# Effect of Gallium on Growth of *Streptococcus mutans* NCTC10449 and Dental Tissues

S.P. Valappila\*, G.J. Owensa , E.J.Milesa, N.L. Farmera, L. Coopera, G. Millerb, R. Clowesc, R.J.M. Lyncha,d, S.M. Highama

aDepartment of Health Services Research and School of Dentistry, University of Liverpool, Research Wing, Daulby Street, Liverpool, L69 3GN, United Kingdom

bDepartment of Chemistry, University of Liverpool, Crown Street, Liverpool, L69 7ZD, United Kingdom

cCentre for Materials Discovery, University of Liverpool, Crown Street, Liverpool, L69 7ZD, United Kingdom

dGlaxoSmithKline, St. Georges Avenue, Weybridge, Surrey, KT13 0DE, United Kingdom.

Short title: Activity of gallium on *Streptococcus mutans* and dental tissues

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**\***Corresponding Author. Mailing address:Sabeel P Valappil, Department of Health Services Research and School of Dentistry, University of Liverpool, Research Wing, Daulby Street, Liverpool, L69 3GN, UK Tel +44 (0)151 706 5299, Fax +44 (0)151 706 5809 Email: S.Valappil@liv.ac.uk

## Declaration of Interests

## There are no potential conflicts of interest for any of the authors, listed in this article, pertaining to this study.

## Abstract

Gallium doped phosphate-based glasses (Ga-PBGs) were assessed for their impact on *S. mutans* and dental mineralisation, firstly by disk diffusion assays followed by biofilms grown on nitrocellulose filter membrane (NFM) and constant depth film fermentor (CDFF). Short time exposure (10 min) effects of Ga-PBG on *S.mutans* biofilm were compared with that of 0.2% chlorhexidine. The effects of Ga-PBG on bovine enamel (which was investigated under pH cycling condition) and dentine were analysed using microradiography (TMR), profilometry and inductively-coupled plasma optical-emission spectrometry (ICP-OES) Disk diffusion assays showed inhibition zones of 24.5 ± 0.5 mm for Ga- PBG compared with controls (C-PBG). Ga-PBG showed statistically significant growth inhibition of *S. mutans* biofilms on NFM (*p*=0.001) and CDFF (p<0.046) compared with hydroxyapatite (HA) and C-PBG., CDFF assay revealed maximum of 2.11 log CFU reduction at 48 h but short time exposure effect were comparable with that of 0.2% chlorhexidine only on older biofilms (maximum of 0.59 vs. 0.69 log CFU reduction at 120 h). TMR analyses of enamel revealed non significant mineral loss (p=0.37) only in the case of Ga-PBG samples compared with controls including sodium fluoride. ICP-OES analyses indicated transient gallium adsorption into dentine by calcium displacement. Results confirmed that gallium inhibited *S. mutans* growth and appears to have the potential to protect the enamel surface under conditions representative of the oral environment. Further work is needed to establish whether it has an application in daily oral hygiene procedures to prevent or reduce caries.

## Introduction

Dental caries occurs as a result of complex interactions, over time, between acid-producing bacteria, fermentable carbohydrates and host factors such as teeth and saliva [Gustafsson et al., 1953]. The high numbers of certain oral streptococci, such as human caries associated *S. mutans*, play a pivotal role in dental caries formation [Loesche, 1986]. Salivary clearance modulating bacterial selection directly [Rudney, 2000] or effect imparted systemically through nutrition [Johansson and Ericson, 1987]; such as reducing the intake of fermentable carbohydrates, comprises some targeted adjunct treatments. Some antimicrobial and biofilm modulatory activities have also been demonstrated by weak organic acids and fluorides albeit with limited success [Marquis et al., 2003]. So far there are various agents developed to interfere with microbial growth or metabolism (often by interfering with a single intracellular target) which include chlorhexidine, cetylpyridinium chloride [Wilson et al., 1996], triclosan [Davies et al., 2004] and alcohol [Borrajo et al., 2002]. Chlorhexidine is a widely used antibacterial agent but lack of evidence on long-term clinical outcomes and reported side effects limits its application for caries prevention [Autio-Gold, 2008]. Moreover, *S.mutans* were reported to develop resistance against other antibacterial agents such as cefuroxime, penicillin and tetracycline used for prophylaxis [Leistevuo et al., 2000]. The potential of appropriate new antimicrobial agents for long-term caries management was highlighted recently [ten Cate, 2009; Filoche et al., 2010].

The emergence of bacterial resistance towards antibacterial agents, such as antibiotics, triggered interest in exploring alternative strategies such as the use of trivalent gallium (Ga3+) which are antibacterial agents that possess a new mode of action [Kaneko et al., 2007; Valappil et al., 2008]. Ga3+ interferes with iron-dependent enzymes which cause concurrent action on multiple targets in bacteria and thus mutation of single intracellular targets might not yield high-level Ga3+ resistance among subjected bacteria [Kaneko et al., 2007]. Ga3+ possesses remarkably similar chemical attributes to Fe3+ [Hubbard et al., 1986] and as a result, biological systems are likely to incorporate Ga3+ in place of Fe3+ in many of the Fe3+ requiring enzymes. However, unlike Fe3+, Ga3+ is unable to undergo redox cycling; the incorporation of Ga3+ therefore results in the collapse of cellular processes essential to maintain viability [Olakanmi et al., 2000]. Apart from its antibacterial role, gallium was reported to inhibit bone resorption by direct effect on osteoclasts [Blair et al., 1992] indicating their potential use in oral applications to control dental mineralisation. However, the effect of Ga3+ exposure on *S. mutans*, a caries-associated bacterium, and dental mineralisation dynamics is yet to be resolved. The present work therefore aims to study the *in vitro* effect of Ga-PBG on *S. mutans*, including biofilm and assess its effect on bovine dental enamel and dentine, under conditions reflective of the oral environment.

## Materials and methods

### *Preparation of Ga-PBGs*

PBGs were created using NaH2PO4 (BDH, ≥98%), P2O5 (Sigma, ≥97%), CaCO3 (BDH, ≥98.5%) and Ga2O3 (Sigma, 99.99%). Each reagent was weighed and transferred in to a Quartz crucible (Fisher Scientific, UK), and placed in a preheated furnace at 1100 °C for 1 h, after which the molten PBG was poured into graphite moulds, preheated to 350 °C. The PBGs were cooled to room temperature, prior to cutting them into disks (diameter, 5 mm; thickness,2 mm) by using an Isomet low speed rotary diamond saw (Buehler Ltd, UK). PBGs of composition (CaO)14(Na2O)38(P2O5)45(Ga2O3)3 hereafter given the abbreviation Ga-PBG were prepared with control PBGs (C-PBG) of composition (CaO)20(Na2O)35(P2O5)45 containing no gallium.

### *Antibacterial analyses of Ga-PBG*

*S.mutans* NCTC 10449strain was grownon brain heart infusion agar (BHI), in an anaerobic (N2:CO2:H2, 80:10:10) environmental chamber (Don Whitley MG1000) at 37°C.

#### Disk diffusion assay

Ga-PBG was investigated for its ability to inhibit growth of *S. mutans using* disk diffusion methodology (BSAC Disk Diffusion Method for Antimicrobial Susceptibility Testing, Version 4, 2005). BHI plates were inoculated with standardised cultures of *S. mutans* (~108 cells/mL). Ga-PBG disks were placed on the inoculated plates. C-PBG was used as a negative control. All BHI plates were incubated anaerobically overnight at 370C. The diameters of any zones forming around the disks were measured in mm using callipers, and compared with controls. All experiments were conducted in triplicate.

#### Biofilm assay

##### *Nitrocellulose filter membranes biofilm*

Biofilms of *S. mutans* were grown on nitrocellulose filter membranes, NFM (47 mm diameter, 0.45 μm pore size; Invitrogen Ltd, Paisley, Renfrewshire, UK) laid atop BHI agar plates. Growth of oral biofilms on NFM has successfully been evaluated in a recent *in vitro* study [Valappil et al., 2012]. Firstly, a NFM was placed onto the centre of each BHI plate, following which 100µL of *S. mutans* suspended in PBS (~108 cells/mL) spread evenly across the surface using a sterile spreader for 1 minute. PBG disks; Ga-PBG and C-PBG, were then placed onto the centre of each inoculated NFM. The resulting BHI plates were incubated at 37ºC in an anaerobic cabinet for 72h. After incubation, NFM from plates were transferred into respective sterile containers with 20 mL PBS to disrupt the biofilm. The resulting cell suspensions were then serially diluted in PBS and cultured on BHI plates to enumerate viable bacteria in terms of total colony forming units (CFU, which were log10 transformed) present per NFM.C-PBG disks were used as a negative control and all experiments were conducted in triplicate.

In order to evaluate the short time exposure effect of the gallium, a positive control (0.2% of chlorhexidine digluconate), a negative control (C-PBG dissolved in 50 mL of sterile water for 72h) and test sample (Ga-PBG dissolved in 50 mL of sterile water for 72h) were prepared for a modified NFM biofilm assay. In these experiments, NFM was first placed onto the centre of each BHI plate, following which 100µL of *S. mutans* suspended in PBS (~108 cells/mL) spread evenly across the surface using a sterile spreader and incubated at 37ºC in an anaerobic cabinet for 72h. After the incubation, NFM from plates were transferred into respective containers with 5 mL of positive control, negative control or test sample for 2 minutes. All treated NFM biofilms were transferred into respective sterile containers with 20 mL PBS to disrupt the biofilm. The resulting cell suspensions were serially diluted in PBS and cultured on BHI plates to enumerate viable bacteria (in terms of log10 CFU) present per NFM. All experiments were conducted in triplicate.

##### *Constant depth film fermentor biofilm*

A constant-depth film fermentor, (CDFF) (Cardiff University, Cardiff, UK), was employed for the production of biofilms [Deng and ten Cate, 2004; Valappil et al., 2012]. The CDFF contained a stainless steel turntable and held up to 15 polytetrafluoroethylene (PTFE) pans; each PTFE pan held 5 PTFE plugs. In each PTFE pan disks of C-PBG (5 mm diameter, used as a negative control) or hydroxyapatite (HA, 5 mm diameter, which was used as a negative control as it is known to aid biofilm growth but does not undergo degradation like PBGs in aqueous medium) or Ga-PBG (5 mm diameter) were placed on each plug and recessed by 200 μm. PTFE pans were then slotted in to flush with the turntable. A cylindrical glass vessel and two stainless steel end plates enclosed the turntable. The top plate contained an air inlet port attached to two 0.2μm HEPA filter air vents (Fisher Scientific, Leicestershire, United Kingdom) and 3 medium inlet ports. Incoming medium (in this case saliva type growth medium, STGM [Pratten, 2005] of the composition; Lab-lemco powder 1 g L-1, yeast extract 2 g L-1, protease peptone 5 g L-1, type III hog gastric mucin 2·5 g L-1, sodium chloride 0·2 g L-1, potassium chloride 0·2 g L-1,calcium chloride 0·3 g L-1) was dripped onto the rotating turntable and distributed over the PTFE pans by two scraper blades, which also served to maintain the required depth of biofilms on the disks. The bottom plate contained a medium outlet port. The CDFF was sterilised in a hot air oven at a temperature of 140°C for 3 h. During operation, the CDFF was incubated at 37°C, and rotated at a speed of 3 rpm throughout.

A 24 h culture of *S. mutans* in BHI broth, 10 mL (Oxoid Ltd, Basingstoke, UK) was inoculated into 0.5 L of STGM and circulated through the CDFF kept in an incubator at 37 °C. After 8 h, the fermentor fed from an 8 L medium reservoir of STGM, with the waste flowing into an effluent bottle. The STGM was delivered at a rate of 0·5mL min-1 (to mimic the resting saliva flow rate of healthy individuals [Dawes, 1996]) using a peristaltic pump (Watson and Marlow, UK). At various time intervals (6, 24, 48 and 120h), the pans were removed aseptically from the CDFF. Disks containing biofilms were placed in 1 mL of PBS, vortexed for 1 min to remove and disperse the attached biofilms into suspension. Serial dilutions of the suspensions were carried out in PBS, with 25 μL aliquots of the diluted suspensions spread onto BHI plates, which were incubated anaerobically at 37°C for 72h. For each type of disk, CFUs were determined in triplicate and presented after transforming it in to log10 values.

In order to evaluate the clinical relevance and short time exposure effect of the gallium, *S.mutans* biofilm were grown only on HA discs as described above. At various time intervals (6, 24, 48 and 120h), the pans were removed aseptically from the CDFF and immersed into either 4mL of 0.2% of chlorhexidine digluconate (positive control), 4mL ultrapure water (negative control) or 4mL test sample (Ga-PBG dissolved in 50 mL of sterile water for either 6, 24, 48 or 120h). After 10 minutes exposure, specimens were rinsed with 20 mL sterile ultrapure water three times to wash away any active compounds remaining. The specimens were then transferred in to respective containers with 15mL of sterile cysteine peptone water (5g yeast extract, 1g peptone, 8.5 g NaCl, 0.5g L-cysteine HCl and 100mL glycerine per litre, adjusted to pH 7.3 [Deng et al., 2004]) at 37°C for 1h. After incubation, sterile glass beads were added to each containers and vortexed for 1min to remove attached biofilm and to disperse them into the suspension. For each specimen, serial dilutions of the suspensions were carried out in PBS, with 25 μL aliquots of the diluted suspensions spread onto BHI plates. The plates were incubated anaerobically at 37°C for 72h. For each treatment, CFUs were determined in triplicate and presented after transforming it in to log10 values.

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### *Assessment of Ga exposure on dental tissues*

#### Preparation of Enamel Blocks

15 Blocks were prepared from sound bovine permanent incisors. The central portions of the labial surfaces were successively abraded to a depth of approximately 0.5 mm using wet 300-grit followed by 1000-grit carburundum paper on a rotary grinder (Silfradent model

#### 801, Silfradent, Sofia, Italy), cut to approximately 6 ×3 mm using a diamond wire saw (Well Diamond Wire Saw SA, Le Locle, Switzerland) and painted with nail varnish (MaxFactor Nailfinity, Proctor & Gamble, UK), to leave only polished enamel windows of approximately 3 × 2 mm exposed. After drying at room temperature, the blocks were mounted in 50 mL Sterilin disposable containers (Sterilin Ltd. Newport, UK) using GreenStick impression compound (Sybron Dental Specialties, Kerr, Italia, S.p.A).

#### Ga exposure on Enamel

#### The pH-cycling method previously described by Alves et al., 2011 was used to determine the effect of Ga-PBG on demineralisation. Enamel blocks were assigned to one of 5 conditions (Ga-PBG, C-PBG, No Glass, NaF and Water) and exposed to 5 days of cyclic exposure to an acidic challenge (2.0 mM CaCl2, 2.0 mM KH2PO4, 0.04 ppm F- form NaF and 0.075 mM acetic acid adjusted to pH 4.7 with 1.0 M NaOH) (6h Demineralisations/18h Remineralisation), followed by 2 days in remineralising solution (1.5 mM CaCl2, 0.9 mM KH2PO4, 150 mM KCl, 0.05 ppm F- form NaF and 20 mM HEPES adjusted to pH 7.0 with 1.0 M NaOH). For the Ga-PBG and C-PBG conditions, glass was mounted alongside the enamel. For the water and NaF conditions samples were immersed in water or 228ppm NaF solution for 5 minutes at each solution change.

#### Proscan analyses

Following the pH-cycling, non-contact surface profilometry, NCSP (Proscan 2000, Scantron Industrial Products LTD) data was collected. Removal of all nail varnish using acetone created an area for baseline measurements compared with exposed area. NCSP data was collected with an S5/03 scan-head (resolution 0.01 μm, measuring range 0.3mm with a spot size of 4μm) at a sensor rate of 300 Hz with the averaging (Kalman) filter set at 1 iteration. Each scan included the window of exposed enamel on the enamel surface. Specimens were scanned in both the x and y directions (step size 0.01, steps x = 350 and steps y = 150 (Reversed for y direction), 3 measurements were performed per specimen, and mean surface roughness, Ra, calculated. The images and data were recorded on a computer hard-drive in PRS file format and analysed using software provided by the manufacturer (Proscan 2000, Version 2.1.1.15).

#### Microradiography analyses

Microradiographic analyses were conducted as described previously [Lynch et al., 2011]. Several thin slices were cut form each block corresponding to either exposed or un-exposed areas of the enamel blocks and samples matched as adjacent pairs. Sections were mounted lengthwise on brass anvils, fixed in place with nail varnish (Maxfactor Nailfinity, Proctor & Gamble, UK) and polished on a diamond impregnated grinding disk (15µm particles, Buehler, Illinois) to a final thickness of 80 µm and mounted on a plastic template along with an aluminium step wedge. Microradiographs of the templates were taken on Kodak type 1A high-resolution plates (Kodak, Rochester, USA) exposed to a CuKα X-ray source operating at 10 mA and 30 kV. Exposure time was 25 minutes, the distance from source to template was 300 mm. Microradiographs were examined microscopically (Leica, Wetzlar, Germany). An image of the central, homogeneous portion of each sample was optically centred, typically capturing an area of 300μm of the enamel surface. Corresponding samples sets then provided a comparison for the effect of exposure (exposed enamel section) with respect to a baseline sample (un-exposed section) with minimal variation. Parameters of integrated mineral loss (ΔZ) were assessed using a computerised image analysis system (TMR2006, Inspektor Research Systems, Amsterdam, the Netherlands). ΔZ represented the product of the depth of detectable mineral loss (lesion depth; LD) relative to sound enamel (units; vol%.µm). (Positive values indicate an increase in mineral content).

#### Preparation of Dentine Samples

Dentine samples were extracted using a bromoform extraction method. Bovine incisors were powdered using by a vibrating sample mill and the powder sieved through particulate filters so that particles were ≥ 40µm and ≤ 100µm. 4g aliquots of powder in polypropylene tubes (SARSTEDT Ltd, Leicester, UK), were filled with 10mL ethanol: bromoform (1:4) mixture and the powder suspended by 30s vortex, allowed to stand for approximately 4 h to allow sufficient time for enamel tissue to settle and dentine to float. The separated suspension was then frozen in liquid nitrogen (-1960C) and cut with a hand-held dental saw (Marathon N7, Hayes Handpiece Franchises Inc., USA) to separate enamel from dentine. The frozen suspensions were then allowed to thaw separately in 100% ethanol and the tissues aspirated though 22µm filter membranes. The filter residue was rinsed 3 times in ethanol and once in dH2O and allowed to dry, with occasional shaking to break-up the powder, in a fume cupboard for 2 days. Inductively-coupled plasma optical-emission spectrometry (ICP-OES) elemental distribution charts were used to establish the purity of the sample.

#### Ga Exposure on Dentine

The effect of gallium pre-treatment on dental tissues was also investigated in a static water medium with ionic gallium delivered in the form of gallium nitrate (Ga(NO3)3). In order to evaluate well defined exposure range of gallium, gallium nitrate was used as the gallium source in these experiments. 200mg samples of dentine were exposed to 20ml Ga(NO3)3 solutions ranging in concentration from 0 – 139.44ppm Ga with 0ppm as a control for 4h. The exposed tissues were then rinsed 3 times in dH20, dried and 40mg resuspended in a remineralising solution (1.5mM CaCal2, 0.9mM NaH2PO4, 130mM NaCl, 20mM HEPES, pH 7.2 adjusted with 1M NaOH) supersaturated with respect to hydroxyapatie (Ca10(PO4)6(OH)2) an analogue of dental tissue. Following 15 or 240 minute exposures the samples were centrifuged for 4minutes at 2612 × *g* and the liquid fraction extracted and analysed by ICP-OES for total gallium, calcium and phosphorus. Measurements of these parameters provided an indication of crystal growth following gallium exposure.

#### ICP-OES Analyses

Following gallium exposure, calcium, gallium and phosphate remaining in solution and that present in dentine were measured by ICP-OES. A Spectro Ciros CCD Spectrometer (SPECTRO Analytical Instruments GmbH, Boschstr, DE) calibrated across concentrations in a predicted range of 0.0–40 ppm by dilutions of 1000 ppm element standards (Sigma-Alderich, Dorset, UK) in dH2O was used to provide these measurement. All samples obtained were diluted in dH2O so as to result in an elemental composition within the predicted concentration range. All samples were also subject to an initial broad spectrum analysis and resulting elemental distribution charts used to confirm the absence of residues from the purification and separation processes (eg. bromoform).

Sample analysis was conducted under standard operating conditions (Power: 1400 W, Coolant Flow Rate: 12 L min-1, Auxiliary Flow Rate: 1 L min-1, Nebuliser Flow Rate: 1 L min-1) with a side-on plasma detection system (SOP) providing minimum detection limits for gallium, calcium, and phosphorus of 50 ppb, 0.5 ppb and 50 ppb respectively. Due to the relatively high calcium content predicted within samples regular acidic washout periods (35% HCl) were scheduled approximately every 4 – 6 sample runs in order to alleviate possible signal attenuation form residual build up within the nebuliser unit. Data was collected with specialist software provided by the manufacturer (Smart Analyser of Spectro Smart Studio, Version 2.11.0631).

#### Nitrogen BET Analyses

The mean surface areas of the extracted dental minerals were determined using nitrogen BET calculation. 1g aliquots of enamel, dentine and hydroxyapatite powders were degassed for 15h under constant vacuumed at 120°C. Samples were then analysed in Nova 4200e BET Surface Area Analyser (Quantachrome UK Ltd., Hook, UK). Data was collected and processed with software provided by the manufacturer (NovaWin, Version 10).

### *Statistical Analysis*

Statistical analyses of data such as biofilm viable counts (CFU), surface roughness (*Ra*) and mineral loss (ΔZ) were conducted using GraphPad software (San Diego, CA, USA). Continuous variables were expressed as the mean ± SD (Standard deviation of the mean). One-way analysis of variance (ANOVA) was conducted. . Samples that were pH cycled were compared with baseline and analysed using unpaired *t*-tests. Tukey-Kramer Multiple Comparisons Test was also used to analyse between groups after pH-cycling condition. P values < 0.05 were considered statistically significant.

## Results

### *Antibacterial analyses of Ga-PBG*

Disk diffusion assays were conducted to determine whether Ga- PBGs exhibited any antibacterial activity against *S. mutans*. The zone of inhibition (i.e., zones of no visible bacterial growth surrounding the disks) was found to be 20.5 ± 0.5 mm for the Ga- PBG compared with C-PBG. Once it was established that Ga-PBG had antibacterial effect against *S. mutans* their effect on biofilm growth of these bacteria was investigated. Firstly a simple biofilm model, NFM, was used to evaluate the effect of Ga-PBG on biofilm of *S. mutans* using C-PBG as a control. Ga-PBG showed statistically significant (*p*=0.001) growth inhibition of *S. mutans* (log10 CFU of Ga-PBG; 7.26±0.62 and C-PBG; 10.25 ±0.27) on NFM biofilm. However, the NFM biofilm assay to evaluate short term effect of gallium on *S. mutans* showed that Ga-PBG (log10 CFU; 10.18±0.34) does not have statistically significant (p=0.14) growth inhibitiory effect on *S. mutans* compared with C-PBG (log10 CFU; 10.84±0.51). However, chlorhexidine digluconate (log10 CFU; 9.39±0.19) exerted statistically significant growth inhibition on *S. mutans* compared with both Ga-PBG (p=0.03) and C-PBG (p=0.01). Antibiofilm test on Ga-PBG was carried out in a CDFF that has been reported to match growth conditions in the oral cavity [Deng and ten Cate, 2004]. In the CDFF experiment, at 6 h, the Ga-PBG showed statistically significant difference in log10 of the mean number of viable cells compared to both the C-PBG and HA (p≤0.004) (Figure. 1). At 24 h, the log10 of the mean number of viable cells was significantly reduced for the Ga-PBG compared to both controls (p ≤ 0.002). This effect of the Ga-PBG on the viable count of the biofilms was more prominent at 48 h, even though very high standard deviations were observed; the viable counts were significantly different from both the controls (p ≤0.0001). The greatest effect of gallium on biofilm growth was observed at 48 h (2.11 log10 CFU reduction compared with C-PBG). However, at 120 h the log10 of the mean number of viable cells on Ga-PBG started to recover from the previous low at 48 h but still showed a statistically significant difference compared with C-PBG (p = 0.045).

Further CDFF experiments were carried out to evaluate clinical relevance and short time exposure effect of the gallium. In these experiments, Ga-PBGs did not show statistically significant (p≥0.05) differences in log10 of the mean number of viable cells compared with the negative control (water) at both 6h and 24h (Figure. 2). On the other hand the positive control (chlorhexidine) displayed a statistically significant (p≤0.006) reduction in log10 of the mean number of viable cells compared with water throughout the experiment (Figure.2) However at time points 48h and 120h, the log10 of the mean number of viable cells were significantly reduced for the Ga-PBG compared with water (p≤0.002) but not with chlorhexidine (p≥0.14).

### *Ga exposure on dental tissues*

In order to mimic *in vivo* condition in the oral cavity, effects of gallium on sound enamel have been assessed, under pH-cycling conditions. The results from non-contact surface profiling (Figure 3) showed a trend that the *Ra* values are higher on all samples except the one in the presence of Ga-PBG (where *Ra* decreased by an average of 0.29). Moreover the analyses of *Ra* (μm) data confirmed no statistically significant difference between pH-cycled enamel samples in the presence of Ga-PBG (p=0.62), C-PBG (p=0.50), no glass (p=0.13), NaF (p=0.31) and water (p=0.10) compared with baseline (Figure 3).

TMR analyses of bovine enamel revealed statistically significant mineral loss for most of the samples that had undergone pH cycling compared with baseline; C-PBG (P=0.027), no glass (p=0.026), NaF(P=0.049) and water (p=0.001). However, pH cycled enamel samples in the presence of Ga-PBG displayed statistically non-significant (p=0.35) mineral loss compared with baseline (Figure 4). Mineral loss was quantified in relation to a patch of sound enamel. TMR images of pH-cycled enamel samples in the presence of Ga-PBG also revealed a most unusual pattern of lesion formation occurred with the appearance of several layers of alternating hyper- and hypomineralisation (Figure 5). ICP-OES analyses of Ga treated dentine showed the gallium presence in the mineral phase (Figure 6) but not in the liquid phase .However, a proportional decrease was seen in the calcium present in liquid phase (data not shown) compared with mineral phase (Figure 6). BET Surface area analyses of the minerals showed the maximum surface area achieved for the minerals was; enamel=15m2/g, dentine= 17.2m2/g and HA =46.996 m2/g.

## Discussion

This paper reports the effect of gallium on *S.mutans* and bovine dental tissues. Gallium, which was recently reported to have antibacterial activity on certain oral bacteria [Valappil et al., 2012], was evaluated on *S. mutans* growth. In order to assess the potential use of gallium as an anticaries agent, the effect it may have on dental tissues such as bovine dental enamel and dentine was also evaluated. The use of gallium as an antibacterial agent has made significant progress recently by using the controlled ion release system, PBGs [Valappil et al., 2008; Valappil et al., 2009; Valappil et al., 2012]. The analyses of the PBG degradation data from these reports suggest the degradation rate of the Ga- PBG, up to 48 h, was 14.50 μg mm-2 h-1 (with ion release rates of; Ca=0.40, Na=1.13, P=26.79 and Ga=1.08 ppm h-1) compared with C-PBG which was 10.99 µg mm-2 h-1 (with ion release rates of; Ca=2.33, Na=7.38 and P=11.92 ppm h-1).

In disk diffusion assays, Ga-PBG demonstrated antibacterial effects against *S.mutans*. The fact that a small zone of inhibition was seen around C-PBG for *S.mutans* which was consistent with a previous report where control PBGs, with similar phosphate content, displayed small zones of inhibition against *S.gordonii* [Valappil et al., 2012]. This could be due to the fact that these PBGs are known to exert alkaline pH to the surrounding media during the early phase of PBG degradation and the bacteria belonging to *Streptococcus* genera are susceptible to such pH fluctuation. Due to the consistent local delivery of gallium from the PBG, the iron binding complexes should not affect the Ga3+concentration. The data obtained from NFM and CDFF biofilm models suggested that both the static and the flow system were capable of maintaining good biofilm growth. In the CDFF biofilm study Ga-PBG achieved a statistically significant growth inhibition of *S. mutans* compared with controls but short time exposure effect were comparable with that of 0.2% chlorhexidine only on older biofilms (with a maximum of 0.59 vs. 0.69 log CFU reduction at 120 h). The result thus suggests that Ga-PBG may complement currently available caries preventive agents. These agents could offer some advantages over conventional therapeutic agents as antibiotic-resistant organisms (even those with multi-drug resistance) are likely to be sensitive to gallium. This is probably due to the fact that gallium works by a wholly different mechanism to regular drugs as the Ga3+ has the potential to disrupt Fe3+ metabolism in a wide range of bacteria by employing a “Trojan horse” strategy [Kaneko et al., 2007]. However, the increase of CFU counts after reaching the lowest point at 48h indicated that the Ga ions might be embedded in surface proteins and extracellular polysaccharide layers in the biofilm thus not reaching the active cells in the core of the biofilm. However, the observed re-growth in CDFF experiment is consistent with previously reported studies [Valappil et al., 2012]. In order to overcome such diffusion limitations in clinical application and to extend the action of Ga, we suggest transforming Ga-PBG into powder form (which could then be incorporated into toothpastes or topical gels) which increases the surface area and constant release of high gallium concentrations. Also, such antimicrobial treatment may be more effective if combined with dietary changes for example to obtain a less cariogenic plaque flora for a prolonged period of time. However, to evaluate the potential *in vivo* efficacy of Ga-PBG, further CDFF experiments should be conducted by utilising a dental caries biofilm model, such as the one described elsewhere [Zaura et al 2011], which combines plaque microcosms and dental hard tissue as the substratum.

Surface profiling analyses of bovine enamel revealed no statistically significant changes between pH-cycled samples in the presence of Ga-PBG and controls under conditions reflective of those of the oral environment. Although statistically not significant, the trend in the *Ra* values obtained by NCSP analyses indicate that the surface of the tooth is smoother after it has undergone acid challenge in the presence of Ga-PBG compared with all the controls. However, an increase of *Ra* seen for all of the pH-cycled enamel samples in the presence of controls (including the positive control fluoride) confirmed that the surface roughness cannot directly be correlated to the presence of Ga-PBG. The fact that enamel samples that had undergone pH cycling in the presence of Ga-PBG displayed statistically non significant mineral loss (compared with controls when analysed using TMR) suggest some gallium deposition on the enamel surface may protect the mineral surface. Evidence to support this can be seen clearly in figure 5, where a most unusual pattern of lesion formation occurred with the appearance of several layers of alternating hyper- and hypomineralisation consistent with periods of re- and demineralisation in the presence of Ga-PBG. However, further experiments, utilising chemical analysis, need to be designed to confirm that gallium does have a role in inhibiting mineral loss in enamel. The fact that positive control, NaF, treatment only lasted 5 min as oppose to Ga-PBG that was mounted alongside enamel during the entire process of pH cycling experiment makes it impossible to directly compare the treatment times in this study. More importantly total number of pH-cycled enamel samples for each condition should be increased in future experiments in order to validate the unusual observation made in this study. Nonetheless, the results in this study so far suggest that Ga-PBG which can inhibit *S.mutans* growth could also help to maintain the integrity of the enamel. Further the ICP-OES analyses of Ga treated dentine showed the gallium presence in the mineral phase but not in the liquid phase most likely due to the concentration falling below the detectable level. Combining this observation with that of the proportional decrease of calcium presence in liquid phase (data not shown) compared with mineral phase indicate transient gallium adsorption into the mineral most likely through the displacement of calcium which in turn arrested the crystal growth following gallium exposure.

This was consistent with previous report where gallium nitrate placed in human root canals to assess if it would diffuse across root dentine and reach concentrations high enough to inhibit osteoclasts [Ghazi et al., 2000]. The report showed that gallium was highest adjacent to the root canal space and fell as more peripheral sites were sampled but then rose slightly at the external boundary of the root which is covered with a thin layer of a tubular cementum [Ghazi et al., 2000]. It was reported that *in vivo* administration of Ga3+ interferes with osseous tissue hydroxyapatite (HA) generation resulting in larger and more perfect (lowered CO3/PO4 ratios) crystalline structures [Bockman et al., 1986]. This observation is attributed to a multifactorial decline in the rate of the crystallisation process [Blumenthal et al., 1989]; in effect stabilising HA during formation. Ga3+ is also known to adsorb to the HA crystal surface [Donnelly and Boskey, 1989] and to exert an inhibitory effect on both crystal mineralisation and dissolution directly [Blumenthal et al., 1989; Donnelly and Boskey, 1989].

The need for more effective caries-preventive agents suggests the increasing demand for alternative strategies to combat this infection. The results from this study suggest that Ga-PBG, may offer an effective alternative to currently available antibacterial treatments or could be used to complement current therapies, by allowing the controlled and local delivery of antibacterial gallium ions at carious sites in the mouth.

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## Conceived and designed the experiments: SPV, RJML, SMH. Performed the experiments: SPV, GJO, EJM, NF, GM, RC. Analyzed the data: SPV, RJML, LC, GJO, EJM, SMH. Wrote the paper: SPV, GJO, EJM.

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**Figure legends**

**Fig. 1.** Results from long-term exposure experiments. Totalnumber of viable *S.mutans* obtained from CDFF experiments are shown in y-axis, with x-axis showing biofilm age and exposure to the glasses. The biofilms were formed on hydroxyapatite (HA), control glasses (C-PBG), and gallium glasses (Ga-PBG). The data are average from 6 samples obtained in 2 CDFF runs under identical condition. The error bars represent standard deviation from the mean. Statistical analyses between C-PBG vs. HA are denoted with the letter ‘a’ while C-PBG vs. Ga-PBG with the letter ‘b’. P values < 0.05 were considered statistically significant and highlighted with an asterisk (\*) after the letter.

**Fig. 2.** Results on biofilms of different ages (x-axis) and exposed for 10 min to the three test conditions.Total number of viable *S.mutans* (from biofilms formed on hydroxyapatite in CDFF experiments) that was treated with 0.2% chlorhexidine (CHX), gallium glasses (Ga-PBG) and water is shown in y-axis. The error bars represent standard deviation from the mean (n=5). Statistical analyses between water vs. CHX are denoted with the letter ‘a’ while water vs. Ga-PBG with the letter ‘b’. P values < 0.05 were considered statistically significant and highlighted with an asterisk (\*) after the letter.

**Fig. 3.** Non- contact surface profilometry analyses showing the surface roughness ISO *Ra* (μm) obtained for pH- cycled enamel samples in the presence of gallium glasses (Ga-PBG) control glasses (C-PBG), no glasses (no-PBG), Fluoride (NaF) and water; baseline (blank bars) and pH-cycled (dark bars). The data are averages from 3 enamel blocks. The error bars represent standard deviation from the mean. Statistical analyses within the same group between baseline and pH cycled were carried out together with analyses between groups after pH-cycling condition. Only the statistically significant interactions are highlighted. Statistical analyses between pH-cycled; Ga-PBG vs. no-PBG is denoted with the letter ‘a’ ; C-PBG vs. no-PBG with the letter ‘b’ and NaF vs. no-PBG with the letter ‘c’. P values < 0.05 were considered statistically significant and highlighted with an asterisk (\*) after the letter.

**Fig. 4.** TMR analyses showing average mineral loss (vol%.µm) obtained for pH- cycled enamel samples in the presence of gallium glasses (Ga-PBG) control glasses (C-PBG), no glasses (no-PBG), Fluoride (NaF) and water; baseline (blank bars) and pH-cycled (dark bars).The data are averages from 3 enamel blocks. The error bars represent standard deviation from the mean. Statistical analyses within the same group between baseline and pH cycled were carried out together with analyses between groups after pH-cycling condition. Only the statistically significant interactions are highlighted. Statistical analyses between baseline and pH cycled condition for Ga-PBG, C-PBG, no-PBG, NaF and water were denoted with the letters ‘a, b, c, d, e’ respectively. Statistical analyses between groups after pH-cycling condition such as; no-PBG vs. water and NaF vs. water were denoted with letter ‘f’ and ‘g’ respectively. P values < 0.05 were considered statistically significant and highlighted with an asterisk (\*) after the letter.

**Fig. 5.** TMR images of pH-cycled enamel samples in the presence of NaF (A) compared with pH-cycled enamel samples in the presence of water (B) and Ga-PBG (C). The atypical lesion observed in pH-cycled enamel samples in the presence of Ga-PBG is highlighted with an arrow.

**Fig. 6.** ICP-OES analyses of Ga treated dentine samples showing the gallium, calcium and phosphate ion presence in the mineral phase. The detection limit of gallium, calcium, and phosphorus were set at 50 ppb, 0.5 ppb and 50 ppb respectively. The error bars represent standard deviation from the mean (n=3).