INVESTIGATION ON THE ANTIBACTERIAL PROPERTIES OF NANOHYDROXYAPATITE COATED GUTTA-PERCHA

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INVESTIGATION ON THE ANTIBACTERIAL PROPERTIES OF NANOHYDROXYAPATITE COATED GUTTA-PERCHA

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Title of Research Report ("this Work"): **INVESTIGATION ON THE ANTIBACTERIAL PROPERTIES OF NANOHYDROXYAPATITE COATED GUTTA-PERCHA**

Field of Study: **Endodontics**

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ABSTRACT

Introduction: Coating gutta-percha (GP) with a material similar to the component of dentine could expand it uses to most types of sealer as well as enhance the bonding to the root dentine. Al-Haddad *et al.* (2015) had invented nanohydroxyapatite coated gutta-percha (HAGP) for root canal filling. The technology relates to the deposition of nanohydroxyapatite and tricalcium phosphate coating onto gutta-percha cones. This coating gives improved characteristics such as sealing ability, bonding strength, and ability to form hermetic seal as a root filling material in root canal treatments. However, the antibacterial property for the HAGP is yet to be analysed.

Aim: This study was conducted to examine the antibacterial property of HAGP compared to the conventional GP.

Methods: This study was divided into three parts: preparation of HAGP with biomimetic technique, gamma irradiation sterilization and screening of antibacterial properties. *Escherichia coli (E. coli)* and *Enterococcus faecalis (E. faecalis*) were used to conduct the antibacterial test. The coating involves the surface pretreatment of GP with sodium hydroxide and immersion of GP in simulated body fluid (Tas-SBF), which contains calcium and phosphate ions. These processes result in biomimetic deposition of a thin and uniform layer coating of calcium phosphates and hydroxyapatite. Subsequently, the prepared HAGP and conventional GP were sterilized using gamma irradiation with a dose of 13 kGy. The screenings of antibacterial activities were performed using agar diffusion assay and adherence assay at three time durations, 24, 48 and 72 hours. A total of three samples GP were used; conventional GP soaked in sodium hypochlorite (positive control), GP soaked in distilled water (negative control) and HAGP. In agar diffusion test, all samples were incubated on agar containing *E. coli* and *E. faecalis* for three different

times to assess the formation of inhibition zone. In adherence assay, all samples were exposed in bacteria suspension for three different times. The colonization of bacteria were collected as colony forming unit (CFU) by serial dilution and droplet method.

Results: Based on the antibacterial analysis, the data showed *E. coli* and *E. faecalis* were both susceptible against HAGP when compared to positive control (GP soaked in sodium hypochlorite). The data showed that HAGP produced no zone of inhibition when incubated with *E. coli* and *E. faecalis*, compared to positive control at 24, 48 and 72 hours. Moreover, the adherence study showed that the HAGP attracts more bacteria colonization compared to negative control. Based on the *E. coli* activity towards HAGP, the highest adherence capacity was found to be at 24 hours with significant differences ($p < 0.05$) when compared to negative control (GP soaked in sterile distilled water) and the percentage of adherence at 182.81% ± 49.13. The capacity of *E. coli* to adhere on HAGP was reduced from 48 to 72 hours with no significant difference in time ($p > 0.05$). However, the maximum adherence capacity for *E. faecalis* was at 48 hours (166.09% ± 3.56) and reduced at 72 hours (107.51% ± 6.42) with significant different in time ($p < 0.05$).

Conclusion: The HAGP has no antibacterial properties against *E. coli* and *E. faecalis*. Therefore, it may not be the best to coat the GP with HA alone.

Keywords: Hydroxyapatite, gutta-percha, antibacterial properties, *E. coli*, *E. faecalis.*

ABSTRAK

Pengenalan: Menyalut gutta-percha (GP) dengan bahan yang serupa dengan komponen dentin dapat memperluas ia digunakan untuk kebanyakan jenis simen serta meningkatkan ikatan pada dentin akar. Al-Haddad et al. (2015) telah mencipta nanohydroxyapatite coated gutta-percha (HAGP) untuk mengisi kanal akar. Teknologi ini berkaitan dengan pemendapan salutan nanohydroxyapatite dan tricalcium fosfat ke konveksi gutta-percha. Salutan ini memberikan ciri-ciri yang lebih baik seperti keupayaan pengedap, kekuatan ikatan, dan keupayaan untuk membentuk meterai hermetik sebagai bahan pengisian akar dalam rawatan kanal akar. Walau bagaimanapun, sifat antibakteria untuk HAGP belum dianalisis.

Matlamat: Kajian ini dijalankan untuk mengkaji untuk masa kini sifat antibakteria HAGP berbanding dengan GP konvensional.

Kaedah: Kajian ini dibahagikan kepada tiga bahagian: penyediaan HAGP dengan teknik biomimetik, pensterilan penyinaran gamma dan pemeriksaan sifat antibakteria. *Escherichia coli (E. coli)* dan *Enterococcus faecalis (E. faecalis)* digunakan untuk menjalankan ujian antibakteria. Pelapisan melibatkan permukaan pretreatment GP dengan natrium hidroksida dan rendaman GP dalam cecair badan simulasi (Tas-SBF), yang mengandungi ion kalsium dan fosfat. Proses-proses ini menghasilkan pemendapan biomimetik lapisan lapisan nipis dan seragam kalsium fosfat dan hidroksiapatit. Seterusnya, HAGP yang disediakan dan GP konvensional disterilkan menggunakan penyinaran gamma dengan dosis 13 kGy. Penyaringan aktiviti antibakteria dilakukan dengan menggunakan ujian penyebaran agar dan ujian pematuhan dalam tiga jam yang berbeza, 24, 48 dan 72. Sebanyak tiga sampel GP digunakan; GP konvensional direndam dalam natrium hipoklorida (kawalan positif), GP direndam dalam air sulingan (kawalan negatif) dan HAGP. Dalam ujian penyebaran agar, semua sampel diinkubasi pada agar

yang mengandungi *E. coli* dan *E. faecalis* dalam tiga masa yang berlainan untuk menilai pembentukan zon inhibisi. Dalam kes kepatuhan, semua sampel terdedah dalam penggantungan bakteria dalam tiga masa berlainan. Penjajahan bakteria dikumpulkan sebagai unit pembentukan koloni (CFU) melalui kaedah pengencangan dan titisan siri.

Keputusan: Berdasarkan analisis antibakteria, data menunjukkan bahawa *E. coli* dan *E. faecalis* terdedah kepada HAGP berbanding dengan kawalan positif (GP direndam dengan natrium hipoklorit). Data menunjukkan bahawa HAGP tidak menghasilkan zon perencatan apabila diinkubasi dengan *E. coli* dan *E. faecalis*, berbanding dengan kawalan positif pada 24, 48 dan 72 jam. Selain itu, kajian kepatuhan menunjukkan bahawa HAGP menarik lebih banyak kolonisasi bakteria berbanding dengan kawalan negatif. Berdasarkan aktiviti E. coli terhadap HAGP, kapasiti kepatuhan tertinggi didapati pada 24 jam dengan perbezaan yang signifikan ($p < 0.05$) berbanding dengan kawalan negatif (GP direndam dengan air suling steril) dan peratusan pematuhan di (182.81 % ± 49.13). Kapasiti *E. coli* untuk mematuhi HAGP dikurangkan dari 48 hingga 72 jam tanpa perbezaan masa yang ketara ($p > 0.05$) Walau bagaimanapun, kapasiti pematuhan maksimum untuk *E. faecalis* adalah 48 jam dengan (166.09% ± 3.56) dan dikurangkan pada 72 jam kepada (107.51% ± 6.42) dengan perbezaan masa yang ketara ($p < 0.05$)

Kesimpulan: Daripada data ini, dapat disimpulkan bahawa HAGP tidak mempunyai sifat antibakteria terhadap *E. coli* dan *E. faecalis*. Oleh itu, ia mungkin bukan yang terbaik untuk melekat GP dengan HA sahaja tetapi dengan menggabungkan HA dengan perak, zink dan strontium ion untuk meningkatkan aktiviti antibakteria pada GP.

Kata kunci: Hidroksiapatit, gutta-percha, antibakteria, *E. coli*, *E. faecalis*

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-Firdaus-

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HAGP	:	Hydroxyapatite coated gutta-percha
HA	:	Hydroxyapatite
GP	:	Gutta-percha
NaOH	:	Sodium hydroxide
NaOCl	:	Sodium hypochlorite
dH_2O	:	Distilled water
RCT	:	Root canal treatment
ZnO	:	Zinc Oxide
H_2O_2	:	Hydrogen peroxide
kGy	:	Kilo gray
CaP	:	Calcium phosphate
$Ca(OH)_2$	:	Calcium hydroxide
SBF	:	Simulated body fluid
Tas-SBF	:	TRIS-buffered simulated body fluid

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Bacteria and their byproducts are the primary cause of pulpal and periapical pathosis (Kakehashi *et al.*, 1965). Bacterial elimination from the root canal is achieved by both chemical disinfection and mechanical preparation of the root canal system. Despite the variety of available chemical irrigants and mechanical strategies, the complete elimination of microbes from the canal system in all cases is impossible (ref). Therefore, the use of root canal filling materials with antibacterial activity is considered beneficial (ref).

Recently, a novel apatite calcium phosphate coated gutta-percha (HAGP) was produced to enhance the adhesion of GP cones to root sealers and to root dentine (Al-Haddad *et al.*, 2015). Theoretically, using bioactive hydroxyapatite can lead to the deposition of inorganic particles on the root surface during the degradation process thereby increasing the sealing ability and inducing the growth of crystals on the material surfaces (Johns *et al.*, 2010). The biocompatibility and biomineralization properties of apatite calcium phosphate coated gutta-percha are added values to increase the uses of this material (Johns *et al.*, 2010). Hydroxyapatite is a bioactive material that could be produced in a uniform thickness to increase the bonding ability of bone and similarly the dentine (Al-Haddad *et al.*, 2015). Since this was the first study that showed the sealing ability of HAGP, it is of interest to further investigate whether the HAGP has an antibacterial property as a root canal filling materials.

The microbiota of infected root canals is polymicrobial and is dominated by Gram-negative anaerobes (Baumgartner & Falkler, 1991; Baumgartner *et al.*, 2003). It has been demonstrated that the presence of residual bacteria in root canal is connected with significantly higher rates of treatment failure (Sjögren *et al.*, 1997). A few of the

most commonly be found bacteria in root canal secondary infection are, *Pseudomonas aeruginosa, Staphylococcus* species, *Escherichia coli* and other enteric rods, *Candida* species, and *Enterococcus faecalis* (Ørstavik & Ford, 2008). *E. faecalis*, a Gram-positive facultative anaerobe, is commonly found in the root canals of failing endodontically treated cases (Rôças *et al.*, 2004). *E. faecalis*, is found in 4 to 40% of primary endodontic infections and in 24 to 77% of persistent endodontic infections (Stuart *et al*., 2006). It has the ability to survive in the root canal as a single organism (Sundqvist *et al.*, 1998; Sundqvist & Figdor, 1998) and to resist nutrient starvation for a long period of time (George *et al*., 2005). Another microorganism which has often been found in infected root canals is *E. coli,* a Gram-negative facultative anaerobe (Ayhan *et al.*, 1999). The microorganisms found in the root canals of primary teeth are similar to those in the root canals of permanent teeth (Önçağ *et al.*, 2003).

Following this, a few studies have developed an improved coated GP to prevent the reinfection in root canal procedures. In 2008, nanosilver (AgNPs) coated GP was produced by Iranian researchers as an attempt to improve the antibacterial effect of GP. This newly AgNPs coated GP, has significantly improved on the antibacterial effects against *E. faecalis, S. aureus, C. albicans* and *E. coli* (Dianat & Ataie, 2008). Subsequently, in 2015, nanodiamond-amoxicillin coated GP was produced by a group of researchers from Korea (Lee *et al.*, 2015) which results in eliminating pre-existing microbes and also shown to prevent reinfection of the root canal system. Whereas, Alves *et al.* (2018) have produced the zinc oxide (ZnO) coated GP to improve antibacterial effect and biocompatibility in endodontics treatment. Based on their outcomes, the deposition of ZnO on GP, has increased its antibacterial activity against *E. faecalis* and *S. aureus*, and may prevent secondary endodontic infections.

Therefore, this study was conducted to investigate whether the HAGP possess the antibacterial activity. The study design was planned and prepared as shown in Figure 1.1. Firstly, the conventional GP was coated with HA via biomimetic coating technique which was adapted from previous study (Al-Haddad *et al.*, 2015). All GPs were sterilized using gamma irradiation procedure prior to the antibacterial study. Two types of bacteria were used for this study, *E. coli* and *E. faecalis*. A total of three samples of GP were used; conventional GP soaked in sodium hypochlorite (positive control), GP soaked in distilled water (negative control) and HAGP. All samples were analysed using agar diffusion assay to obtain the formation of zone of inhibition. In brief, all samples were incubated on agar containing *E. coli* or *E. faecalis*. Subsequently, both of the bacteria were subjected for adherence assay in order to investigate the adherence capacity on HAGP compared to positive and negative control. The antibacterial study was conducted at three different times, 24, 48 and 72 hours. Finally, the results were collected and analysed using statistical analysis. Based from the collected data, independent t-test and one-way ANOVA methods were used to analyse the data. All experiments were conducted in triplicates.

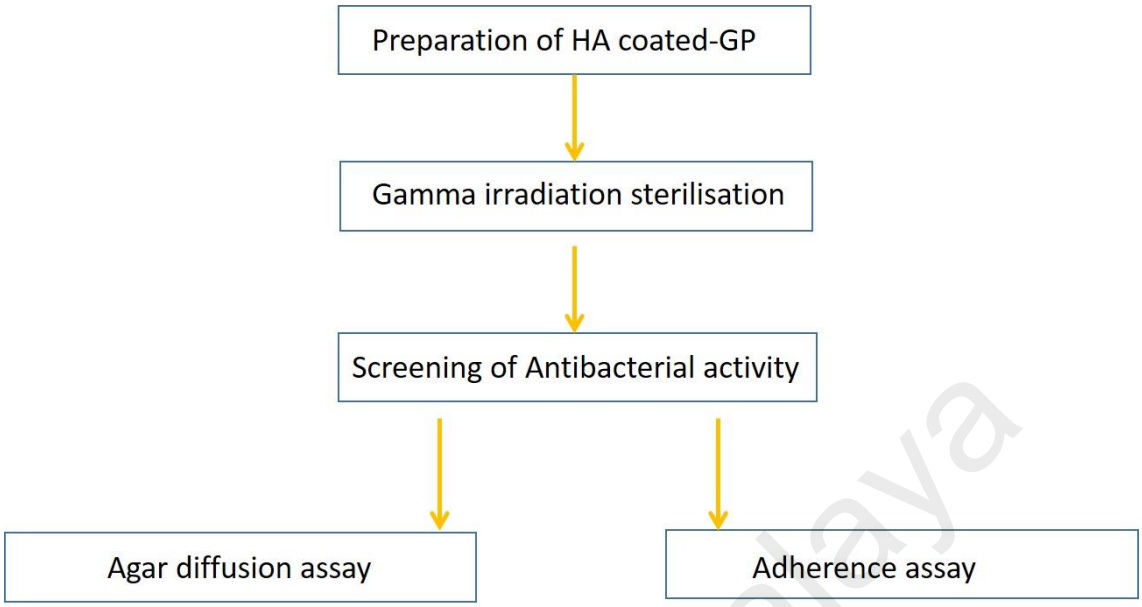

Figure 1.1: An outline of research design

1.2 Aim of the study

The aim of the study is to investigate the antibacterial effect of HAGP. Therefore, to achieve the aim, this study has three objectives as followings:

1.3 Objectives of the study

1. To investigate the antibacterial properties of HAGP on *E. faecalis*, and *E. coli* using agar diffusion assay.
2. To determine the adherence effect of HAGP on *E. faecalis*, and *E. coli*
3. To determine the influence of exposure time on the adherence capacity of bacteria on HAGP.

1.4 Null hypothesis

There is no differences in the antibacterial property between HAGP and conventional gutta-percha.

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of Root Canal Treatment (RCT)

According to American Association of Endodontists (AAE), root canal treatment is the treatment and prevention of disease and injuries of the pulp and associated periradicular tissues (ref). The goal of this treatment is to prevent or cure apical periodontitis and to maintain the treated teeth in function (Marquis *et al.*, 2006). This treatment is comprised of three essential phases. The first phase is diagnostic phase, which is to delineate the disease and to establish the treatment plans (Cohen & Liewehr, 2002). Subsequently is preparatory phase which is to clean the canals by removing the inflamed or infected pulp tissue, microbes, and microbial products by chemo-mechanical preparation and to shape the canals into a continuously tapered form to facilitate root obturation (Schilder, 1974). Success in endodontic treatment depends almost completely on how well the root canal is shaped and cleaned (Byström & Sundqvist, 1981). Finally, is the obturation phase, which is to prevent reinfection by providing a three dimensional hermetic seal as close as possible to cemento-enamel junction with an inert material (Schilder, 2006).

2.2 RCT obturation materials

Root canal obturation materials have three primary functions: sealing the canal against leakage of bacteria and fluids from the oral cavity, entombing the microorganisms that survive the preparation procedures, and filling the canal at a microscopic level to prevent stagnant fluid from accumulating and serving as nutrients for bacteria (Sundqvist & Figdor, 1998). The classic list of desirable properties of root filling materials was adapted from the 'Grossman's Criteria' (ØRstavik, 2005). Orstavik listed 10 requirements for an ideal root filling material (Table 2.1). The current obturation materials composed of two

parts: a core that fills the majority of the canal space namely gutta-percha and the sealer that fills the gap between the core and the root dentine.

Table 2-1: Requirements for an ideal root filling cement. Grossman Criteria, 1978 (Orstavik, 2005)

It should be easily introduced into the canal
It should seal the canal laterally as well as apically
It should not shrink after being inserted
It should be impervious to moisture
It should be bacteriostatic or at least not encourage bacterial growth
It should be radiopaque
It should not stain tooth structure
It should not irritate periapical tissue
It should be sterile, or quickly and easily sterilized before insertion
It should be easily removed from the root canal if necessary

2.2.1 **Gutta-percha**

Gutta-percha (GP) has been widely used as root obturation material (Tsukada *et al.*, 2004) and it was first introduced in 1847 (Prakash *et al.*, 2005). GP is a rubber-based material obtained from the sap of the Taban tree (Isonandra percha), native to Malaysia (Combe *et al.*, 2001). GP polymer is a trans-1,4-polyisoprene, thus GP polymer is harder, brittle and less elastic than natural rubber (Gurgel-filho *et al.*, 2003). The chemically pure GP exists in two distinctly different crystalline forms, alpha and beta, that can be converted interchangeably depending on the temperature (Rootare & Powers, 1977). The GP comes directly from the tree in alpha phase form. Most of the commercially available GP is the beta crystalline form at 37ºC, which transforms to the alpha phase when heated to 46-48ºC (Combe *et al.*, 2001). The two forms have a few differences in physical

properties. The melting point of pure GP is 80°C, and it can be reduced to 60°C based on the percentage of wax and resin added to commercial GP (Tsukada *et al.*, 2004).

The GP used in dentistry is composed of approximately 20% of weight GP (matrix), 65-75% zinc oxide (filler), and 5-10% heavy metal sulfates (radiopacifier), waxes and/or resins (plasticizer) (Spangberg, 2002). GP is the standard obturation material to which other materials are compared and it has many advantages such as biocompatibility, non-staining and radiopacity. It also can be easily removed from the canal when necessary (Nguyen, 1994). GP expands slightly on heating and it shrinks as it returns to body temperature. This property requires use of pressure to compact the heated GP as it cools to prevent contraction gaps from developing.

GP is manufactured in the form of cones in both standardized and non-standardized sizes. GP is also available in either pellet form or in cannules to be used in injectable thermoplastic obturation techniques. It is available in a heatable syringe and carrier coated with a layer of α-phase gutta-percha (Prakash *et al.*, 2005).

GP is non-irritant and non-toxic in nature; however, cytotoxicity from dental GP materials is related to the high content of zinc oxide (Pascon & Spngberg, 1990). Additionally, it possesses poor sealing ability as resultant of poor bonding to dentine and sealers. It is also not able to reinforce the roots of endodontically treated teeth because its low value of cohesive strengths and moduli of elasticity (Williams *et al.*, 2006). GP has been proven to demonstrate slight antibacterial property, mainly attributed to the zinc oxide in its components (Moorer & Genet, 1982). However, Bodrumlu &Alaçm (2006) demonstrated that GP has insignificant antibacterial properties.

2.2.2 **Coated GP with antibacterial properties**

In view of the high prevalence of anaerobes and facultative anaerobes in unsuccessful endodontic therapy, it is crucial that the antibacterial activity of root canal obturation material help to eliminate residual microorganisms unaffected by either chemomechanical preparation or intracanal medication. Therefore, it has been advocated that the root filling material should have antibacterial properties. The first study was demonstrated by (Podbielski *et al.*, 2000) where they had developed a GP contained with calcium hydroxide, and it showed greater antibacterial effects against *E. faecalis*. Subsequently, Barthel *et al.* (2002) evaluated antibacterial effect of GP contained chlorhexidine and it showed an improved antibacterial activity. However, Tanomaru *et al.* (2007) tested both GPs against calcium hydroxide paste, and the result was GP contained chlorhexidine showed greater effect against antibacterial activities.

In 2008, nanosilver (AgNPs) coated GP was produced by Iranian researchers as an attempt to improve the antibacterial effect of GP. This newly AgNPs coated GP, has significantly improved on the antibacterial effects against *E. faecalis, S. aureus, C. albicans* and *E. coli* (Dianat & Ataie, 2008). Besides that, Shantiaee *et al.* (2011) have showed that this nanosilver coated GP presented similar cytotoxicity to conventional GP in *in vivo* study. Subsequently, in 2015, nanodiamond-amoxicillin coated GP was produced by a group of researchers from Korea (Lee *et al.*, 2015). This type of GP was invented to overcome some of the limitations occur during root canal procedures, including leakage, root canal reinfection and poor mechanical properties. They have demonstrated that the nanodiamond-amoxicillin coated GP is improved in terms of mechanical robustness, thus improving the handling properties during clinical implementation. In addition, the nanodiamonds used were incorporated with amoxicillin to eliminate pre-existing microbes and to prevent reinfection of the root canal system. Recently, Alves *et al.* (2018) have produced the zinc oxide (ZnO) coated GP for improved

antibacterial effect and biocompatibility in endodontics treatment. Based on their outcomes, the deposition of ZnO on GP has increased its antibacterial activity against *E. faecalis* and *S. aureus*.

2.3 Hydroxyapatite (HA)

Hydroxyapatite (HA) is considered one of the most biocompatible and bioactive materials, and has gained wide acceptance in medicine and dentistry in recent years. It is mainly found in the form of a nonstoichiometric calcium-deficient carbonate apatite (Eick *et al.*, 1997). Nano-hydroxyapatite in sized (1-100 nm) has been used as the main component of bone and dentine (Wei & Ma, 2004). Synthetic HA is the most recognized calcium phosphate ceramic that is used in bulk form or as coating material. All forms of synthetic HA have excellent biocompatibility and bioactivity to react with surrounding hard and soft tissue (Hannig & Hannig, 2010). The similarity in chemical and structural compositions of synthetic HA, bone and dentine makes it the most used material for medical and dental applications such as; bone graft, coatings to metal implants, and as scaffold for tissue regeneration (Hannig & Hannig, 2010).

2.3.1 Dental applications of HA

HA has been used in the dental field for various purposes such as coating of dental implants (Lee *et al.*, 2000), restoration of edentulous atrophic ridges (Piecuch, 1986), intrabony periodontal pockets (Meffert *et al.*, 1985), periodontal defects (Yukna *et al.*, 1989), and ridge augmentation prior to implant placement for metal prosthetics (Hallman *et al.*, 2002).

The use of HA as in restorative or filling materials offers several promising advantages, including enhancing radiopacity, polishability and wear performance (Arcís *et al.*, 2002). Adding HA of different particles size (nano and micro) as fillers for dental resin composites was found to improve the mechanical properties of composite (Domingo

et al., 2003). The use of HA nano-rods filler in adhesive systems also improve the mechanical properties of adhesive and its bond strength to dentine (Sadat-Shojai *et al.*, 2010).

HA has also been used successfully for endodontic treatment. Hayashi *et al.* (1999) reported that HA is an effective direct pulp capping material and the wound healing process after HA application is more appropriate than that of $Ca(OH)_2$. The biocompatibility of HA is established and that makes it a suitable material for apical barrier formation and repairing of both periapical defect (Pissiotis & Spngberg, 1990) and bifurcation perforation (Chau *et al.*, 1997). Furthermore, HA has been used as filler for root canal sealer. The primary reasons for use of HA or its precursor calcium phosphate as a root canal sealer were its biocompatibility, enhanced the healing of bone in case of apical periodontitis and its sealing ability to a furcation perforation (Cherng *et al.*, 2001).

Calcium phosphate was first introduced as possible restorative dental cement by (LeGeros, 1988). However, the first documented use of bioceramic materials as a root canal sealer was not until two years later when K. F. Krell and Wefel (1984) compared the efficacy of experimental calcium phosphate cement with Grossman's sealer in extracted teeth. They found no significant difference between either sealer in terms of apical occlusion, adaptation, dentinal tubule occlusion, adhesion, cohesion or morphological appearance. Nonetheless, the experimental calcium phosphate sealer failed to provide apical seal compared to the Grossman's sealer (K. Krell & Madison, 1985). Chohayeb *et al.* (1987) later evaluated the use of calcium phosphate as a root canal sealer in animal model using adult dog teeth. They reported that the calcium phosphate-based sealer produced a more uniform and tighter adaptation to the root dentine as compared to GP (Chohayeb *et al.*, 1987).

2.3.2 **Methods of coating with HA**

HA is a bioactive material and it is commonly used as a coating material to improve the surface properties and biocompatibility of the substrate (Kokubo *et al.*, 2004). Among the various methods for depositing a layer of HA on the surface of the substrate (dental implant and orthopaedic alloy), the most used methods are; electrophoretic deposition, sol-gel, dip coating, sputter, plasma spray, biomimetic, mixing dynamic, hot isostatic pressing and pulsed laser deposition (Jiang *et al.*, 2009).

2.3.3 **Biomimetic coating**

Briefly, there are two essential steps for biomimetic coating. Firstly, pre-treatment of the substratum surface for modification of the surface topography, the chemical composition or form a new surface layer (Zhao *et al.*, 2011). Secondly, changing the simulated body fluid (SBF) solution which is critical to maintain the level of pH and to prevent decrease in calcium phosphate (CaP) precipitate (Marques *et al.*, 2003). SBF solution is a supersaturated preparation of calcium and phosphate solution used to form HA-coating on a substratum through soaking of the substratum. This artificially prepared supersaturated CaP is known as SBF and is prepared at physiological temperature and pH (S Jalota *et al.*, 2006).

Pre-treatment of the substratum can be performed through various methods including physical methods, chemical and surface-induced mineralization. The physical method is to produce coarse and porous surface using grit blasting (Habibovic *et al.*, 2002). Chemical methods such as; alkali or alkali-heat treatment, acid-alkali treatment, hydrogen peroxide (H_2O_2) treatment or a combination of these methods (Zhao *et al.*, 2011), and surface–induced mineralization by introducing surface-functional end groups including -COOH, -SO3H, -PO4, -CH3, and -NH2 to the substratum (Campbell *et al.*, 1996).

Simulated body fluid (SBF) solutions are able to induce apatite calcium phosphate formation on metals, ceramics or polymers immersed in them with proper surface treatments (Jalota *et al.*, 2006). As reported by Kokubo *et al.* (2004) calcium phosphate is meta-stable in SBF and it eventually transforms into crystalline apatite. Once apatite nuclei are formed at the initial stage of coating, they spontaneously will grow by consuming the calcium and phosphate ions from the SBF to form a dense and uniform apatite layer (Kokubo *et al.*, 2004). Different SBF solutions have been used to deposit apatite calcium phosphate on substrates such as conventional-SBF, revised-SBF and Tas-SBF (Kokubo & Takadama, 2006). The difference between these solutions is the concentration of bicarbonate Tas-SBF solution was reported to have a HCO3- concentration mimicking the human plasma (27 mM) and it was able to produce even carbonated calcium phosphate coating compared to conventional-SBF and revised-SBF. In addition, it can induce a nano porous apatite formation on metal and bone substitutes under the biomimetic conditions of 37^{o}C and pH 7.4 (Jalota *et al.*, 2006; Jalota *et al.*, 2008). Apatite-inducing ability of Tas-SBF on a polymer substrate has been reported success by Al-Haddad *et al.* (2015)

2.4 Bacteria in endodontics

Microorganisms and their products are considered to be the primary etiological agents in endodontic diseases (Narayanan & Vaishnavi, 2010; Pinheiro *et al.*, 2003). Endodontic infections are considered polymicrobial and more than 150 bacterial species are usually found in combinations with 3 to 6 species in each canal. Species commonly associate with secondary infections include, *P. aeruginosa, Staphylococcus* species, *E. coli* and other enteric rods, *Candida* species, and *E. faecalis.* All of them not usually found in primary infection (Ørstavik & Ford, 2008). In this study, two types of bacteria have been used namely *Enterococcus faecalis* and *Escherichia coli*.

2.4.1 *Enterococcus faecalis*

Enterococcus faecalis (E. faecalis) is frequently isolated from root canals in cases of pulp infections and also recalcitrant infections after endodontic treatment. Reports show that chronic failure of an endodontically treated tooth is due to ability of *E. faecalis* to bind to the collagen of the dentinal tubule and remain viable within the tubules and as many as 38% of the failed root canal systems were contaminated with *E. faecalis*. These bacteria also reported to be found in 4 to 40% of primary endodontic infections and in 24 to 77% of persistent endodontic infections (Stuart *et al.*, 2006). These microorganisms have the ability to grow even in a low-nutrient environment and can survive in the root canals as a monoinfection (Cambala *et al.*, 2019). It is a facultative anaerobic Gram-positive coccus that can be a normal inhabitant of the oral cavity. It is also found in a much higher incidence in failed root treatment cases compared to primary infections (Figdor *et al.*, 2003). In addition, *E. faecaelis* has been shown to be able to form biofilms in root canals and has the ability to survive in environments with scarcity of nutrients. Sedgley *et al.* (2005) demonstrated that these bacteria are able to recover from a prolonged starvation state in root-canal-treated teeth. Therefore, *E. faecalis* is often used as a model organism to evaluate the antimicrobial effectiveness of different irrigants, medicaments, and sealers (Lima *et al.*, 2012). In conclusion, all these properties assist explain the significantly high prevalence of *E. faecalis* in root canal treated teeth (Sedgley *et al.*, 2005).

2.4.2 *Escherichia coli*

E. coli is Gram-negative, facultative anaerobic, rod-shaped, coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Tenaillon *et al.*, 2010). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination (CDC National

Center for Emerging and Zoonotic Infectious Diseases 2012) . The harmless strains are part of the normal microbiota of the gut, and can benefit their hosts by producing vitamin K_2, and preventing colonization of the intestine with pathogenic bacteria, having a symbiotic relationship (Hudault *et al.*, 2001). *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards (Russell & Jarvis, 2001).

Secondary intraradicular infection is caused by microorganism that were not present in the primary infection, but that were introduced into the root canal system at some time after professional intervention. The moment can be during treatment, between appointments, or even after the conclusion of the endodontic treatment. *E. coli* and *E. faecalis* are both bacteria which are also commonly associated with secondary infections. *E. coli* is sometimes recovered from root canals and represents a standard organism used in antibacterial testing (Haapasalo *et al.*, 1983; Siren *et al.*, 1997).

2.5 *In vitro* assay for antibacterial properties

Antibacterial assays are important tools to test and screen the inhibitory effects of myriad compounds against microorganisms before establishing their inhibitory spectra (broad vs. narrow). Knowledge of the inhibitory spectra of antimicrobial compounds before their application in the fields of agriculture, biotechnology, and medicine is crucial. Various conventional and contemporary methods are available, but they vary in their sensitivity and efficacy. In this study, our aim was to measure the efficacy of HA using an agar-based diffusion assay and adherence assay.

2.5.1 Agar-based diffusion assay

The agar-based diffusion assay or Kirby-Bauer Test is the most commonly used technique to assess antibacterial activity. The activity is evaluated by measuring the kinetics of bacterial growth. Insoluble materials can be tested with this quantitative assay.

The size of this zone depends on many factors, one being how effective the compounds or molecules is, at stopping the growth of the bacterium. Another factor that will influence the size of a zone is the diffusion of the compounds or molecules within the agar medium and varies based on the molecular configuration of the antibiotic. Once the zone diameter is measured it must be compared to a database of zone standards to determine if the bacterium being studied is susceptible, moderately susceptible or resistant to the antibiotic. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration on the agar is greater than or equal to the effective concentration which is referred to as zone of inhibition. The zone of inhibition with the rate of antibiotic diffusion is used to estimate the bacteria's susceptibility to that particular antibiotic. The agar diffusion test used in this study is one of the most frequently used methods for assessment of the antimicrobial activity of endodontic materials (Bodrumlu & Alaçm, 2006). It allows direct comparisons of the filling materials against the test microorganisms, indicating which material has the potential to eliminate bacteria in the local microenvironment of the root canal system. In this study, it involves the transfer by diffusion of the antibacterial agent from the gutta-percha to an agar plate. After a few hours of diffusion, the gutta-percha is removed and the agar plate is further incubated. The growth inhibition zones appear in the places, where the antibacterial compounds contact with the agar layer.

2.6 Disinfection in dental root canal

The most commonly used root canal filling material for many years is a biocompatible latex compound commonly called Gutta-percha (GP), which comprises polyisoprene, or trans-polyisoprene with a chemical composition of 1,4-trans-polyisoprene (TPI). The filling material is made into cone/point shape with different diameter sizes at the tip of the cone. Typically, the filling cones are supplied in bulk (with 50 to 100 cones within a plastic container) and are non-sterile. It has been long desired by endodontists (e.g., by

pioneer endodontist Dr. Louis I. Grossman's) that these filling cones should be sterilized prior to insertion into root canals of in vivo teeth. Failure to completely sterilize the root canal sealing cone could lead to future bacteria colonization inside the root canal system, and re-infection and possible loss of the tooth. Due to the low melting temperature threshold for GP material, the most widely used and very effective heat based sterilization method such as heat steam autoclave is however off limits. Throughout the years, clinicians and researchers have tried so-called "cold sterile' technique with different chemical agents, such as sodium hypochlorite, glutaraldehyde, and chlorhexidine. Research papers have shown that these agents require sufficient time to be adequate to achieve sterilizing effect, sometimes hours for these chemical agents to take effect. This raises two fundamental questions: (a) are these chemical agents bactericidal or bacteriostatic; and (b) are the results achieved by using these chemical agents simply disinfection or true sterilization. Generally, disinfection and sterilization are both decontamination processes. Disinfection generally refers to the process of eliminating or reducing harmful microorganisms from inanimate objects and surfaces. Sterilization generally refers to the process of killing substantially all microorganisms, these being the main difference between sterilization and disinfection.

2.6.1 **Sodium hypochlorite disinfection**

Sodium hypochlorite is one of the cold disinfection chemical solutions besides ethyl alcohol, hydrogen peroxide and chlorhexidine liquid. It is one of the most widely used endodontic solutions, either as an irrigant or for dental dam and cone decontamination. Its concentration ranges from 0.5% to 5.25%. Sodium hypochlorite has antibacterial activities related to the liberation of active chlorine. Gomes *et al.* (2005) also showed that sodium hypochlorite was able to disinfect bacteria spores at 5.25% of concentration. Taken from this study and other related studies, it can be concluded that the antibacterial

activity of sodium hypochlorite was related to its concentration, i.e., higher concentrations took less time to inhibit bacterial growth than lower concentrations.

2.6.2 Gamma irradiation sterilization

For irradiation sterilization purpose, gamma ray power is measured as absorbed dose units, in Kilo Gray (kGy). The irradiation dosage may be in the range between 5 kGy and 20 kGy. Preliminary tests have shown that irradiation dosage in the range of 8 kGy to 13 kGy applied to dental gutta-percha material can achieve desired sterilization result and yet preserve physical property of the product for intended use. This dosage range is also commonly used for sterilizing other medical disposable single use devices, such as syringes, needles, and etc. Gamma rays are a form of electromagnetic radiation-like X-rays, but with higher energy. The primary industrial sources of gamma rays are radionuclide elements such as Cobalt 60, which emit gamma rays during radioactive decay. Gamma rays pass readily through plastics and kill bacteria by breaking the covalent bonds of bacterial DNA. They are measured in units called Kilo Grays (kGy). Gamma irradiation provides a number of benefits in cost and sterility assurance. It can be applied under safe, well-defined, and controlled operating parameters, and is not a heat or moisture generating process. Consequently, there is no heat stress and condensate drainage or outgassing is not required. Most importantly, there is no residual radioactivity after irradiation. Since dental root canal filling material is made of variety of polymer material, just like plastic polymer, gamma ray can certainly penetrate into root canal obturation cones, just like penetrating plastic layer (Martin, 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacteria

- *Enteroccoccus faecalis* (American Type Culture Collection (ATCC) 19433)
- *Escherichia coli* (American Type Culture Collection (ATCC) 11775)

3.1.2 Culture media

- Brain Heart Infusion (BHI) agar and broth (Merck, Germany)

3.1.3 Consumables

- Falcon tube 50 mL (Corning)
- Latex Examination Glove (Clean Guard)
- 10cm Petri dish (Corning)
- Disposable pipette tips (Tarson)
- Beaker 250mL (Pyrex)
- Bunsen burner (HMbG)
- Solvent: Ethanol, Acetone (Sigma Aldrich)
- Sand paper
- Schott bottle (Pyrex)
- Conical flask (Pyrex)
- Gutta-percha #40, 0.06 taper (Denstply)
- #1000 SiC paper (FEPA P#1000, Struers)
- size X4, #40, 0.06 taper GP cones (ISO coded Dentsply, USA)

3.1.4 **Equipments**

- Lamina flow hood (ESCO)
- Water bath (Memmert)
- ultrasonic bath (Wise Clean, Korea)
- Centrifuge (Eppendof)
- -80°C freezer (Telstar)
- Scanning electron microscope (Quanta FEG)
- Fume hood (Labconco)
- Incubator (Memmert, Germany)
- Spectrophotometer (Shimadzu)
- Shaker (LAbnett)

3.2 Experimental Design

It was reported in earlier study that the used of GP which have been coated with HA in root canal procedure have resulted in its better adhesion to walls of the canal (ref). The present study was designed to investigate if this HAGP would also exhibit antibacterial activity on two bacteria commonly isolated from the endodontic environment, *E. coli* and *E. faecalis*. In the study, conventional GPs were coated with HA using the biomimetic technique. Following sterilization, the HAGP were tested for antibacterial property based on their susceptibility and adhesion capacity towards the two bacteria. The former was performed using the agar diffusion assay, while the later was determined based on the population of adhering bacteria to the HAGP. Figure 3.1 is an outline of the experimental design for the study.

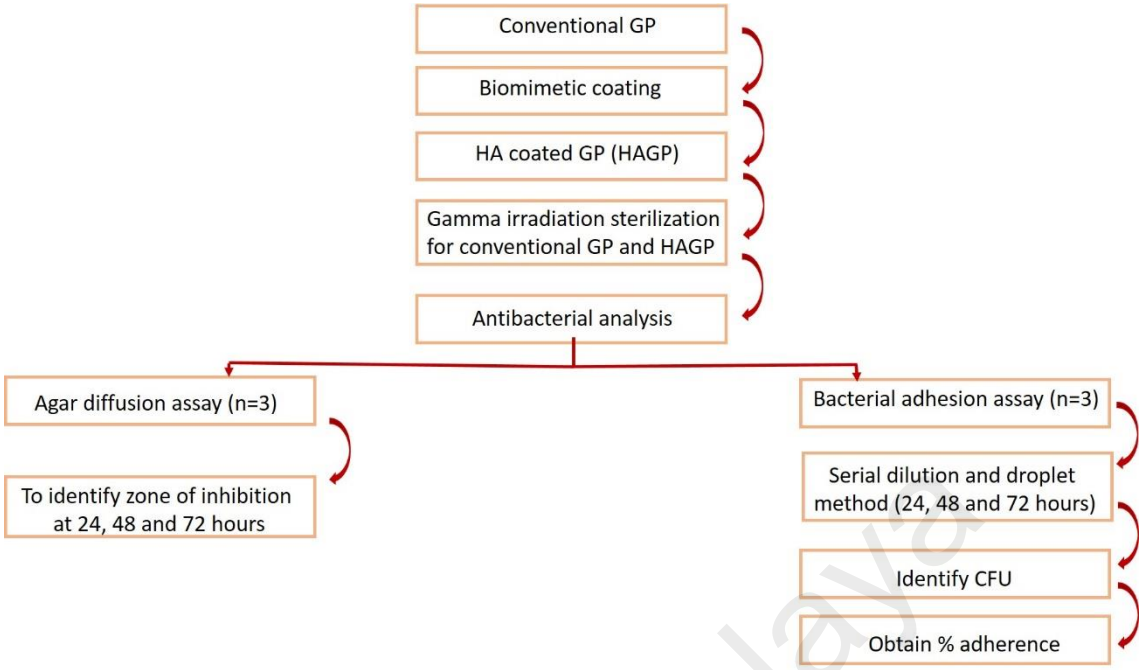

Figure 3.1: Flowchart of experimental design of the study

3.2.1 Preparation of HAGP using Biomimetic coating technique

The biomimetic coating technique was based on a procedure by Al-Haddad *et al.* (2015) which was a modification of a previously published work by Jalota *et al*. (2006). Size X4, #40 and 0.06 taper GP cones (ISO coded Dentsply, Maillefer, USA) were used as GP. The cones were first abraded with a #1000 SiC paper (FEPA P#1000, Struers), and then washed three times, with acetone, ethanol, and deionized water in that order in an ultrasonic bath (Wise Clean, Korea). Following this, each one of the GP cones was then vertically placed in a glass bottle using stain steel wires, immersed in 50 mL of a 5 M NaOH (EMSURE, Germany) solution at 60 °C for 24 hours. The cones were then washed with deionized water and dried at 40 °C. 1.5 mL of TRIS-buffered saline, 27 mM stimulated body fluid solutions (1.5 X Tas-SBF) was freshly prepared by adding the reagents in the sequence and quantity given in (Table 3.1). Subsequently, NaOH-treated GP cones were soaked vertically at 37 °C in 50 mL of 1.5 X Tas-SBF (pH 7.4) in tightly sealed Scott® media bottles (USA) of 100 mL-capacity, for a period of 10 days (Figure

3.2). The SBF solution was changed and replaced every 48 hours. Lastly, GP cones were removed from the SBF solution at the end of the respective soaking times, and washed with deionized water, followed by drying in an oven atmosphere at 40°C.

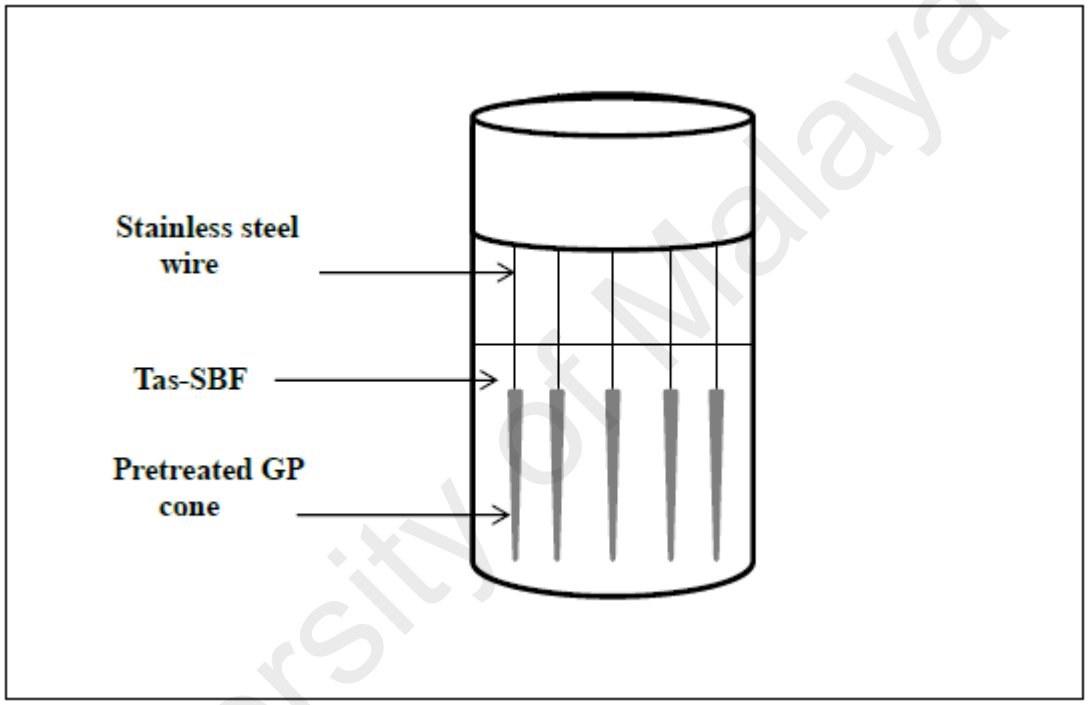

Figure 3.2: Schematic illustration of GP cones immersed vertically in Tas-SBF solution in a Schott bottle. (Adapted from Al-Haddad *et al.*, 2015)

3.2.2 **Sterilisation of HAGP using gamma rays**

Gamma cell irradiation was employed to sterilize the HAGP and the dosage used was 13 kGy. The gamma irradiation sterilization unit is located at the Department of Physics, Faculty of Science, University of Malaya. In brief, this method used the radioisotope cobalt 60 as an energy source and the irradiation process took place in a specially designed cell. A key characteristic of gamma irradiation is the high penetration capability that enabled sterilization of HAGP without jeopardizing its molecular structure. The unit was expressed as kGy and measured as an absorbed dose, determined by product density, pack size, dose rate, exposure time and to some degree by plant design. In this study, the sterilization dose for all the GPs was at 13kGy and performed over a period of six days. Below is an example of a calculation of dosage based on the following equation:

1 month = 30 x 24 x 60 x 60 = 2592000 seconds
The dose rate after one month $D_t = D_0\ e^{-\lambda T}$, where $\lambda = 4.1681 \times 10^{-9}\ s^{-1}$.

D_0 is the initial dose rate for Gammacell 220 and D_0 = 0.525 Gy/sec (November 1995).

D_t = $0.525\ e^{(-4.1681 \times 10^{-9} \times 2592000)}$
= 0.519 Gy/sec (December 1995).

Dose rate/Gy = minute/second.

eg : $\frac{10Gy}{2.04}$ = 4.9 minute

(Table 3.1) indicates the dose rate for Gammacell 220 from January 2017 until December 2019. Based on the table, the dose employed in this study for GP sterilisation was on December 2018 with 1.57 gy/min. In this study, since the dosage used for sterilization on the GP was 13 kGy, therefore 6 days of sterilization period was required to sterile all GPs.

Table 3.1: Table Dose Rate for Gammacell 220 from January 2017 until December 2019

2017		
Mon	Gy/sec	Gy/min
Jan	0.0339	2.04
Feb	0.0335	2.01
Mar	0.0331	1.99
Apr	0.0327	1.96
May	0.0323	1.94
June	0.0320	1.92
July	0.0317	1.90
Aug	0.0314	1.88
Sept	0.0311	1.86
Oct	0.0307	1.84
Nov	0.0303	1.82
Dec	0.0300	1.80

2018		
Mon	Gy/sec	Gy/min
Jan	0.0297	1.78
Feb	0.0294	1.76
Mar	0.0291	1.74
Apr	0.0288	1.72
May	0.0284	1.70
June	0.0281	1.68
July	0.0278	1.66
Aug	0.0275	1.65
Sept	0.0272	1.63
Oct	0.0269	1.61
Nov	0.0266	1.59
Dec	0.0263	1.57

2019		
Mon	Gy/sec	Gy/min
Jan	0.0260	1.56
Feb	0.0257	1.54
Mar	0.0254	1.52
Apr	0.0251	1.50
May	0.0248	1.48
June	0.0245	1.47
July	0.0242	1.45
Aug	0.0239	1.43
Sept	0.0236	1.41
Oct	0.0233	1.40
Nov	0.0230	1.38
Dec	0.0227	1.36

3.2.3 Determination of Antibacterial Property of HAGP

3.2.3.1 Preparation of bacterial suspension

Gram negative *E. coli* (ATCC 11775) and Gram positive *E. faecalis* (ATCC 19433) were selected and purchased for use in this study. The bacteria which was obtained in freeze dried form was revived in Brain Heart Infusion (BHI) broth overnight at 37 ºC. On the next day, 100 µL of *E. coli* and *E. faecalis* broth suspension were inoculated by streaking on nutrient agar plate and incubated at 37 ºC for 24 h to allow for colonies growth. The agar plates were then removed and using a sterile inoculation loop, a single bacterial colony of *E. coli* and *E. feacalis* was respectively transferred into separate culture tubes containing 100 mL of sterile BHI broth. The culture tubes were vortexed for 60 secs and the suspension was incubated at 37 ºC for 16 h in a shaking incubator set at 200 rpm. Following incubation, 10 mL of the growth suspension was transferred into fresh 100 mL BHI broth. Optical density of the bacterial suspension was measured and the absorbance was adjusted to 0.144 at a wavelength of 600 nm. This absorbance represented an equivalent bacterial concentration of 1×10^6 cells/mL as a standardized

cells concentration. Figure 3.3 illustrated the procedure for the preparation of *E. coli* and *E. faecalis* working suspension.

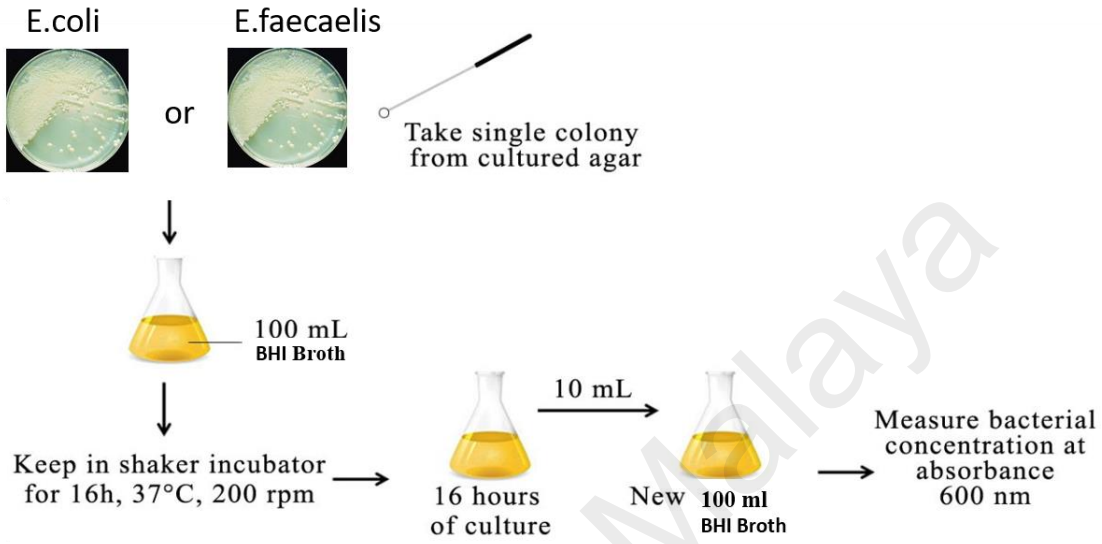

Figure 3.3: Schematic illustration of the procedure for preparation of bacterial suspension

3.2.3.2 Susceptibility of *E.coli* and *E. faecalis* to HAGP using agar diffusion technique

This test was performed on both *E. coli* and *E. faecalis* to indicate their susceptibility to HAGP. Including a positive and a negative control, three types of GPs were used in this study namely (i) HAGP, (ii) conventional GP soaked in sterile distilled water (dH_2O) as negative control and (iii) conventional GP soaked in 5.25% sodium hypochlorite (NaOCl) as positive control. All GPs were soaked for one minute in sterile distilled water or sodium hypochloride. To perform the susceptibility assessment, 100 µL aliquots of E. *coli* and *E. faecalis* suspension were each aseptically inoculated and spread on three sterile BHI agar plates. The inoculated plates were air-dried for 15 minutes under a laminar flow following which, each type of GP was aseptically placed on three separate

agar plates. The test was performed in triplicates. All plates were incubated for 24, 48 and 72 h at 37 ºC. At each of the incubation period, the plates were removed and the diameter of growth inhibition zones that forms around the GPs were measured. Comparative to the positive and negative controls, the susceptibility of *E. faecalis* and *E. coli* to HAGP was analysed.. Figure 3.4 illustrated procedure for the agar diffusion susceptibility test.

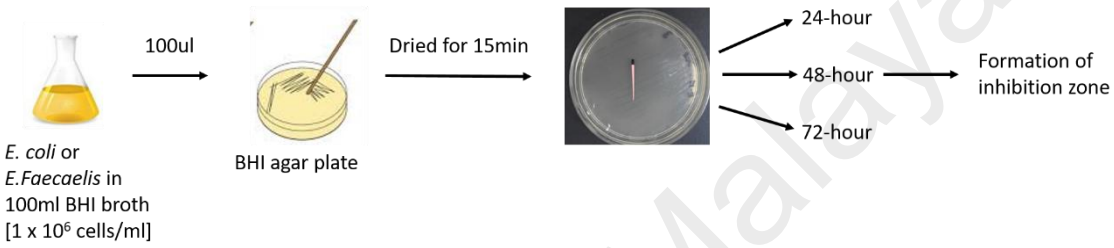

Figure 3.4: Schematic illustration of the agar diffusion technique to measure the susceptibility of *E. coli* and *E. faecalis* to HAGP

3.2.3.3 **Adherence assay**

The HAGP was further assessed for its capacity for bacterial adherence and the test included a positive and negative control, similar to those used in section 3.2.3.2. All three GPs namely, (i) HAGP, (ii) GP soaked in 5.25% NaOCL and (iii) GP soaked in dH_2O were further evaluated for bacterial adherence using the drop plate method with BHI broth as the growth medium. All GPs were prepared in triplicate. Firstly, the GP specimens were aseptically and separately placed in 2 mL of *E. coli* and *E. faecalis* suspensions prepared in section 3.2.3.1. The GPs were exposed to the bacterial suspension for 24, 48 and 72 hours to allow for bacterial adherence to its surfaces. The incubation was performed in a shaking incubator set at 200 rpm.

Following incubation, the GPs were removed, rinsed twice with phosphate buffered saline (PBS) and placed in 2 mL of fresh BHI broth. The adherent bacteria cells on surfaces of the GPs were detached by vortexing the tube for 60 secs. The bacterial suspension containing the detached cells was serially diluted to six dilution series. Enumeration of bacterial population was done using the drop plate method (Naghili *et al.*, 2013). The diluted bacteria were then grown on BHI agar by using a drop plate method. A volume of 10 µL of bacterial suspension at each dilution factor was dropped on BHI agar plates which have been segmented to six parts. The plate was then incubated at 37 °C for 24 h (Figure 3.5).

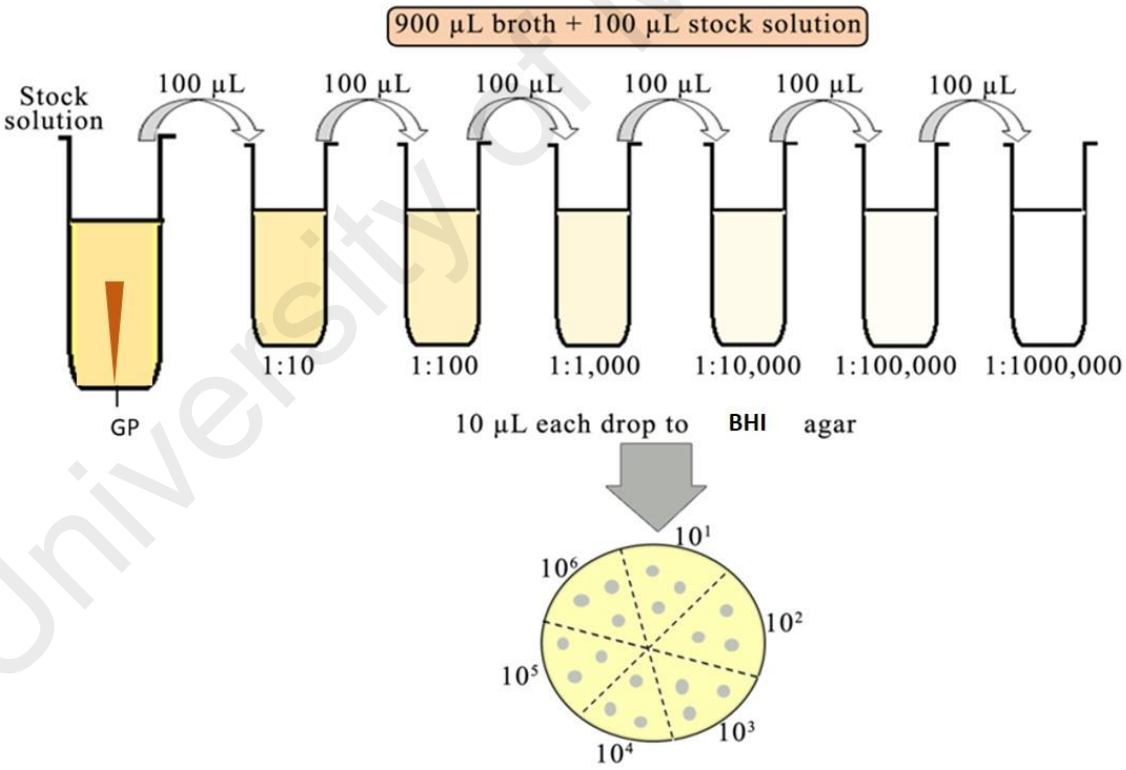

Figure 3.5: Schematic illustration of the serial dilution steps and drop plate method

Following incubation, the bacterial colonies formed on the agar were manually counted and expressed as colony forming unit (CFU). Taking into consideration of the dilution factor and CFU values, the percentage of bacterial adherence was determined following the equation in section 3.2.3.4.

3.2.3.4 **Determination of bacterial adherence**

The population of adherent bacteria to each of the (i) HAGP, (ii) positive and (iii) negative control GPs was determined as:

$$\text{Adherent cell population} = \frac{\text{Number of CFU}}{\text{(Dilution factor) x volume used (ml) to lawn the agar plate}}$$

The percentage of *E. coli* and *E. faecalis* adherence (X) to HAGP therefore, was compared to the population adhering to the negative control GP, following the equation:

$$X = \frac{\text{Adhered cells (test or positive control) x 100}}{\text{Adhered cells (negative control)}}$$

3.3 Data recording

The diameter of bacterial growth inhibition zones was measured with a millimeter ruler of digital caliper with an accuracy of 0.5 mm in two perpendicular locations for each sample by an independent observer.

3.4 Statistical analysis

The data were computed and analysed using SPSS software. Statistical analysis was performed using an Independent t-test to compare two different samples for mean CFU and one-way ANOVA for time differential among test materials. Statistically significant differences among groups were set at $p < 0.05$.

CHAPTER 4: RESULTS

4.1 Preparation of HAGP

The biomimetic coating technique was successful in coating the GP with HA. A micrograph image was taken to show the morphology of the HAGP surface (Figure 4.1).

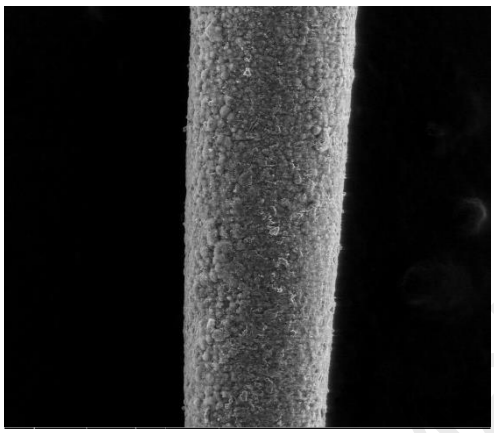

Figure 4.1: FESEM micrograph showing surfaces of a HA-coated GP. (50x)

4.2 Antibacterial property of HAGP against *E. coli* and *E. faecalis*

The antibacterial activity of HAGP was based on its growth inhibitory effect on *E. coli* and *E. faecalis.* A bacterium that is susceptible to HAGP would show the presence of a growth inhibitory zone around the coated GP while, a bacterium that is resistant to HAGP produces no growth inhibitory zone. As shown in Table 4.1, *E. coli* was found resistant to the HAGP at all incubation periods of 24, 48 and 72-hour. The GP soaked in sodium hypochlorite (positive control) showed an equivalent strong growth inhibitory activity against *E. coli* at all three incubation periods with zone of growth inhibition of (30 ± 0.1), (29.0 ± 0.2) and (29.0 ± 0.1) mm at 24, 48 and 72-hour, respectively. The GP soaked in distilled water (negative control), showed no growth inhibitory effect on *E. coli* at all three incubation periods. Almost similar results were obtained for *E. faecalis,* which was resistant towards HAGP and the negative control (Table 4.2). The susceptibility of *E. faecalis* to the positive control was equivalent to that of *E. coli* at, (30.0 ± 0.3), (30.0 ± 0.2) and (30.0 ± 0.2) mm at 24, 48 and 72-hour of incubation periods.

Table 4-1: Susceptibility of *E. coli* to (i) positive control and (ii) negative control GPs and (iii) HAGP. All values were expressed as mean ± SD of three determinations performed in triplicate (n=9).

Incubation period (Hour)	Zone of growth inhibition (mm) (Mean ± SD)		
	Positive control GP	**Negative control GP**	**HAGP**
24	30.0 ± 0.1	0	0
48	29.0 ± 0.2	0	0
72	29.0 ± 0.1	0	0

Table 4-2: Susceptibility of *E. faecalis* to (i) positive control and (ii) negative control GPs and (iii) HAGP. All values were expressed as mean ± SD of three determinations performed in triplicate (n=9).

Time (Hour)	Zone of growth inhibition (mm) (Mean ± SD)		
	Positive control GP	**Negative control GP**	**HAGP**
24	30.0 ± 0.3	0	0
48	30.0 ± 0.2	0	0
72	30.0 ± 0.3	0	0

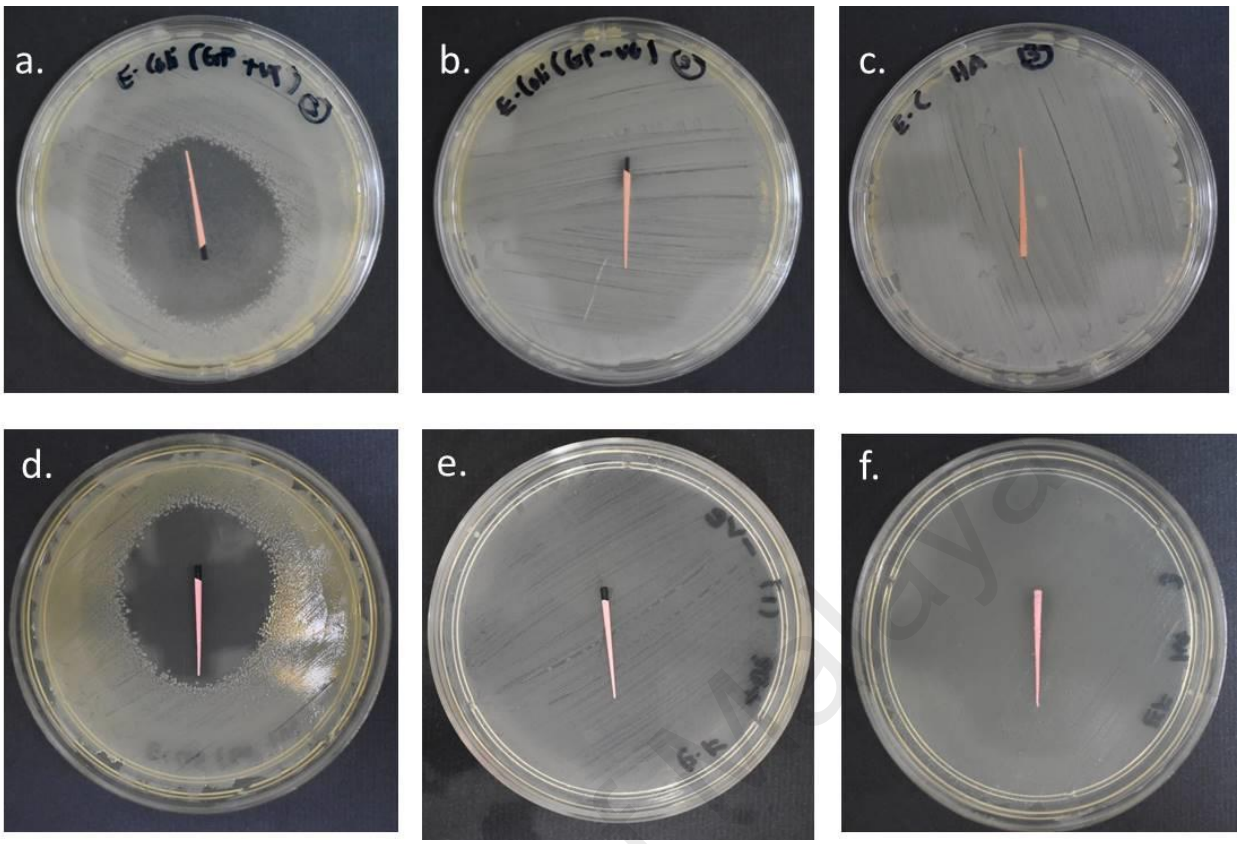

Figure 4.2: The agar diffusion test showing the susceptibility of the three different GPs to *E. coli* (a,b,c) and *E. faecalis* (d,e,f). susceptibility assay.

Figure 4.2 showed the antibacterial response of *E. coli* to (a) GP soaked in sodium hypochlorite, (b) GP soaked in distilled water and (c) HAGP, and that of *E. faecalis* to (d) GP soaked in sodium hypochlorite, (e) GP soaked in distilled water and (f) HAGP.

4.3 Adherence capacity of HAGP for *E. coli* and *E. faecalis*

Figure 4.3 and 4.4 comparatively showed the maximum adhering capacity of *E. coli* and *E. faecalis* to all the three GPs; (i) HAGP, (ii) GP soaked in sodium hypochlorite (positive control) and (iii) GP soaked in distilled water (negative control) The adhering capacity was indicated by the number of bacteria adhering to the GPs' surfaces following incubation periods of 24, 48 and 72 hours. At 24-hour of incubation, a higher adhering capacity of *E. coli* to the negative control was observed compare to *E. faecalis*. The adherence of both bacteria was also found to be significantly ($p < 0.05$) increased when the GP was coated with HA, and this was observed at all three incubation periods (Figure 4.3 and 4.4). In other words, HAGP was shown to enhance the adherence on both *E. coli* and *E. faecalis* at all three incubation periods. It was also observed that the adhering capacity of *E. coli* was reduced ($p < 0.01$) at the 48 to 72-hour of incubation when compared to negative control (Figure 4.3). As for *E. faecalis*, changes in the adhering capacity were only slightly affected at the three incubation periods (Figure 4.4).

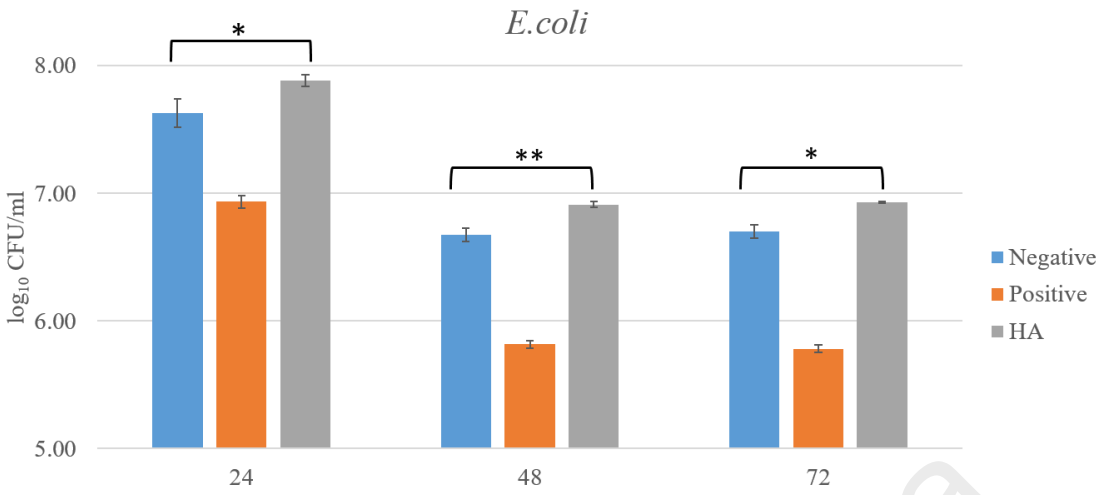

Figure 4.3: The adhering capacity of *E. coli* to (i) HAGP, (ii) Positive control and (iii) negative control. Results were expressed as mean ± SD of triplicate in three determinations (n=9). $p < 0.05$ (*), $p < 0.01$ (**).

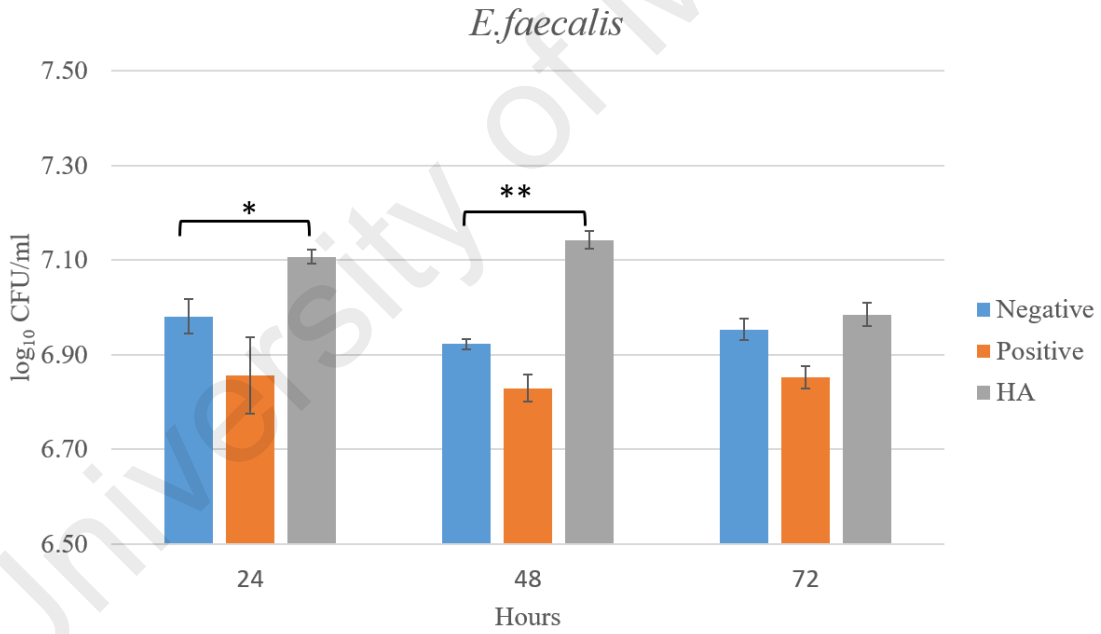

Figure 4.4: The adhering capacity of *E. faecalis* to (i) HAGP, (ii) Positive control and (iii) negative control. Results were expressed as mean ± SD of triplicate in three determinations (n=9). $p < 0.05$ (*), $p < 0.01$ (**).

4.4 Effect of exposure time on adherence of *E. coli* and *E. faecalis*

Data from Figure 4.3 and 4.4 was further analysed to produce Figure 4.5 and 4.6. Bacteria counts adhering to the negative controls was made as a reference point to show the maximum (100%) adherence capacity of GP for *E. coli* and *E. faecalis*. Both graphs clearly showed hydroxyapaptite coating of GP enhanced the adherence of more bacteria to the GP. HAGP was seen to allow higher adherence of *E. coli* by about 82.81%, 73.14% and 69.77% following a 24, 48 and 72 hours exposure time, respectively (Figure 4.5). The difference in adhering bacteria at these three time frames was however, not significantly different (Table 4.3). As for *E. faecalis*, similar adherence promoting effect of HAGP was observed. However, HAGP increased the adherence of *E. faecalis* at a lesser degree than *E. coli* by about 34.03%, 66.09% and 7.51% at 24, 48 and 72 hours, respectively. From Figure 4.5 and 4.6, it was also obvious that soaking GP in sodium hypochlorite (positive control) had worked well in reducing the adherence of both bacteria to GPs, although it worked better towards *E. coli* compared to *E. faecalis*.

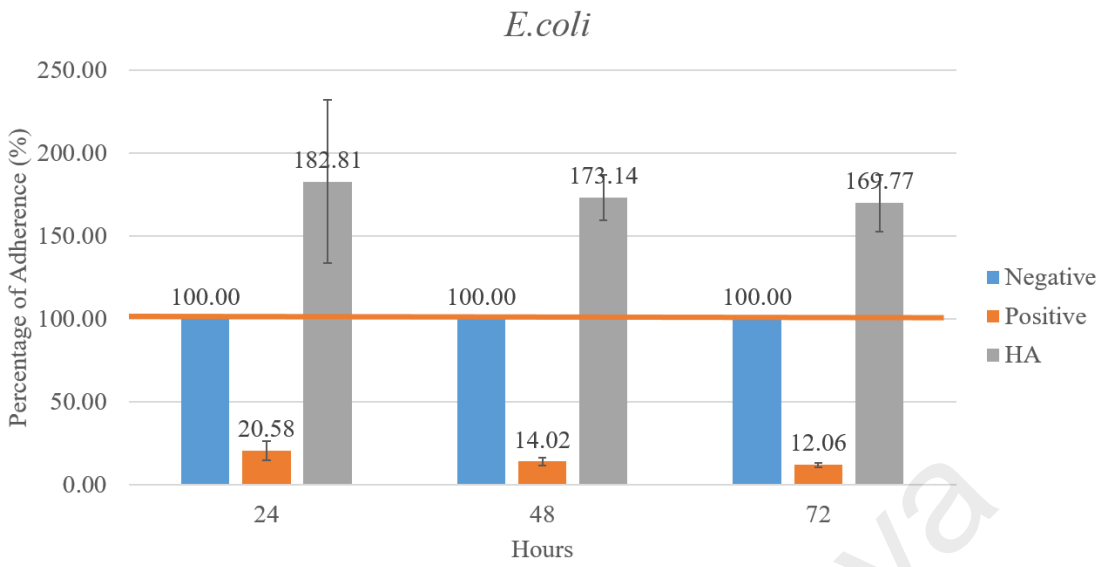

Figure 4.5: The percentage of adherence of *E. coli* on HAGP as compared to the positive and negative control GPs.

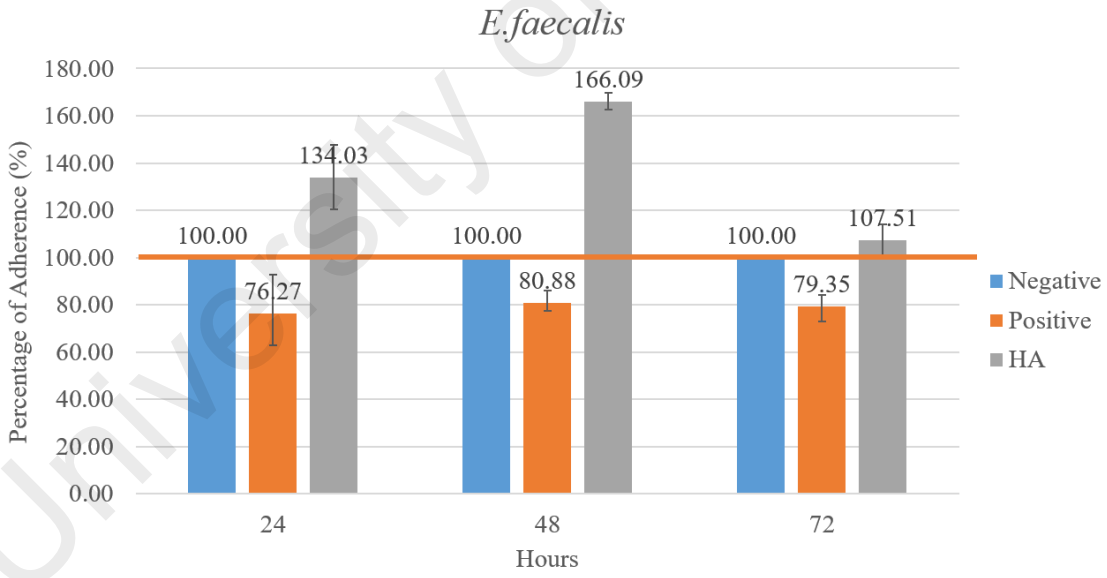

Figure 4.6: The percentage of adherence of *E. faecalis* on HAGP as compared to the positive and negative control GPs..

Table 4-3: Percentage of bacteria adherence on HAGP at three different times

Bacteria	Mean % of adherence ± SD of different hours at HAGP			P value
	24	48	72	
E. coli	182.81±49.13	173.14±13.73	169.77±17.02	0.870
E. faecalis	134.03±13.49	166.09±3.56	107.51±6.42	0.001

One-way ANOVA

Significant $p < 0.05$

CHAPTER 5: DISCUSSION

One of the key goals of endodontic therapy is complete obturation of the root canal system. The success of obturation is directly related to the elimination of microorganisms through mechanical cleaning and shaping, supplemented by antibacterial irrigants, adequate filling of the empty space, and use of antimicrobial dressings (with calcium hydroxide) between appointments, if necessary (Sundqvist *et al.*, 1998) However, these procedures do not result in complete sterility of the root canal space. In view of the high prevalence of anaerobes and facultative anaerobes in unsuccessful endodontic therapy, it is important that the antimicrobial activity of root-canal obturation material help to eliminate residual microorganisms unaffected by chemomechanical preparation or intracanal medication, as well as persistent root canal infection. Therefore, it has been advocated that the root-filling material should have antibacterial properties.

The concept of coating the GP has been introduced to the endodontic field in line with the increased interest in adhesive dentistry. The coating of GP with different materials is used to enhance its bonding to the root sealer and consequently to the root dentine to form one unit 'monoblock'. However, the current systems have not been able to provide an airtight seal and good adhesion thus providing scope for the development of better root canal filling materials to improve these properties. In 2015, Al-Haddad *et al.* (2015) has demonstrated that hydroxyapatite (HA) coated GP (HAGP) showed a promising result to be used as root canal filling material in combination with bioceramic sealer. Their study showed that the HAGP possessed an improved sealing ability when compared to the commercially available coated GP such as EndoREZ (Ultradent Products Inc, USA), ActiV GP (Brasseler USA, Savannah, GA), BCGP (Brasseler USA, Savannah, GA) and conventional GP. Furthermore, HAGP was able to use with any type of sealers for example resin-based, calcium hydroxide base, glass-ionomer base as it has the same

component of materials as dentine. Elimination of bacteria from the root canal system is essential for long-term success of endodontic treatment. Additionally, the root filling prevents infection by acting as a barrier to further microbial challenges, entombing any surviving bacteria within the root canal system and stopping periapical tissue fluids from reaching bacterial cells in the root canal (Figdor *et al.*, 2003). However, several studies have demonstrated that bacteria are the main aetiological agent of pulpal infection and periadicular lesion formation (Sundqvist *et al.*, 1998). The microbiota of infected root canals is polymicrobial and is dominated by Gram-negative anaerobes (Baumgartner *et al.*, 2003). It has been demonstrated that the presence of residual bacteria in root canal is connected with significantly higher rates of treatment failure (Sjögren *et al.*, 1997). Since elimination of bacteria in root canals is the key to treatment success, endodontic materials should ideally provide some antibacterial property (Torabinejad *et al.*, 1995), in order to improve the prognosis of endodontically treated teeth. Hence, this study was conducted to investigate whether the HAGP possess antibacterial property.

In this study, two of the common bacteria in endodontics study were used, *E. coli* and *E. faecalis*. *E. faecalis* is an anaerobic Gram positive bacteria, and is also the most prevalent species isolated from human. This bacterium is often isolated from failed endodontic treatment cases and is and this makes it a good reason to be tested in this study (Lotfi *et al.*, 2011; Rôças *et al.*, 2004). Several studies have shown that it is prevalence in persistent infection in dental disease. Another microorganism which has been often found in infected root canals is *E. coli* (Ayhan *et al.*, 1999), a Gram-negative facultative anaerobe, although research literature on this particular microorganism is sparse. The microorganisms found in the root canals of primary teeth are similar to those in the root canals of permanent teeth (Önçağ *et al.*, 2003). *E. coli* is sometimes recovered from root canals and represent a standard organism used in antibacterial testing (Haapasalo *et al.*,

1983; Siren *et al.*, 1997). *E. coli* and *E. faecalis* are both bacteria which are also commonly associated with secondary infections (Ørstavik & Ford, 2008).

In this study, the susceptibility of *E. coli* and *E. faecalis* on HAGP were investigated. The results showed that *E. coli* and *E. faecalis* produced positive susceptibility on HAGP compared to the GP soaked in sodium hypochlorite. From this result, it is suggested that the HAGP has no antibacterial activity when incubated with *E. coli* and *E. faecalis*. However, in 2008, a study conducted by Dianat and Ataie (2008) demonstrated that nanosilver coated-GP produced a significant effect against *E. faecalis, S. aureus, C.albican* and *E. coli*. Moreover, Shantiaee *et al.* (2011) has shown that the nanosilver coated-GP has lower cytotocity levels compared to the normal GP. Whereas, Alves *et al.* (2018) have showed that Zinc oxide coated-GP possessed an antibacterial activity when compared with the conventional GP. They also observed that the cytotoxicity level of zinc oxide coated-GP was comparably lower than the normal GP whereas the cytotoxicity level of HAGP used this study is still yet to uncover. However, this study used sodium hypochlorite as the positive control since it is the common root canal irrigant besides hydrogen peroxide, citric acid and chlorhexidine. As expected, from the results, GP soaked-sodium hypochlorite showed positive inhibitory activity against *E. coli* and *E. faecalis*. These results are in accordance with the study performed by Kaushik *et al.* (2013), which showed that sodium hypochlorite is effective against *E. faecalis* and *E. coli*.

Since the HAGP has a non-significant effect on the antibacterial activity, the adherence effect of the bacteria on HAGP was conducted to investigate the colonization of the bacteria. From the adherence study, it can be seen that HAGP attracts more colonization of *E. coli* and *E. faecalis* compared to the GP soaked in sodium hypochlorite (positive control) and GP soaked in distilled water (negative control). Hydroxyapatite (HA) has

shown to have an excellent biocompatibility in human tooth and bones (MM *et al.*, 2007). However, MM *et al.* (2007) demonstrated that, when HA coating contains silver ion, it suppressed *S.mutans* growth. Another study also showed that HA produced with silver ions particles produced distinctly improved antibacterial properties (Lamkhao *et al.*, 2019). Kolmas *et al.* (2014) suggested that HA integrated with strontium ions has a potential antibacterial activity. So far, none of the studies have shown the ability of HA alone possessed any antibacterial effects. However, one study has demonstrated that bacteria adhered rapidly to hydroxyapatite, especially *E. coli* and *Enterococcus* (Berry & Siragusa, 1997) which might explain the reason why *E. coli* and *E. faecalis* were found to adhere more on the HAGP. The interaction between HA and the bacteria is non-specific and it could be due to electrostatic interactions. As in "opposites attract" bacteria tend to be negatively charged and it is then may be attracted to HA (Berry & Siragusa, 1997). HA is a form of calcium phosphate and it is important for teeth and bones. Most probably, if the calcium concentration is increased in HA, this can then attract more bacteria to HA. In addition, since HA is an important part of tooth enamel, dental plaque usually starts forming as soon as finishing brushing the teeth. Therefore, coating HA only to GP might not be the best resolution for the antibacterial properties for root canal treatment.

5.1 Limitations of the study

The study was conducted using GP cones which were conical in shape. These cones were used in agar diffusion assay to investigate the zone of inhibition. Due to its shape, the point of measurement for the zone of inhibition was not equally alike. Therefore, to standardise the measurement of zone of inhibition, centre point of the GP cones was used to measure the zone. Unfortunately, the HAGP did not produce any antibacterial activity in this study.

CHAPTER 6: CONCLUSIONS

Results from the study found that both *E. coli* and *E. faecalis* was not affected and in fact, resistant to hydroxyapatite-coated gutta-percha (HAGP). The HAGP was also found to support rather than reduce the adhesion *E. coli* and *E. faecalis*, and this effect was independent of the duration of exposure time. It is strongly suggested that its usage need to be assisted by investigating the effect of HA for antibacterial property by addressing three important objectives of the study. From this study, it can be concluded that:

i- there is no antibacterial property observed in HAGP.

ii- *E. coli* and *E. faecalis* are both susceptible on HAGP.

iii- the growth of *E. coli* was faster than *E. faecalis*.

In conclusion, HA attracts more bacteria colonization on the GP, and HA alone does not possess any antibacterial property.

CHAPTER 7: RECOMMENDATION

Several studies have shown that HA integrated with silver, zinc and strontium ions have an improved antibacterial activity. Therefore, it is suggested that HAGP integrated with zinc, silver and strontium ions be used as coating materials on GP, rather than coating with hydroxyapatite alone. On a separate note, a comparison study between the integrated HAGP and bioceramic coated-GP can be conducted, as a few studies have shown that the calcium silicate-based root canal sealers exerted antibacterial effects against *E. faecalis* (Alsubait *et al*., 2019). Thus, this study can further demonstrate whether the integrated HAGP or bioceramic coated-GP possess better antibacterial activity for root canal treatment.

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