placed in new Petri dishes and microorganisms' suspensions were added and 9 wells were punched into the agar. The glass ionomer specimens were taken out of their previous Petri dishes and placed in the new wells. The plates were then incubated with active microorganisms at 37˚c ± 1˚c for 24h for *Strep. mutans* and for 48h for *L. acidophilus*, and the inhibition zones around the specimens were measured in millimeters with a digital caliber the day after. This procedure was done for GICs without chlorohexidine (GIC CHX0), GICs with chlohexidine diacetate powder (GIC CHX1) and GICs with chlorohexidine digluconate liquid (GIC CHX2) and repeated with fresh agar plates inoculated with fresh

microorganisms on all control days (14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84 and 91 days) where long term antibacterial effect was carried out.

Statistical Analysis

Statistical analysis was carried out using SPSS program, One way analysis of variance (SPSS, analysis, compare means, one way ANOVA) was used to test the effect of material, treatment or techniques on free bacterial area within each time. Duncan Post-Hoc Multiple comparisons at p≤ 0.05 was used for means comparison (SPSS Inc., Chicago, IL)

RESULTS

Figures **1** and **2** revealed significant difference among *Streptococcus mutans inhibition* zones of the three GICs at day zero as Fuji IX GI1CHX0 showed the highest inhibition zone followed by ketac molar GI2CHX0 and Riva GI3CHX0. It showed also that Fuji IX GI1CHX0 has the highest durability (› 35 days) in comparison to other types. For *Lactobacillus acidophilus,* Fuji IX GI1CHX0 and Ketac molar GI2CHX0 showed statistically significant larger inhibition zones than Riva GI3CHX0 and still give inhibition zones till day 28.

Figures **3** and **4** revealed significant difference of *Streptococcus mutans* inhibition zone of the three GICs formulation with Chlorhexidine diacetate. Fuji IX with Chlorhexidine diacetate GI1CHX1 showed the largest inhibition zone in comparison to Ketac molar GI2CHX1 and Riva GI3CHX1 in most of the tested period which extend to day 84, whereas there was no any inhibition zone for the three GICs formulation with Chlorhexidine diacetate at day 91. For *Lactobacillus acidophilus* Fuji IX with Chlorhexidine diacetate GI1CHX1 showed the largest inhibition zones and durability (day 77) in comparison to Ketac molar GI2CHX1 and Riva GI3CHX1.

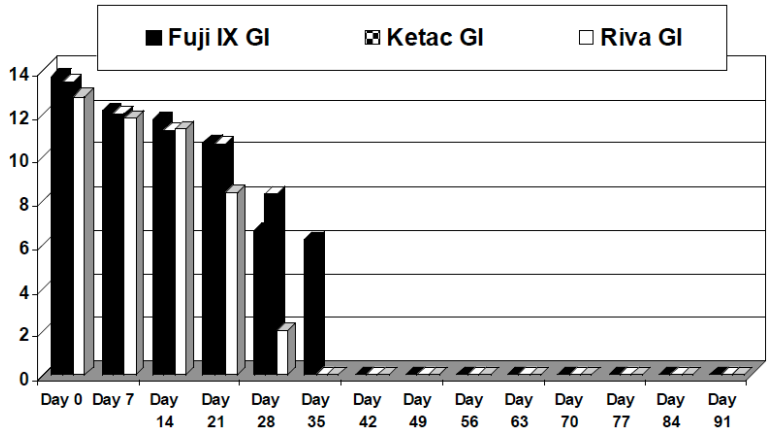

Figure 1: Mean free *Streptococcus mutans* area in different materials within each time using 0 CHX treatment.

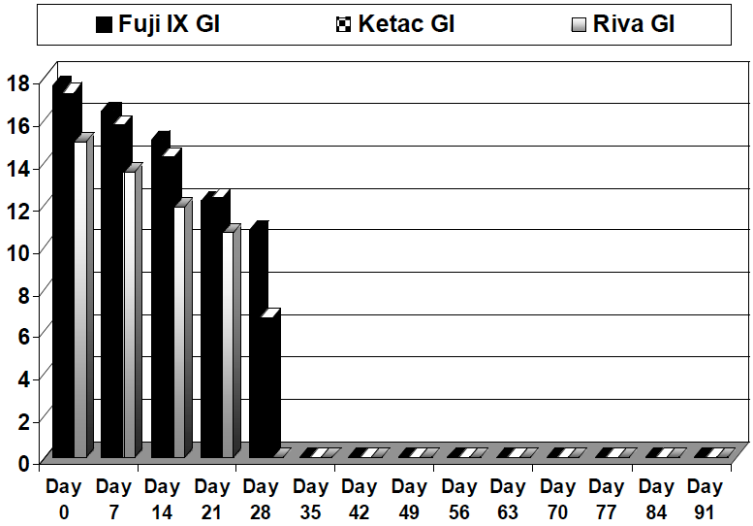

Figure 2: Mean free *Lactobacillus acidophilus* area in different materials within each time using 0 CHX treatment.

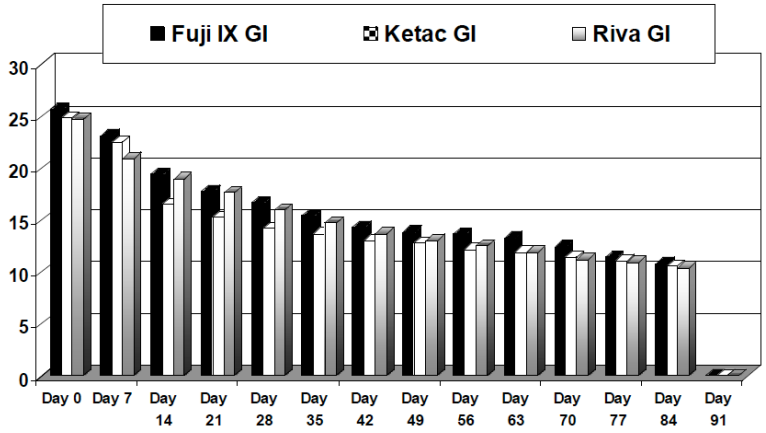

Figure 3: Mean free *Streptococcus mutans* area in different materials within each time using 1% CHX1treatment.

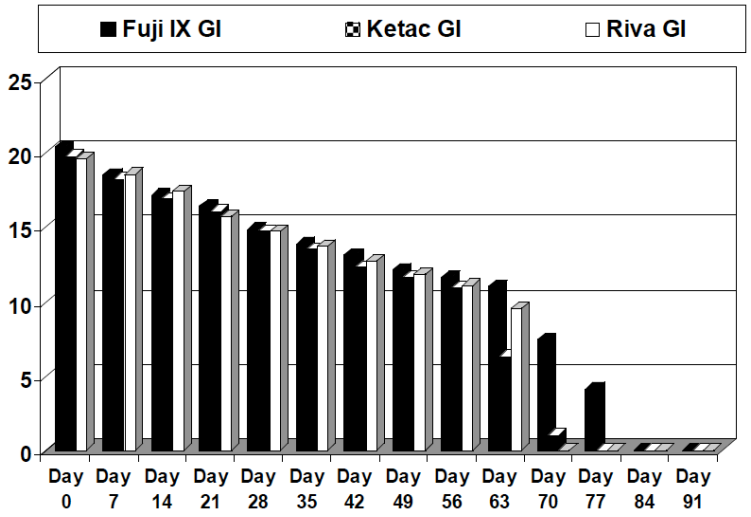

Figure 4: Mean free *Lactobacillus acidophilus* area in different materials within each time using 1% CHX1 treatment.

Fuji IX with Chlorhexidine digluconate showed the largest inhibition zone in comparison to both Ketac molar GI2CHX2 and Riva GI3CHX2 (Figures **5** and **6**) For both *Streptococcus mutans* and *Lactobacillus acidophilus*. In addition, Fuji IX with Chlorhexidine digluconate showed higher durability for *Streptococcus mutans* than that shown by *Lactobacillus acidophilus*.

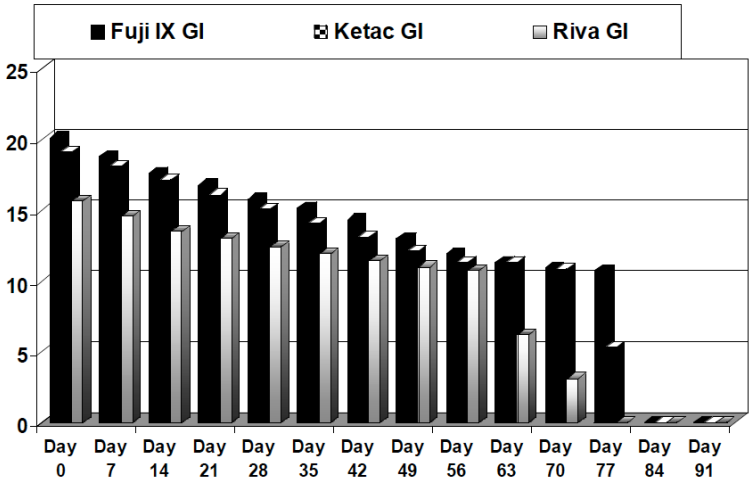

Figure 5: Mean free *Streptococcus mutans* area in different materials within each time using 1% CHX2 treatment.

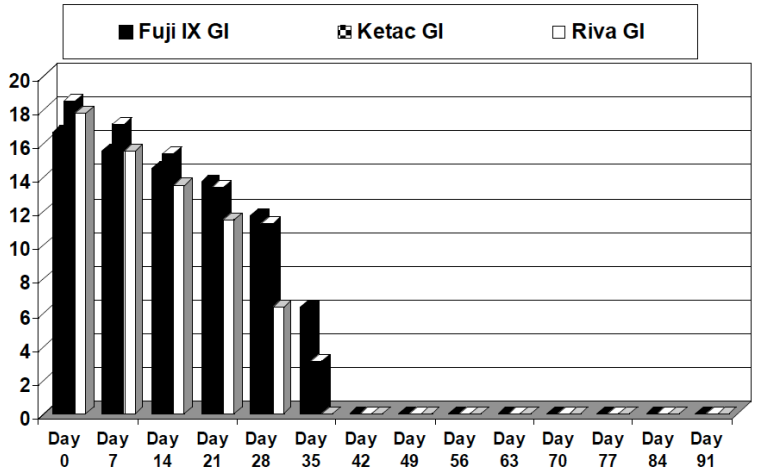

Figure 6: Mean free *Lactobacillus acidophilus* area in different materials within each time using 1% CHX2 treatment.

Table 1: Descriptive Statistics and Test of Significance for the Effect of Technique (Material and Treatment) on Free *Streptococcus Mutans* Area within Selected Time Intervals According to Relation between Area and Time

	Technique \ Time	Day 0	Day 28	Day 77	Day 84	Day 91
		Mean ±S.D.	Mean± S.D.	Mean± S.D.	Mean± S.D.	Mean± S.D
Streptococcus mutans	Fuji IX + 0CHX	13.7±0.18 ^{f}	6.6±6.02 ^{c}	0.0±0.00 ^{c}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
	Ketac + 0CHX	13.4±0.86 ^{fg}	8.3±4.64 ^{c}	0.0±0.00 ^{c}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
	Riva + 0CHX	12.8±0.43 ^{g}	2.0±4.56 ^{b}	0.0±0.00 ^{c}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
	Fuji IX + CHX1	25.7±1.18 ^{a}	16.7±0.67 ^{a}	11.4±0.69 ^{a}	10.8±0.27 ^{a}	0.00±0.00 ^{a}
	Ketac + CHX1	24.9±0.39 ^{b}	14.1±0.57	11.0±0.61 ^{a}	10.6±0.46 ^{ab}	0.00±0.00 ^{a}
	Riva + CHX1	24.7±0.64 ^{b}	16.0±0.82 ^{a}	10.9±0.52 ^{a}	10.3±0.45 ^{b}	0.00±0.00 ^{a}
	Fuji IX + CHX2	20.1±0.66 ^{c}	15.8±0.42 ^{a}	10.8±0.30 ^{a}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
	Ketac + CHX2	19.1±0.67 ^{d}	15.1±0.74 ^{a}	5.3±5.56 ^{b}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
	Riva + CHX2	15.7±0.50 ^{e}	12.4±0.52 ^{b}	0.0±0.00 ^{c}	0.0±0.00 ^{c}	0.00±0.00 ^{a}
Lactobacillus acidophilus	Fuji IX + 0CHX	17.6±0.30 ^{d}	10.8±0.42 ^{b}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Ketac + 0CHX	17.3±0.39 ^{d}	6.6±6.06 ^{c}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Riva + 0CHX	15.0±0.41 ^{f}	0.0±0.00 ^{d}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Fuji IX + CHX1	20.4±0.54 ^{a}	14.8±0.58 ^{a}	4.1±5.26 ^{a}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Ketac + CHX1	19.7±0.40 ^{b}	14.7±0.82 ^{a}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Riva + CHX1	19.5±0.48 ^{b}	14.7±0.48 ^{a}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Fuji IX + CHX2	16.7±0.60 ^{e}	11.7±0.69 ^{b}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Ketac + CHX2	18.4±0.86 ^{c}	11.2±0.37 ^{b}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}
	Riva + CHX2	17.7±0.68 ^{d}	6.3±5.43 ^{c}	0.0±0.00 ^{b}	0.0±0.00 ^{a}	0.0±0.00 ^{a}

Dt (a, b, c, d, e, f, g)= Duncan's Multiple Range Test for the effect of treatment.
Means with the same letter within each time for the same microorganism are not significantly different at p=0.05.

Table **1** showed that glass ionomer cements in combination with chlorohexidine diacetate showed the largest inhibition zones in comparison to glass ionomers free of chlorohexidine and glass ionomers in combination with Chlorhexidine digluconate for both *Streptococcus mutans* and *Lactobacillus acidophilus*.

They also showed that glass ionomers in combination with chlorohexidine diacetate showed higher efficacy against *streptococcus mutans* than for *Lactobacillus acidophilus* and also give zones of inhibition till day 84 while its effect on *Lactobacillus acidophilus* extended to day 77 in case of Fuji IX with Chlorohexidine diacetate and to day 28 in case of Ketac molar and Riva with Chlorhexidine diacetate.

DISCUSSION

Dental caries constitutes one of the most common infectious diseases. It is a multi-factorial disease related to the presence of cariogenic bacteria embedded in the dental plaque which are particularly the *Streptococcus mutans* and *Lactobacillus acidophilus* [22, 23]. Therefore several experiments have been conducted to incorporate an antibacterial agents into dental filling materials as resin composites and glass-ionomers, in order to inhibit bacterial attachment and thus plaque accumulation. However, the antibacterial activity is considered to depend upon release of the antibacterial agent [13, 24-28]. Glass-ionomer cements were selected in this study due to their major advantages of adhesion to tooth structure, fluoride uptakes and release which can inhibit caries, further more the variety of the clinical application of GICs [9, 29, 30]. High viscosity GICs were commonly used for atraumatic restorative treatments (ART) and conservative simple cavities in posterior teeth. Reports have shown that the newer, more viscous GICs release substantially less cumulative fluoride ions than less viscous conventional restorative GICs and resin-modified GICs [31-33]. The less fluoride release may contribute to less antibacterial effect, this was one of the contributing factors to evaluate the antibacterial activity of these high viscosity GICs. Chlorhexidine is one of the antimicrobial agents available for dental use. It is the most thoroughly researched in terms of ability to control cariogenic activity [34-37]. In our study we selected CHX, in the form of a powder (Chlorhexidine diacetate) and a liquid (Chlorhexidine digluconate) to be easily incorporated into the conventional GICs (Fuji IX, Ketac molar easy mix and Riva). The agar diffusion test was used to evaluate the antibacterial activity for each type of glass-ionomer cements against the tested microorganisms. This method was chosen for this study because it is relatively inexpensive and can be performed rapidly and easily with a large numbers of specimens; also it had been widely accepted as a simple screening assay to assess the antibacterial properties or restorative materials [6]. However there are limitations associated with the agar diffusion test [38]. One of the main limitations is the inability to distinguish between bacteriostatic and bactericidal effects, so the test does not provide any information about the viability of the tested microorganisms within the inhibition zones [39, 40] and also this assay does not reflect the actual status in the oral cavity where the bacteria exist as a biofilm which exhibits an increased resistance to antibacterial agents [41]. Turkun *et al.*, [42] reported that chlorhexidine diacetate was more effective against both *S. mutans* and *L. acidophilus* and has longer durability (up to 90 days for *S. mutans* and up to 60 days for *L. acidophilus*) than chlorhexidine digluconate that agree with our study. It was found that Fuji IX GIC showed higher antimicrobial activity against the tested microorganisms which agree with results obtained by Coogan and Creaven [43] and DeSchepper *et al*., [44]. on the other hand, Botelho [10] showed that Fuji IX GIC has no antibacterial activity. Botelho [45], proved also that *Streptococcus mutans* is more sensitive to chlorhexidine than other oral bacteria which is in agreement with the findings of this study.

CONCLUSION

Addition of chlorhexidine diacetate and chlorhexidine digluconate to GICs has the ability to provide a long term antimicrobial activity against *S. mutans* and *L. acidophilus*. Fuji IX with Chlorohexidine diacetate has the highest antibacterial activity and durability.

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