Characterisation of Pulpal Responses to Bacterial Challenge and Novel Antimicrobials for Management of Bacterial Contamination of Infected Pulps

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I dedicate this to my parents, my family and my friends who have always lovingly supported me.

Summary

Dental pain from pulpal infection and inflammation are the common cause of dental emergencies. Therefore, evaluation of modalities to treat them would be beneficial. This work aims to characterise and validate the previously developed *ex vivo* pulp infection model and make it reliable and reproducible in terms of quantifying cell viability, area of bacterial colonisation and expression of inflammatory markers.

The method developed and used in this work demonstrated that SAG infection reduced cell viability and IL-10 levels. SAG infection also Increased area of SAG colonisation of pulp, expression of IL-β, TNF-a and IL-18 in a time depended manner compared to uninfected control pulp. These findings were consistent with clinical observations, making it a reliable model for the observed characteristics. There were differences in the response to two SAG strains, with similar overall trends, but with greater response to *S. constellatus*.

Differences in response to the two strains was confirmed by evaluating the effect of the SAG supernatants using the model. The response observed confirmed the deleterious effects of the supernatant on cell viability, and increase in expression of inflammatory markers, with a greater response of pulp observed to *S. constellatus*. Analysing the supernatants of SAG strains revealed the difference could be due to hyaluronidase produced by *S. constellatus*.

Triclosan's anti-microbial and anti-inflammatory effect in the treatment of pulpal infection was demonstrated using the SAG model through increased cell viability, reduced area of colonisation and inflammatory marker expression levels form triclosan treatment. Triclosan showed no anti-hyaluronisase activity.

A poly-microbial infection model was developed with *E. faecalis* and *S. anginosus* on tooth slices. It was observed that *E. faecalis* perpetuated *S. anginosus* growth in mixed culture and caused greater cell-death compared to *S. anginosus* mono-infection. The proportion of *E. faecalis* and its affinity to blood vessels could influence the response of the model.

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Conference presentations, abstracts and publications

Conference and Mode of presentation	Publication details
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Abbreviations

ACE	Adhesin to collagen of *E. faecalis*
AS	Aggregation substance
BCA	Bichinchoninic acid
BHI	Brain heart infusion
BMP	Bone morphogenic factor
BSP	Bone sialoprotein
Ca(OH)2	Calcium Hydroxide
CFU	Colony-forming unit
CGRP	Calcitonin gene-related peptide
DGP	Dentine glycoprotein
DMC	Dentine matrix components
DMEM	Dulbecco's Modified Eagles's Medium
DMP	Dentine matrix protein
DNA	Deoxyribonucleic acid
DPC	Direct pulp capping
DPCs	Dental pulp cells
DPP	Dentine phosphoprotein
DPSC	Dental pulp stem cells
DSP	Dentine siloprotein
DSPP	Dentinesialophosphoprotein
EDTA	Ethylenediaminetetraacetic acid
EFAD	Essential fatty acids deficient
ENR	Enoyl-acyl-carrier reductase
Esp	Extracellular protein
FAA	Fastidious anaerobic agar
FBS	Foetal bovine serum
FDA	Fluoresine diacetate
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
H&E	Haematoxylin and eosin
IEE	Inner enamel epithelium
IGF	Insulin like growth factor
IL	Interleukin
IPC	Indirect pulp capping
LBP	Lipopolysaccharides binding protein
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MSCRAMM	Microbial surface component recognising adhesive matrix molecule
MTA	Mineral trioxide aggregate
MTCM	Modified tissue culture media

MV	Matrix vesicles
NCPs	Noncollagenous proteins
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OPN	Osteopontin
PAMPs	Pathogen–associated molecular patterns
PBS	Phosphate buffered saline
PNMLs	Polymorphnuclear leukocytes
PRR	Pathogen recognition receptors
qPCR	Quantitative polymerase chain reaction
RDT	Remaining dentine thickness
ROS	Reactive oxygen species
SAG	*Streptococcus anginosus* group
SDS- PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIBLINGs	Small integrin-binding ligand N-linked Glycoproteins
TBS	Tris-buffered saline
TGF	Transforming growth factor
TLR	Toll-like receptor
TNAP	Tissue non-specific alkaline phosphatase
TNF	Tumour Necrosis Factor
TRP	Transient receptor potential
TSA	Trypticase soy agar
UV	Ultra-violet
VEGF	Vascular endothelial growth factor
VPT	Vital pulp therapy

Chapter 1

1 General Introduction

1.1 Context of the research

One of the commonest reasons for patients presenting at dental emergency clinics is toothache**.** Studies on the disability weight for the burden of oral disease which uses Disability Adjusted Life Years (Murray 1994; Murray and Lopez 1994) have shown that pulpal disease had the highest disability weights of all oral diseases (Brennan and Spencer 2004). The system used to grade oral diseases using scores from 0 - 1 to indicate the disability weight of the condition. Zero meaning conditions with no symptoms to score 1 for a terminal condition.

This is mainly the sequela of dental caries that was not treated. Caries is a disease caused by microorganisms in the oral cavity which ferment sugars to produce acids and liberate enzymes which cause demineralisation of the inorganic content of the tooth and destruction of the organic content of the tooth structure (Holst 2005). If caries is not treated during its early stages it progresses into the tooth and infects the dental pulp, the soft connective tissue that contains the nerves and blood vessels at the centre of the tooth. As the lesion progresses it stimulates various levels of inflammation in the pulp (Trowbridge 1981). This is the commonest cause of pulpal inflammation called pulpitis (Wu, S *et al* 2018).

Pulpitis was traditionally classified, clinically as either reversible or irreversible. In recent years new classifications system for pulpal disease has been recognised based on the clinical signs and symptoms elicited by the pulp in association with the treatment modalities which mainly aim to preserve as much of the pulp as possible (Wolters *et al* 2017). According to this classification the initial and mild form of pulpitis can be resolved by indirect pulp treatment and moderate pulpitis and sometimes sever pulpitis can be treated by coronal pulpotomy (Wolters *et al* 2017). The challenge till recently was being able to differentiate clinically the transition between the different states of the pulpal inflammation.

It was for a long time believed that there is no reliable way to correlate consistently the histological status of the pulp to the clinical signs and symptoms of the pulpitis (Seltzer *et al*.

1963; Dummer *et al*. 1980; Reit 2010; Trowbridge 2012). Further, it was also thought that there is no obvious association between clinical investigation of asymptomatic deep carious lesion and pulpal inflammation status (Mejare *et al*. 2012). Even though spontaneous pain was considered to be a clinical indicator of irreversible pulpitis histologically there can be no evidence of pulp exposure or necrosis indicating irreversible state of the pulp (Mejare *et al*. 2012). Therefore, till recently it was challenging to make a decision to attempt to maintain the vitality of the pulp on the basis of a fairly imprecise clinical diagnosis. More recent histological studies have shown that there is good correlation between clinical symptoms of pulpitis and corresponding histological state of the diseased pulp (Ricucci *et al*. 2012). Further the grading pulpitis based on clinical signs and symptoms (Wolters *et al* 2017) have made diagnosis and treatment of pulpal diseases less complicated.

Most commonly, teeth that are diagnosed clinically as having irreversible pulpitis are treated by extraction or pulp extirpation and root canal treatment (Levin *et al*. 2009). If the tooth is extracted it may need to be replaced by a denture, bridge or implants (Al-Quran *et al*. 2011). Such restorations require long term maintenance and thus have substantial financial implications (Hannahan and Eleazer 2008; Al-Quran *et al*. 2011); in addition, tooth loss may affect the quality of life (Brennan *et al*. 2008).

The success of primary and secondary root canal treatment is around 80% and 75%, respectively (Ng *et al*. 2007, 2008) based on absence or reduction in size of apical radiolucency. If teeth with irreversible pulpitis are left untreated the pulp undergoes necrosis and this leads to the formation of periapical lesions (Yu and Abbott 2007). The prevention of apical periodontitis is higher when irreversible pulpitis prevents is treated compared to treatment of pulpal necrosis and apical periodontitis (Ng *et al*. 2011). Irreversible pulpitis as mentioned above is usually treated by pulp extirpation and root canal treatment or extraction of the tooth. It is very important to maintain the vitality of the pulp; however, it presents a great challenge. As endodontic treatment replaces the pulp tissue with an inert material, it should be emphasised that maintenance of the vitality of the pulp is very important (Zhang and Yelick 2010). With the loss of vitality, the tooth loses its ability to respond to environmental changes such as caries progression. This affects the crown-root ratio and also the dentine wall of the roots of these teeth are thin, predisposing them to vertical root fracture. Filling materials used for root canal treatment can cause discolouration of the tooth, creating aesthetic problems (Goto et al. 2009). Maintaining the vitality of the

pulp prevents apical periodontitis by preventing bacterial colonisation of the periapical area (Lin et al. 2006; Rocha *et al.* 2008*)*. Last but no least vital teeth have been shown to detect pressure and minimises damage to the teeth from loading compared to pulpless teeth (Randow and Glantz 1986). Studies have shown success rates of up to 90% with conservative treatments of VPT in cases of irreversible pulpitis (Asgary and Eghbal 2013) which is comparable to conventional root canal treatment. Therefore, it is important to maintain the health of the pulp through vital pulp therapy (VPT). Further, the option of root canal treatment still remains if VPT fails.

The success rate of VPT is affected by the degree of infection and inflammation of the pulp, the level of tissue resection and the type of materials used (Yamaguchi *et al*. 2004; Cooper *et al*. 2010; Cooper *et al*. 2014; Ghoddusi *et al*. 2014; Wolters *et al* 2017). Further, the pressure and swelling from the oedema of the inflamed pulp (Heyeraas *et al*. 1994; Heyeraas and Berggreen 1999) and the non-yielding wall of the pulp chamber stimulates the nerve endings causing severe pain in the acute phase of inflammation (Yu and Abbott 2007; Estrela *et al*. 2011). Therefore, if pulpitis, the most common cause of which is infection, can be controlled the pulp can repair by the formation of a barrier of reparative dentine (Bjorndal *et al*. 2010; Colombo *et al*. 2014). This will prevent the pulp from losing vitality and the sequela of the spread of infection. There is some evidence that dentine bridge has tunnel defects which may eventually lead to bacterial infection and this can be prevented by using bonding agents. (Kitasako *et al*. 2008)

Streptococcus anginosus group (SAG) bacteria are normally commensal flora in the oral cavity (Poole and Wilson 1979), which can cause opportunistic infection in other parts of the body (Neumayr *et al*. 2010; Moazzam *et al*. 2015). They have also been isolated from dentoalveolar abscesses (Cachovan *et al*. 2014). They are one of the early colonisers of the exposed dental pulp, which facilitate colonisation by other species of bacteria (Love and Jenkinson 2002) and as the infection progresses and suitable conditions develop they are instrumental in the development of dentoalveolar abscesses (Cachovan *et al*. 2014). SAG is one of the frequently identified bacteria from these abscesses (Lewis *et al*. 1990; Robertson and Smith 2009). Therefore, understanding pulpal infection is very important in order to be able to identify the avenues for treatment of early pulpal infection and thereby control the inflammation.

If infection can be controlled and inflammation of the pulp modulated it can tip the balance towards regeneration and repair rather than pulpal necrosis (Colombo *et al*. 2014). If not treated at this stage by root canal treatment it will lead to subsequent periapical lesions and potentially loss of the tooth. Therefore, in order to understand pulpal disease a suitable model for modelling pulpal infection and inflammation and also for evaluating treatment modalities to treat pulpal disease is needed. Role of SAG in pulpal infection has not been extensively investigated in the past. Further, there is a wide gap in our understanding of the role of SAG in the pulpal disease process, from a carious lesion to the development of a periapical abscess. Studies have shown that when subcutaneous infections in mice were created with dental plaque containing low levels of SAG they resulted in formation of abscesses with increased level of SAG (Thurnheer *et al*. 2001; Okayama *et al*. 2005). Due to the complexity, systemic influence, expenditure and ethical issues of *in vivo* models, added to their over simplified nature, lack of interaction between different cell types, and lack of organisation into truly living and perfused/innervated tissues, *in vivo* models have serious limitations. Critically when considering dental pulp models, odontoblasts are terminally differentiated cells and cannot be cultured *in vitro*.

An *ex vivo* model may be fitting to address the issues with *in vivo* and *in vitro* models. The organotypic *ex vivo* tooth slice culture system maintains the structure of the dentine pulp complex, along with the phenotypic morphology and secretory activity for up to 14 days (Sloan *et al*. 1998). This model paved the way for the development of the *ex vivo* pulp infection model which helps to study the effect of SAG bacterial infection of the pulp, which was previously not possible (Roberts *et al*. 2013). Even though the work established the ideal culture conditions for co-culture of SAG and pulp tissue, the model was characterised and validated semi-quantitatively (Roberts *et al*. 2013), with respective to viable cell counts, evaluation of colonisation of the pulp by SAG and with regard to expression of inflammatory markers. These may not be the true representation of the response of the entire pulp tissue and may not be reliably reproducible. Therefore, it is necessary to develop a highly reproducible method to characterise and validate the model, which this work intends to do.

The work will also evaluate the effect of SAG bacterial toxins using this model to identify potential proteins that are deleterious to the pulp in an effort to evaluate therapeutic approaches to target these proteins selectively and thereby control tissue destruction and facilitate the pulp to repair and regenerate.

As the current gold standard agent for VPT is MTA (Olatosi *et al*. 2015; Caliskan and Guneri 2016). The material is quite expensive (Caliskan and Guneri 2016) and not used extensively in general dental practice, even in developed countries (Chin *et al.* 2016). Therefore, there is a need to develop less expensive alternatives. Further, MTA has a setting time of 24 h and poor handling properties. Triclosan is being used in dental products as an antimicrobial agent; however, it has been shown to possess anti-inflammatory properties (Gaffar *et al.* 1995) and is inexpensive. Furthermore, its use in VPT has not been evaluated. This work will use the *ex vivo* pulp infection model to evaluate the effectiveness of triclosan in VPT.

Even though the SAG pulp infection model simulates reasonably closely the clinical situation it does not completely reproduce it, as a clinical pulpal infection is polymicrobial (Rocas *et al*. 2016). This work will try to create a polymicrobial pulp infection model.

1.2 Development and structure of the tooth

The tooth comprises of different tissues. The outermost layer is made up of mineralised tissue called enamel in the crown and cementum in the root **(Figure1.1)**. Enamel and cementum are supported by another mineralised tissue called dentine. Dentine is also a mineralised vital tissue containing cell process extending from the cells of the dental pulp, which is the soft non-mineralised loose connective tissue that the dentine encases at the centre of the tooth. The pulp contains blood vessels and nerves and can respond to infection or injury through immuno-inflammatory responses. The molecular and genetic mechanisms that take place during early tooth development share similarities with those that take place during the reparative processes following caries and trauma (Mitsiadis and Rahiotis 2004). Therefore, an understanding of the development of processes involved in the formation of the dental tissues will be useful in the understanding of the repair and regenerative process that occur in the tooth when subjected to disease and trauma. Therefore, the following section will discuss the events that take place during tooth development.

The development of the teeth involves a well-orchestrated reciprocal interaction of the oral epithelium and mesenchyme (Lumsden 1988; Tummers and Thesleff 2009), resulting in the formation of the tooth germ (Thesleff 2003). The tooth germ consists of an enamel organ that forms the enamel. A ball of mesenchymal cells condenses around these epithelial

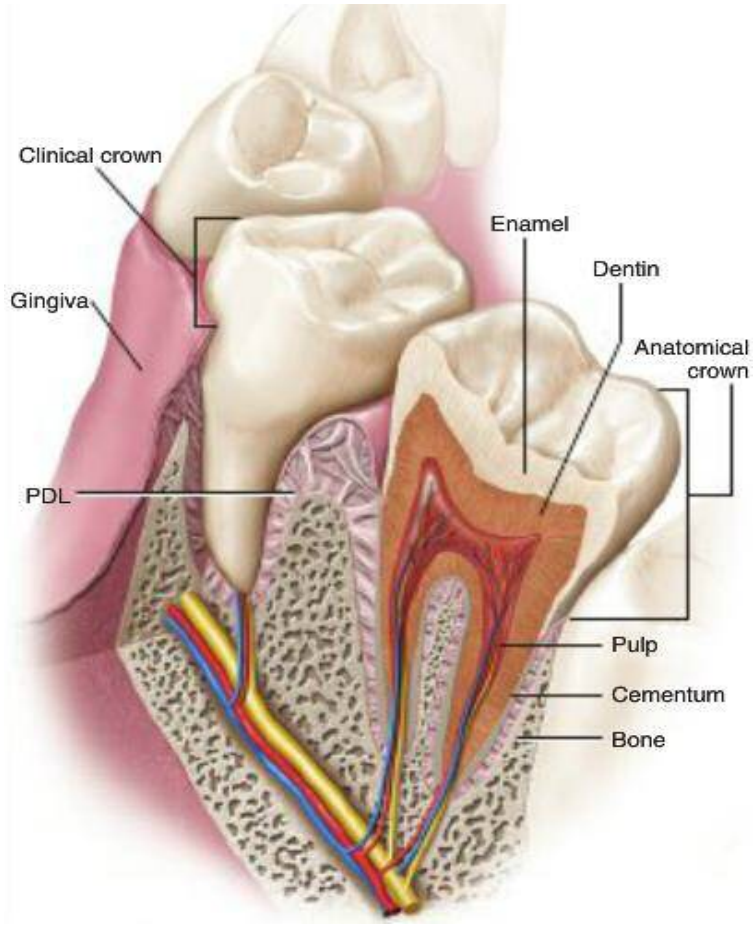


Figure 1.1: The tooth tissues: enamel, dentin and pulp, and the supporting structure: periodontal ligament (PDL), bone and cementum. (Nanci 2018)

outgrowths to form the dental papilla which gives rise to the dentine and pulp. Condensation of ectomesenchymal cells limiting the dental papilla and encapsulating the enamel organ is called the dental follicle. The dental follicle is responsible for forming the supporting structures of the tooth cementum, periodontal ligament and the alveolar bone. At the cytodifferentiation stage, the inner enamel epithelium (IEE) and the basement membrane of the enamel organ influence the dental papillae adjacent to the IEE to differentiate into odontoblasts. The IEE cells differentiate into ameloblasts (Tucker and Sharpe 2004). The odontoblasts are terminally differentiated cells. Before terminal differentiation, the pre-odontoblasts divide into two daughter cells. The daughter cell in contact with the basement membrane differentiates into odontoblasts and the other cell forms the cell rich layer of Höhl in the pulp.

1.2.1 Enamel

Enamel is a non-vital tissue and it is not capable of repair once damaged by carious cavitation, trauma or tooth wear. It is the hardest tissue in the body and is made up of 96% inorganic and 4% organic material. The process of formation of enamel is called amelogenesis. This process has three functional stages, namely the presecretory, secretory and maturation stages. The presecretory stage begins with the laying down of the dentine matrix against the basal lamina between the IEE and the odontoblasts that is yet to be mineralised, the cells of the IEE are still dividing while forming the crown of the tooth. Cell division stops and the cells of IEE differentiate into ameloblasts with polarisation of the nuclei and features for protein synthesis. It has been shown by (He *et al*. 2010) that opposing matrix proteins are expressed reciprocally by preameloblasts and preodontoblasts. The ameloblasts also become compartmentalised with a body and an extension called Tomes' process against which enamel forms. Once cells of the IEE differentiate into ameloblasts, they start secreting the enamel, which is only 30% mineralised. When the full thickness of immature enamel has been secreted, the maturation phase begins with cell modulation of post-secretory ameloblasts. This process hardens enamel through growth of the pre-existing crystal. Cell modulation activity facilitates the removal of water and matrix proteins and allows introduction of more inorganic material.

1.2.2 Dentine – development, type and structure

Dentine is a mineralised connective tissue like bone. Dentine comprises of 70% inorganic matter, 20% organic matter and 10% water. Dentine that is formed before completion of the crown and root is called primary dentine. Physiological deposition of dentine after completion of crown and root is secondary dentine and spans a lifetime. The secretion of dentine in response to external stimulation is tertiary dentine (Kuttler 1959).

The process by which dentine forms is called dentinogenesis. Various growth factors are known to play an important role in odontoblast differentiation and dentinogenesis. Growth factors such as TGF-β and BMP can initiate cytological and functional differentiation of odontoblasts from dental papilla cells and formation of dentine and enamel (Begue-Kirn *et al*. 1992; Heikinheimo *et al*. 1993; Heikinheimo 1994; Aberg *et al*. 1997). In addition, they maintain the embryonic stem cell pluripotency and facilitate their renewal and

differentiation (Watabe and Miyazono 2009; Beyer *et al*. 2013). IGF-1 has been shown to play an important role in odontoblast proliferation and differentiation (Yan *et al*. 2017). Early and limited exposure to FGF promotes differentiation of dental pulp cells into odontoblasts (Sagomonyants *et al*. 2015). These factors are also produced by pulpal and inflammatory cells during pulpal injury and inflammation; modulating them could help in repair and regeneration of the pulp (Bergenholtz 1981).

The differentiating odontoblasts starts producing dentine, which commences with the secretion of the organic matrix. The capillary plexus close to the cell-rich layer plays a key role in transporting nutrients to facilitate secretion and mineralisation of the dentine matrix (Tobin 1972). Molecular signalling of this angiogenic activity is mediated through VEGF (Tran-Hung *et al*. 2006; Goncalves *et al*. 2007). Inhibition of VEGF has been shown to cause degeneration of odontoblasts, decreased mineralization of dentine, inflammation and necrosis of the pulp (Fletcher *et al*. 2010). VEGF has been shown to possess mitogenic potential (Grando Mattuella *et al*. 2007). The organic matrix of the first deposited dentine contains type III collagen associated with fibronectin. This is followed by laying of type I collagen, which is parallel to the dentinoenamel junction. Dentine is made up mainly of type I collagen (Yamakoshi 2009). This dentine is called mantle dentine. Mineralisation of the matrix starts with the formation of small membrane-bound vesicles called matrix vesicles (MV), which bud off from the cell. MV contain tissue non-specific alkaline phosphate (TNAP), nucleotide pyrophosphate phosphodiesterase and annexin V (Golub 2009). The MV phospholipids, calcium and phosphate ions interact to form nucleation sites for hydroxyapatite crystal formation and thereby initiation of mantle dentine mineralisation (Bonucci 2002; Genge *et al*. 2007a, b).

With the completion of mantle dentine formation and elimination of the extracellular compartment between the odontoblasts, odontoblasts form a tightly packed layer of cells. With the full differentiation of the odontoblasts the dentine now produced is called circumpuplal dentine. The odontoblasts lie on the formative surface of the matrix and move centrally towards the pulp, leaving a cytoplasmic extension called the odontoblastic process within the dentinal tubule in the matrix. Collagen acts as a binding site for calcium and phosphate facilitating nucleation for hydroxyapatite crystal growth (Silver and Landis 2011). Control of the mineralisation process is now taken over by the non-collagenous proteins (NCPs). The mineralisation lags behind the formation of organic matrix. This unmineralised

matrix is called predentine. Gene mutation studies of NCP gene have confirmed their role in matrix organisation and mineralisation (McKnight *et al*. 2008). Non-collagenous matrix proteins such as SIBLINGs, DPP, DSP, DGP, DMP-1, osteocalcin, BSP, OPN and phosphoglycoprotein, proteoglycan and serum proteins are present in dentine (Hargreaves 2002). These proteins are packed between the collagen fibrils. The non-collagenous matrix proteins through their role as promotors, inhibitors and stabilisers regulate the mineralisation of dentine (Linde and Goldberg 1993; Butler and Ritchie 1995). The dentine that forms mineralises and thickens to form the primary or circumpulpal dentine. The dentine matrix mineralisation around the odontoblastic process gives the tubular structure to dentine. This dentine between the odontoblastic processes is called intertubular dentine and forms the bulk of circumpulpal dentine. The dentine in immediate proximity to the odontoblastic processes in the walls of the dentinal tubules is called peritubular dentine. Peritubular dentine is rich in NCPs and is deposited throughout life (Arana-Chavez and Massa 2004).

Deposition of secondary dentine is slower than primary dentine and it is deposited in an asymmetrical pattern. The tubules of secondary dentine are continuous with primary dentine, which is suggestive of the same odontoblast being responsible for primary and secondary dentine deposition. Focal secretion of dentine in response to external stimulation is called tertiary dentinogenesis. Based on type of cells involved and the intensity of the stimulation, whether mild or strong, tertiary dentine is sub-classified into reactionary and reparative dentine (Lesot. *et al*. 1993; Smith *et al.* 1995) respectively. Reactionary dentine forms in response to mild stimulation as in the case of a slowly progressing caries. Reactionary dentine is secreted by primary odontoblasts, which are still viable (Couve *et al*. 2014). The tubules are continuous with those of secondary dentine but narrower and fewer compared to primary dentine (Charadram *et al*. 2013). Reparative dentine is formed by new generation odontoblast-like cells (Tziafas *et al*. 2000) in response to intense stimulation as in the case of rapidly progressing caries which causes death of the postmitotic odontoblasts responsible for primary and secondary dentinogenesis. The dentinal tubules in reparative dentine are not continuous with those of secondary dentine. Reparative dentine can also be atubular. Tertiary dentine is laid so rapidly that it may include the cells that laid down the matrix, thereby giving an osteotypic appearance (Goldberg *et al*. 2011).

1.2.3 Pulp

As mentioned in (**section 1.1**), the aim of endodontic treatment, where possible, should be to maintain the vitality of the dental pulp and facilitate reparative and regenerative dentinogenesis. Therefore, it is important to understand the development and structure of the dental pulp to be able to appreciate it's immune-inflammatory and repair response to trauma and infection. The pulp develops from the dental papilla and is the soft connective tissue that supports dentine. Histologically the pulp has an outermost layer of odontoblasts, beneath which is the cell-free zone of Weil, and below this is the cell-rich zone and the innermost core of blood vessels and nerves. The pulp contains cells, collagen matrix and ground substance. Odontoblasts are post-mitotic cells, which poses a problem. When the pulp is exposed to the oral environment the affected odontoblasts die and repair takes place by formation of new dentine. This is due to new odontoblasts-like cells that must have differentiated and migrated to the sub-odontoblast region of the pulp. The differentiation of new odontoblasts-like cells occurs under the influence of the IEE but the stimulus for new odontoblast formation in this circumstance is different (Li *et al*. 2011) and not fully understood. The fibroblasts are the most abundant cells in the pulp especially in the cell-rich zone, and their role is to form and maintain the matrix of collagen and ground substance. They produce non-collagenous proteins and express growth factors such as TGF-β/BMP (Nakashima *et al*. 1994; Sloan *et al*. 2000). *In vitro* experiments have shown that fibroblasts stimulated by inflammatory cytokines and bacterial products cause degradation of connective tissue during pulpal inflammation (Nakata *et al*. 2000). Undifferentiated ectomesenchymal cells, when appropriately stimulated, form odontoblasts and fibroblasts and are found to be related to blood vessels. The pulp is also shown to contain multipotent dental pulp stem cells (DPSC) (Gronthos *et al*. 2002), which tend to proliferate in the pulp and migrate to the site of injury following pulp exposure (Tecles *et al*. 2005). Pulp defence is through dendritic cells (Jontell *et al*. 1998) macrophages, lymphocytes and leukocytes.

Extracellular matrix comprises of type I and III collagen (van Amerongen *et al*. 1983) in which the cells are scattered. The ground substance, which is a medium of transport between vasculature and cells, is made up mainly of glycosaminoglycans such as like chondroitin sulphate, dermatan sulphate, hyaluronic acid (Mangkornkarn and Steiner 1992), a variety of glycoproteins and water. The high viscosity of the proteoglycans creates a mechanical barrier to bacteria. NCPs of the pulp include fibronectin, osteonectin, BSP, OPN. Fibronectin binding

sites have been identified on collagen, glycosaminoglycans and cell adhesion molecules, making it a mediator of cell-cell and cell-matrix adhesion, thereby, affecting proliferation, differentiation and organisation of cells. Fibronectin mediates interaction between fully differentiated odontoblasts and extracellular fibres and facilitates maintenance of cell morphology (Yoshiba *et al*. 1994). Fibronectin is also involved in cell migration and anchorage during wound healing in the pulp and reparative dentinogenesis (Yoshiba *et al*. 1996). The pulp lacks several non-collagenous proteins found in dentine such as DSP, DPP, DMP and osteocalcin and this could be the reason why pulp does not mineralise like dentine.

1.3 Dynamics of pulp dentine complex-tertiary dentinogenesis

Dentine secreted in response to external influence, including dental caries, tooth wear, trauma and other tissue injury is called tertiary dentine (Kuttler 1959). The response can result in the production of dentine with a regular tubular matrix similar to primary and secondary dentine or dysplastic atubular dentine.

Reactionary dentine is tertiary dentine secreted by upregulation of primary odontoblasts in response to mild stimuli. Reparative dentine formation in contrast is a complex biological event of tertiary dentine production by newly differentiated odontoblast-like cells in response to a strong stimulus that has caused the death of the primary odontoblasts. Furthermore, reparative dentinogenesis may be a sequela of reactionary dentinogenesis and both may be observed in the same lesion. Due to the difference in cellular and molecular events in the two processes it would be useful to understand them in order to be able to exploit their potential in pulp healing and dentine repair.

1.3.1 Reactionary dentinogenesis

The chronological sequence of events after injury within the pulp-dentine complex helps to explain reactionary dentinogenesis, as it involves primary odontoblasts. This involves focal upregulation of the secretory activity of the surviving odontoblasts as an extension of the physiological behaviour of the odontoblasts, but by a stimulus distinct to that of normal physiological secretory behaviour. The intensity of the response will depend on the degree and duration of the stimuli and the extent limited to the odontoblast in communication with the stimuli through the dentinal tubules.

An *in vivo* study (Smith *et al*. 1994) showed that when dentine matrix components (DMC) were isolated and implanted in cavities prepared in ferret teeth without pulp exposure, odontoblasts survived and reactionary dentine matrix was secreted in a time-dependent manner. When DMC was not added, reactionary dentine was not observed. This supports the fact that the bioactive signalling molecules in dentine could be released and delivered to the odontoblast by diffusion of the injurious agents (Smith *et al*. 1994). Affinity chromatography of dentine matrix components revealed a high content of growth factors, in particular TGF-β which is implicated in reactionary dentinogenesis (Smith *et al*. 1995). When TGF-β1-3 containing agarose beads were implanted on tooth slices in culture, they were associated with showed upregulation of odontoblast secretion, manifested as an increase in predentine secretion compared to unstimulated controls, with mitogenic activity in the subodontoblastic layer closer to the TGF-β3 application site (Sloan and Smith 1999). Continuous application of TGF-β1, in a localised manner, to the dentine-pulp complex of human tooth slices *via* a tube glued to the dentine of the tooth slice revealed collagen production by odontoblast and subodontoblast cells. This suggests the potential of TGF-β1 to diffuse into the dentinal tubules to stimulate reactionary dentinogenesis. This study found that healthy pulpal cells had increased staining for TGF-β3 compared to the pulp cells of carious teeth, that showed TGF-β1 at higher levels. This again suggests the sublimation and release of TGF-β1 to stimulate reactionary dentinogenesis. Only TGF-β1 is sequestrated into the dentine matrix (Cassidy *et al*. 1997), suggesting the existence of a reservoir of growth factors in dentine that are readily released by degradation of the matrix. Tissue injury may release adrenomedullin (Tomson *et al*. 2007; Musson *et al*. 2010) and other bioactive molecules like IGF-1-2 (Finkelman *et al*. 1990), BSP (Decup *et al.* 2000; Goldberg *et al.* 2001) and dentonin, a synthetic peptide derived from matrix extracellular phosphoprotein (Six *et al*. 2007), which may play a role in reparative processes. This suggests that a diverse cocktail of powerful bioactive molecules may be released by injury and play an important role in reactionary dentinogenesis.

The different steps in treatment of a carious lesions can also influence the type of tertiary dentinogenesis, including cavity preparation, its dimension, remaining dentine thickness (RDT) after caries excavation, the type of etch used or restorative material and the method of application. An *in vivo* study reported that if the RDT was between 0.25-0.5 mm then an increased thickness of reactionary dentine was observed. $Ca(OH)_2$ was associated with a greater thickness of reactionary dentine and zinc oxide-eugenol the least. Cavities closer to

the pulp injured the odontoblasts and reduced their number to just over 50% (Murray *et al.* 2002). These studies were conducted on sound not carious teeth, which makes it difficult to understand the interplay of inflammation on regeneration that is important in understanding the interaction between injury and repair events (Cooper *et al*. 2011). Etching with ethylenediaminetetraacetic acid (EDTA) for 60s stimulated increased secretion of reactionary dentine (Murray *et al*. 2008). The effect of EDTA may be due to the solubilisation of bioactive molecules from dentine. Furthermore, the increased reactionary response to $Ca(OH)_2$ may be due to its ability to release growth factors from dentine (Graham *et al*. 2006).

1.3.2 Reparative dentinogenesis

Reparative dentinogenesis could be an independent reparative process of the dentine-pulp complex to severe insult causing death of the primary odontoblasts or a sequela of reactionary dentinogenesis. As the primary odontoblasts are destroyed, reparative dentinogenesis involves a more complex process of recruiting progenitor cells from the pulp, inducing them to differentiate into odontoblasts-like cells and upregulating them to secrete reparative dentine. The last step is the same in reparative and reactionary dentinogenesis and it is the first two steps that help in differentiating the two processes.

Histologically, the matrix of reparative dentine has a wide spectrum of appearances, from regular tubular to dysplastic and atubular. Variation in morphology of the secretory cells correlates with the type of secretory activity, leading the heterogeneity of structure and composition of the matrices. This heterogeneity also reflects the specificity of the dentinogenic process taking place. When the environment within the dental pulp is favourable for tissue healing, it allows the expression of intrinsic potential of pulp cells to differentiate into odontoblast like cells (Tziafas 1995; Inoue *et al*. 1981), which facilitates physiological dentine formation with tubular matrix, secreted by columnar and polarised odontoblast-like cells. In contrast, when an inappropriate environment exists, the process of fibrodentinogenesis by cuboidal/spindle-shaped fibrodentinoblasts produces atubular matrix called fibrodentine during repair (Tziafas 1995) The osteotypic form of fibrodentine is called osteodentine (Tsuji *et al*. 1987), elaborated by osteodentinocytes and observed when the pulp was surgically exposed in rats. Pulp exposures in germ-free animals has shown fibrodentine was to be replaced by tubular matrix produced by differentiating odontoblast during reparative dentinogenesis (Baume 1980; Inoue and Shimono 1992). The cells involved

in fibrodentinogenesis resemble undifferentiated pulp cells, pointing to a non-specific wound healing response. It is thought that the fibrodentine matrix may immobilise the bioactive molecules from dentine that can stimulate odontoblast differentiation. The difference in tubularity of the reparative dentine influences its permeability and thereby its potential to protect the pulp from the effect of bacterial and restorative materials. Therefore, understanding the cellular behaviour that contributes to tubular and atubular dentine formation would be useful to exploit its potential in regenerative treatment.

The presence of the dentine-pulp tissue environment is important in the differentiation of DPSCs into odontoblast-like cells (Batouli *et al*. 2003; Liu *et al*. 2005). Stem cells from human exfoliated primary teeth have been reported to have the potential to differentiate into odontoblasts (Cordeiro *et al*. 2008). The exposure of the undifferentiated cells of the cell-rich zone of Höhl and the cell from the preodontoblast division have been traditionally considered as progenitor cells for odontoblast-like cells. Notch signalling pathways have been identified on subodontoblastic cells during reparative responses to injury and associated with vascular structures in the apical area of the root, indicating that these cells may have a role in reparative dentinogenesis. Notch signalling pathway is critical in the regulation of stem cells to differentiate into odontoblast-like cells (Mitsiadis *et al* 2011; Oh and Nör 2015). Other cells implicated as progenitor cells for odontoblast-like cells are undifferentiated mesenchymal cells, perivascular cells and fibroblasts (Fitzgerald *et al*. 1990). This makes it clear that no single stem or progenitor cell is responsible for reparative dentinogenesis. Therefore, it is important to understand the cell recruitment in reparative dentinogenesis in order to be able to make use of the appropriate stem and progenitor cells in reparative therapy.

TGF-β1 has been shown to be chemotactic and mitogenic to cells in the subodontoblastic layer (Veis *et al*. 1989; Melin *et al*. 2000; Goncalves *et al*. 2016). There are several studies (*in vitro, in vivo and ex vivo*) which have demonstrated the significance of the TGF-β family in odontoblast-like cell differentiation (Tziafas *et al*. 1998; Sloan and Smith 1999; Dobie *et al*. 2002). The role of TGF-β1 in odontoblast-like cell differentiation has been confirmed by blocking the TGF-β1 with antibodies, which prevented odontoblast differentiation (Begue-Kirn *et al*. 1992). Demineralised dentine (Tziafas and Kolokuris 1990), predentine (Tziafas *et al*. 1992b), dentine-matrix derived bioactive molecules like BMP, osteogenic proteins and demineralised dentine placed on exposed pulps in animal teeth have shown to induce regular tubular dentine secretion by odontoblast-like cells (Smith *et al*. 1990; Nakashima 1994;

Rutherford *et al*. 1994). *In vitro* studies have shown that the pulpal response to $Ca(OH)_2$ and MTA may be mediated by the release of growth factors sequestrated in the dentine matrix (Tomson *et al*. 2017). Fibronectin has been suggested to control the matrix-cytoskeletal interaction, which could cause cytological modification and polarisation in normal odontoblast differentiation (Lesot *et al*. 1992). Further, allogenic plasma fibronectin, when implanted in dog pulp tissue, induced thick layers of dentine and odontoblast-like cells differentiation (Veis 1985; Tziafas *et al*. 1992a). This response was observed only in young pulp and not old pulp tissue. Injury to the pulp can directly release bioactive molecules. Death of odontoblasts can release intracellular contents and inflammatory cells attracted to the site of injury can also release growth factors (Cooper *et al*. 2011), all of which can directly or indirectly influence reparative dentinogenesis. Therefore, it would be useful to understand pulpal infection and the immuno-inflammatory responses of the pulp more fully.

1.4 Pulpal infection and inflammation

1.4.1 Oral microbiology

The oral microbiome is an ecological community of commensal, symbiotic and pathogenic microorganism. This supports the fact than 500-700 bacterial species have been detected in the oral cavity (Jenkinson and Lamont 2005; Paster *et al*. 2006). Endodontic disease is microbially mediated and is initiated and propagated by a complex community of microorganisms that are common members of the commensal oral microflora (Pourhajibagher *et al.* 2017). The predominant groups of bacteria found in the oral cavity include *Streptococcis, Neisseria, Veillonella, Actinomyces* and obligate anaerobes (Avila *et al*. 2009). Most organisms present in the oral cavity are beneficial to health but some of them can transition from commensal to pathogen due to environmental triggers, thereby causing infection (Chino *et al.* 2009).

1.4.2 Microbiology of caries and pulpal Infection

The caries process begins with the formation of a conditioning film called the acquired pellicle, which is composed of biologically active proteins and glycoproteins derived from saliva. Initially, bacteria attach reversibly, but soon the adhesins on these early colonisers (such as *S. mitis* and *S. oralis*) bind irreversibly to receptors on the acquired pellicle (Whittaker

et al. 1996). These initial bacteria then multiply and their metabolites modify the micro-environment, facilitating the attachment of obligate anaerobes to receptors of bacteria already attached by the process of coaggregation (Kolenbrander *et al*. 2006) and making biofilms more diverse with a mixture of cocci, rods and filaments (Jenkinson and Lamont 2005). The bacteria then secrete extracellular polymers, this along with nucleic acid from dying microbes stabilise and consolidate attachment of the biofilm (Fong and Yildiz 2015).

The role of bacteria and sugar in dental caries have been confirmed in classic animal studies where germ-free rats failed to develop caries even when fed a high sucrose diet (Orland *et al*. 1954; Horton *et al*. 1985). The bacteria produce organic acids through the metabolism of fermentable carbohydrates causing an acidified environment lowering the pH of the plaque below the critical value of 5.5, resulting in dissolution of the hydroxyapatite crystal of the tooth (Featherstone 2004; Selwitz *et al*. 2007). The most frequently implicated gram-positive bacteria in the initiation of early caries development are *Mutans Streptococcus, Actinomyces viscosus* and species from *Lactobacillus* genus (Brown *et al*. 1986; van Houte *et al*. 1994). The main gram-negative bacteria identified were *Veillonella Sp. Bacteroides* (*Prevotella* and *Porphyromonas* spp) and *Fusobacterium* sp. (Brown *et al*. 1986; van Houte *et al*. 1994). The lactic acid produced by the bacteria dissolves the hydroxyapatite crystals by demineralisation (Featherstone 2000). It has been demonstrated that even if demineralisation has progressed deep into the enamel, the surface remains intact. This is due to neutralisation of the acids and remineralisation action of the saliva (Featherstone 2004). If this demineralisation is not treated, it causes dental caries and eventually cavitation of the enamel.

The porosity in enamel from demineralisation makes it more permeable to acids and bacteria, facilitating their entry into the relatively less mineralised dentino-enamel junction. At this point the carious lesion spreads laterally and pulpally by the penetration of bacteria via the dentinal tubules in the demineralised dentine causing cavitation. Cavitation makes the pulp-dentine complex vulnerable to a vast number of oral bacteria. With the progression of the carious lesion, the environment changes due to reduction in carbohydrate availability, increase in pH and reduction in oxygen. These changes cause a change in the microflora from the ones responsible for initiation to ones of progression, allowing microaerophilic opportunistic bacteria such as *Streptococcus anginosus* with moderate cariogenic potential (Drucker and Green 1977) to thrive.

Early dentine invasion was predominated by *lactobacilli*, *Actinomyces* spp., *veillonellae*, and *mutans streptococci* (Love and Jenkinson 2002). A high number of obligate anaerobes such as *Eubacterium* sp., *Propionibacterium* spp., *Bifidobacterium* sp., *Peptostreptococcus micros*, and *Veillonella* sp. have been observed in dentinal tubules (Love and Jenkinson 2002). *Lactobacilli* have been demonstrated to be predominant species at the advancing front of caries in dentine (Byun *et al*. 2004). Deep carious lesions also demonstrate gram-positive rods, anaerobic *Lactobacilli* sp. and gram-positive cocci such as *Peptococcus* and *Peptostreptococcus* (Hahn *et al*. 1991). In the same study two types of deep carious lesion, based on the presence of high or low numbers of *lactobacilli,* as well as the demonstration of low *Streptococcal* numbers, which predominate in early lesions. This suggests that *Streptococci* cannot survive the highly acidic environment of *lactobacilli* and therefore may be responsible for enamel caries (Bowden *et al*. 2000). As the lesion progresses deeper, the microflora is predominated by gram-negative anaerobic rods such as *Prevotellae, Porphyromonas* and *Fusobacteria* (Massey *et al*. 1993). The dentine responds to bacterial insult by peritubular dentine formation to occlude the dentinal tubules or through the formation of reparative or regenerative tertiary dentine based on the rate of progression of caries and the type of insult to the pulp, as discussed in the previous section.

As the caries progresses it elicits inflammatory and immunological responses in the pulp, which could be symptomatic or asymptomatic. Samples from teeth with vital pulps and deep carious lesions close to the pulp demonstrated a predominance of *Prevotella intermedia* and *Prevotella melaninogenica* (Massey *et al*. 1993). The histology of the pulp from the same teeth showed mononuclear infiltration irrespective of the variability in microbial flora. This study cultured bacteria by plating them on non-selective media and incubation under anaerobic and microaerophilic conditions, which may not give an accurate representation of the full bacterial microflora present. Martin *et al.* (2002) conducted a similar experiment but used real-time PCR and specific probes to identify bacteria to overcome the disadvantages of culturing bacteria. They reported a high number of *Prevotella* and *Fusobacterium* spp in addition to *M. micros*, *P. endodontalis* and *P. gingivalis* in most carious samples. A significant positive correlation was detected between the presence of *M. micros* and *P. endodontalis* and inflammatory degeneration of pulpal tissues (Martin *et al*. 2002). This study suggested that the presence of a high levels of these bacteria in the carious lesion might indicate irreversible pulpitis. Further, Hahn *et al*. (1991) demonstrated through culture that in teeth with deep carious lesions causing irreversible pulpitis, there were greater numbers of

bacteria in the carious lesion than in the pulp and that the number of branching rods and *A. viscosus* were significantly higher in the pulp compared to the caries site, there was a high number of *Lactobacilli* spp in the deep caries with less *S. mutans*. This study also identified the presence of *Streptococcus constellatus* in the carious lesions. To overcome the disadvantages of the culture method of identification, Rôças *et al*. (2016) used 16S rRNA gene sequencing and bioinformatics analysis to explore the microbiome of deep carious lesions and symptomatic irreversible pulpitis. This study, like that of Hahn *et al*. (1991), confirmed the existence of high and low lactobacillus carious lesions. The study also identified *Pseudoramibacter, Olsenella, Streptococcus,* and *Stenotrophomonas* as being dominant, demonstrating high bacterial diversity in deep carious lesions associated with irreversible pulpitis. These bacteria were also detected in infected root canals. Lactobacilli have been suggested to have an important role in pulp death (Hahn *et al*. 1991). If the necrotic pulp is not removed then the infection spreads to the periapical area where it can cause abscesses, which are polymicrobial and consist of strict anaerobes (such as anaerobic cocci, *Prevotella*, *Fusobacterium* species) and facultative anaerobes (such as viridans group *Streptococci* and the *Streptococcus anginosus* group) (Shweta and Prakash 2013). A systematic review demonstrated the presence of the commensal bacterium *Enterococcus faecalis* in persistent intraradicular infections and untreated chronic periapical periodontitis (Zhang *et al*. 2015)**.**

Commensals such as SAG are identified as having moderate cariogenic potential (Drucker and Green 1977); are often identified in pulpitis (Hahn *et al*. 1991) and periapical abscesses (Shweta and Prakash 2013) and may be some of the early colonisers of the pulp (Roberts 2010). Therefore, it would be worthwhile to investigate their effect on dental pulp in greater detail.

1.4.3 Streptococcus anginosus group bacteria (SAG)

Also known as *S. milleri* group, a subgroup of *Viridans Streptococci* which area large group of commensal *streptococci*. SAG are considered normal commensals in humans and are commonly isolated from the oral cavity, oropharynx, gastrointestinal tract and vagina (Whiley *et al*. 1992). They have the potential to cause bacteraemia and abscesses (Whiley *et al*. 1992). More recent molecular analysis shows three discrete DNA homologies within SAG, corresponding to three distinct species: *Streptococcus constellatus, Streptococcus intermediu* and *Streptococcus anginosus* (Whiley and Beighton 1991; Jensen *et al*. 2013). *S.*

constellatus has three subspecies: *S. constellatus* subsp. *constellatus*, *S. constellatus* subsp. *pharyngis*, and *S. constellatus* subsp. *viborgensis* (Whiley *et al*. 1999). *S. anginosus* has two subspecies: *S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whiley* (Jensen *et al*. 2013). They are recognised as a commensal in the oral cavity and gastrointestinal tract.

SAG are Gram-positive, catalase-negative cocci, non-motile facultative anaerobes and can be beta-, alpha-, or gamma-haemolytic (Spellerberg and Brandt 2017). They can grow in the presence of CO_2, whereas some strains grow in anaerobic conditions. They can be grown in the laboratory on blood agar or fastidious anaerobic agar (Facklam 2002). Members of the *S. anginosus* group often exhibit Lancefield antigens A, C, F, or G or none of these (Whiley and Beighton 1998). The members of this group can be differentiated from other *Streptococci,* as they are positive for three biochemical reactions: acetoin production from glucose, arginine and sorbitol. *S. intermedius* differs from other strains by its ability to produce detectable levels of alpha-glucosidase, beta-galactosidase, beta-D-fucosidase, beta-N-acetylgalactosaminidase, beta-N-acetylglucosaminidase, and sialidase with 4-methylumbelliferyl-linked fluorogenic substrates and by its ability to produce hyaluronidase. By contrast, *S. constellatus* produces alpha-glucosidase and hyaluronidase and *S. anginosus* produces beta-glucosidase. All three strains have been identified in the oral cavity (Whiley *et al.* 1990; Spellerberg and Brandt 2017).

The establishment of an infection is dependent on various factors and the coordinated expression of various virulence genes. The pathogenicity of the SAG varies from strain to strain and also between different isolates of the same strain based on the virulence factors (Asam and Spellerberg 2014) . Bacteria need to adhere to the host cell to colonise the tissue and cause infection. *In vitro* studies have shown that attachment of SAG to hydroxyapatite beads to be in the same range as *S. mutans*, but the ligands involved were different, as adherence inhibitors of the latter did not affect SAG (Tarsi *et al*. 1998). SAG has been shown to adhere to extracellular matrix components fibronectin, fibrinogen and laminin and to a lesser extent with collagen (Allen *et al*. 2002a). SAG strains isolated from infection show greater affinity to fibronectin, adhere to epithelial cells and saliva coated hydroxyapatite than other *viridans Streptococci* (Willcox and Knox 1990). Homologues of a number of adhesins found in streptococci have been identified in SAG (Asam and Spellerberg 2014) but their functions have not yet been investigated.

In polymicrobial infections, *Eikenella corrodens* has been shown to co-aggregate with SAG and tostimulate their growth (Young *et al*. 1996). One *in vitro* study showed that human polymorphnuclear leukocytes (PMNL) when incubated with SAG stimulated less chemotaxis and resistance to killing by the PMNL compared to *S. aureus,* but the virulence factor for this phenomenon is unclear (Wanahita *et al*. 2002). Capsule production by bacteria contributes to their virulence by protection against antibodies. This was demonstrated by infecting mice with encapsulated and unencapsulated strains of *S. constellatus*. It was observed that the capsule inhibited phagocytosis and killing of SAG by PMNLs (Kanamori *et al*. 2004). The same effect was demonstrated using a murine model of pulmonary infection (Toyoda *et al*. 1995). This was also confirmed by the identification of the gene locus for capsular polysaccharide synthesis in SAG (Tsunashima *et al*. 2012; Olson *et al*. 2013). It has been shown that *S. intermedius* exerts immunosuppressive effects through the expression of B-cell mitogenic protein (P90) (Lima *et al*. 1992).

Hydrolytic enzymes such as hyaluronidase and chondroitin sulphatase are very important for extracellular matrix breakdown, thus facilitating bacterial invasion (Jacobs and Stobberingh 1995; Olson *et al*. 2013; Sunwoo and Miller 2014). Nucleases ribonuclease and deoxyribonuclease destroy nucleic acids contributing to SAGs' pathogenicity (Jacobs and Stobberingh 1995). A strain of SAG isolated in India expressed superantigen and extracellular DNase coding genes identical to corresponding virulence genes of *S. pyogenes* (Babbar *et al*. 2017). These virulence factors contribute to evasion of the neutrophil extra-cellular trap immune mechanisms. *S. intermedius* has been shown to produce a cytolysis intermedilysin (a β-haemolysin), which has been identified as a virulence factor for formation of deep seated abscesses (Nagamune *et al*. 2000).

Streptococci inhibit host colonisation by related bacteria by expression of peptides called bacteriocins, which are considered to be virulence factors. The genes coding for bacteriocins are located close to the Streptococcus Invasion Locus (*Sil*) system and these bacteriocin/immunity genes are found adjacent to the *sil* system seen in most SAG (Mendonca *et al*. 2016). Quorum sensing mechanism is responsible for regulating bacterial growth in biofilms, virulence regulating, genetic competence in bacterial (Asam and Spellerberg 2014). Gene *lux*S operon associated with quorum sensing has been identified in SAG and has been demonstrated to influence antibiotic susceptibility (Ahmed *et al*. 2007). Further, natural competence which is the ability of bacteria to transport environmental

fragments of DNA across their cell envelope that could then replace homologue segments of the bacterial chromosome. This is controlled by the gene, competence regulation operon (*com* operon). SAG has been demonstrated to express *com* operon required for natural competence (Olson *et al*. 2013).

1.4.4 Enterococcus faecalis

Like SAG, *Enterococcus faecalis* is part of the normal human commensal flora found in the intestine, oral cavity and vagina (Sedgley *et al*. 2006). *E. faecalis* is frequently identified from persistent root canal infections, with a prevalence of up to 77% (Stuart *et al*. 2006). More recent evidence suggest that other species may be predominant in primary and secondary root canal infection and persistent apical periodontitis (Tennert *et al*. 2014; Sanchez-Sanchueza *et al*. 2018) It has also been identified in periradicular abscesses (Rocas *et al*. 2004). Clinical study has identified the presence of *E. faecalis* in 80% of samples from deep carious lesions (Radman *et al*. 2016). Studies have also identified *E. faecalis* in irreversible pulpitis (Kutllovci *et al*. 2015). Clinical findings mentioned above suggest that *E. faecalis* could be present as an early coloniser in the pulp along with SAG and subsequently become pathogenic when suitable conditions arise and the microenvironment in the dental pulp changes**.** Most literature has evaluated the role of *E. faecalis* in failed root canal treatments. It might be worthwhile evaluating the role of *E. faecalis* in pulpitis, as there is limited information in this area.

The species *E. faecalis* belongs to the genus Enterococci previously classified as group D *Streptococci*. It is a non-motile, Gram-positive, facultatively anaerobic, catalase-negative coccus arranged in pairs or short chains, and grows at a range of 10 to 45^{o}C at pH 9.6 in broth containing 6.5% NaCl or 40% bile (Murray *et al*. 2016). It catabolises a variety of energy sources including glycerol, lactate, malate, citrate, arginine, agmatine and many keto acids. It resists bile salts, detergents, heavy metals, ethanol, azide, and desiccation and can survive up to 30 min at 60^{o}C (Gijo *et al*. 2015).

The pathogenicity of *E. faecalis* is due to a number of virulence factors. Its virulence is due to the ability to adhere to host forming biofilms, production of various enzymes and their high antibiotic resistance (Murray *et al*. 2016). Its ability to withstand acid and alkali is due to the impermeability of its membranes and its resistance to a range of temperature is due to the

membrane lipid and fatty acid (Fisher and Phillips 2009). Adherence and colonisation of abiotic surfaces by *E. faecalis* is mediated through cell surface adhesins called *enterococcal* surface proteins (Toledo-Arana *et al*. 2001; Paganelli *et al*. 2012). AS, another plasmid encoded adhesion, facilitates plasmid exchange between bacteria and mediates binding to extracellular matrix proteins, including collagen type I. This could be the mechanism for attachment of *E. faecalis* to dentine which is important for endodontic infection (Gijo *et al*. 2015). AS expressing *E. faecalis* resists killing by neutrophils despite their marked phagocytic ability and the neutrophil activation (Rakita *et al*. 1999), leads to phagosoaml oxidant production which causes tissue damage. *E. faecalis,* having surface AS, stimulates T-cells and macrophages, which cause tissue damage by nitric oxide production mediated by TNF-α produced by these cells (Gijo *et al*. 2015).

E. faecalis has been known to produce gelatinase which can hydrolyse gelatine, collagen and contributes to adherence and biofilm formation (Franz *et al*. 2003; Kayaoglu and Ørstavik 2004). Sex pheromones produced by *E. faecalis* are responsible for virulence traits like antibiotic resistance and cytolysin production (Clewell and Weaver 1989). Cytolysin is a potent bacteriocin produced by *E. faecalis* which exacerbates infection (Van Tyne *et al*. 2013). Hyaluronidase production by *E. faecalis* facilitates tissue invasion (Fisher and Phillips 2009). Hyaluronidase breaks down hyaluronan in dentine, enabling *E. faecalis* survival in dentinal tubules (Gijo *et al*. 2015). Lipoteichoic acid produced by *E. faecalis* is thought to contribute to its resistance against lethal conditions (Gijo *et al*. 2015).

E. faecalis has a natural resistance to penicillin and isolates from root canals have been shown to possess 100% resistance (Radeva and Karayasheva 2017). Furthermore, the treatment of acute endodontic infection with penicillin may suggest that *E. faecalis* could be an innocent bystander than a pathogen in endodontic infections. The presence of *E. faecalis* in endodontic infections and the virulence factors contributing to its pathogenicity warrants further investigation to tackle it at the early stages of pulpal infection before it becomes established and causes infections that are difficult to treat.

1.4.5 Inflammatory response of the pulp

Clinically, inflammation of the pulp is known as pulpitis. Based on the signs and symptoms pulpitis is classified into reversible pulpitis, symptomatic irreversible pulpitis and

asymptomatic irreversible pulpitis (Glickman 2009). In reversible pulpitis, if the cause of inflammation is removed the pulp heals. In the case of irreversible pulpits, the tooth is treated by partial or complete removal of the pulp.

The major cause of inflammation in the human dental pulp is oral bacteria that are responsible for carious lesions in dentine (Love and Jenkinson 2002). Pulpal immunoinflammatory and neurogenic inflammatory processes are initiated by host innate and adaptive immune responses to bacteria and the components they release into the dentinal tubules (Park *et al*. 2015b). Therefore, a thorough understanding of the pulpal inflammatory process is necessary for development of treatment procedures and immunotherapeutic agents.

1.4.6 Process in pulpal infection and inflammation

Inflammation is part of the body's normal response to infection to eliminate the injurious pathogens; uncontrolled inflammation can result in acute, chronic and systemic inflammatory disorders (Serhan and Petasis 2011). The following factors can cause pulpal inflammation: microorganisms through caries, chemical irritations from etchants, bonding agents and restorative materials, cracks, restorative procedures, periodontal disease leading to inflammation of the pulp, trauma including occlusal trauma and orthodontic movement.

Even though the dental pulp is a connective tissue it behaves differently from other connective tissues. It was believed that as pulp is present in a low-compliance environment with only a neurovascular supply from the nerves and vessels through the apical foramen. This could cause self-strangulation of the pulp from inflammation, due to its lack of ability of collateral perfusion, but this has been disproved by (Van Hassel 1971). Furthermore, there is evidence to suggest that there is a reduction in pulpal blood flow on application of inflammatory mediators (Kim *et al*. 1992) and mechanical feedback causing lymphatic absorption of interstitial fluid to reduce pressure (Heyeraas and Berggreen 1999), thereby making pulpal inflammation a longstanding condition. Therefore, removal of the cause could facilitate healing. In the dental pulp, innate and adaptive immune responses try to eliminate or neutralise the bacteria and their components and dampen their effects, thereby reducing the secretion of pro-inflammatory mediators (Hahn and Liewehr 2007a,c). The innate immune system depends on the release of local mediators and phagocytic cells such as

macrophages, monocytes, neutrophils and DCs and it is the first line of defence which initiates the recognition of pathogens by cells (Hahn and Liewehr 2007a; Crozat *et al*. 2009). Adaptive immunity is mediated through antigen-specific T and B cells.

The inflammatory response in the pulp is initiated by recognition of the pathogens by PRR which are important in initiating the innate immune response through recognition of pathogen-associated molecular patterns (PAMPs) (Jang *et al*. 2015). PRRs identified in the pulp cells include Toll-like receptors (TLR) TLR2, TLR4 and TLR9, nucleotide-binding oligomerization domain proteins 1 and 2 (NOD1, NOD2), and the Nod-like receptor family member pyrin domain containing 3 complex (inflammasome) (NALP3) (Jang *et al*. 2015). These receptors have been identified on odontoblasts, pulpal fibroblasts, pulp stem cells, neurons, endothelial cells and they can detect DNA and membrane components of bacteria (Staquet *et al*. 2008; Hirao *et al*. 2009; Lin *et al*. 2009; Farges *et al*. 2011; Horst *et al*. 2011; Lee *et al*. 2011; Staquet *et al*. 2011). The signal transduction pathways that are activated via PRRs converge on a common set of signalling modules including nuclear factor- (NF-) κB, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK). These modules drive the production of proinflammatory cytokines/chemokines such as interleukin- (IL-) 1, tumour necrosis factor- (TNF-) α, and IL-6 (Akira *et al*. 2006; Kawai and Akira 2009; He *et al*. 2013). The activation of NF-κB and p38MAPK also leads to down regulation of dentine formation, production of pro-inflammatory mediators IL-6 and chemokines CCL2, CXCL1,2 and CXCL8 and accumulation of immature dendritic cells close to the odontoblast layer (Farges *et al*. 2009).

The odontoblasts are the first line of cellular defence, which come into contact with pathogens and their toxins entering through dentine. They have been shown to be involved in environmental sensing and innate immunity through TLR-1 to 6 and -9 (Staquet *et al*. 2011). Ligand binding to these receptors activates NF-κB and p38 MAPK pathways which orchestrate an inflammatory response (Farges *et al*. 2011; Horst *et al*. 2011). NOD is present in odontoblasts and helps in intracellular recognition of bacterial components, which causes IL-8 and MCP-1 cytokine production (Lee *et al*. 2011). The TLR2, 3, 4 and 5 and NOD1 and 2 receptors are present on pulpal fibroblast and pulpal vascular endothelial cells and on binding of ligands secrete chemokine cytokines, adhesion molecules, prostaglandin E2 and COX-2 (Hirao *et al*. 2009). *In vitro* experiments have shown that TLR2 acts synergistically with NOD2 to stimulate pro-inflammatory mediator secretion by pulpal fibroblasts (Keller *et al*. 2010).

The chemokines and cytokines released by odontoblasts and pulpal fibroblasts initiate a complex signalling network which amplifies the cellular and molecular response, leading to a cascade of cellular interaction and recruitment (Taub and Oppenheim 1994). Resident immune cells play a sentinel role in the pulp. As the infection progresses, the inflammation becomes more intense, demonstrated by increases in the levels of pro-inflammatory cytokines INF-α, IFN-γ, IL-1β, IL-6, CXCL8, and IL-18 (Farges *et al*. 2011; Farges *et al*. 2013; Goldberg *et al*. 2015) and the number of T- and B- lymphocytes, plasma cells, neutrophils and macrophages increase in the infected pulp (Izumi *et al*. 1995). IL-10 plays a critical role in limiting the immune response to pathogens. These cytokines form a chemotactic gradient for recruitment and activation of immune cells. The migration and antimicrobial activity of immune cells (Cooper *et al*. 2017) cause collateral host tissue damage through degenerative enzymes and release ROS from bacterial destruction. ROS along with other potent enzymes cause tissue damage. ROS activates the NF-κB, p38 MAPK pathway, which exacerbates the inflammatory response (Morgan and Liu 2011). The pulp is richly innervated and the sensory nerve fibres contain substance P, CGRP and neurokinin A, which when released can cause neurogenic inflammation (Okiji *et al*. 1997).

As mentioned earlier, chronic unresolved inflammation causes inflammatory infiltration, collagenous fibrosis and premature aging of the pulp, which reduces its capacity to defend against future insults (Farges *et al*. 2015). Conversely, if inflammation is controlled and modulated then this will establish homeostasis that will tip the inflammatory process favourably to promote repair and regeneration (Eming *et al*. 2007). Therefore, it is essential to understand the interplay of inflammation in repair and regeneration of the pulp-dentine complex.

1.4.7 Inflammation in pulp repair and regeneration

The dental pulp will attempt to repair and regenerate by initiating a defence response to injury and infection. Therefore, elimination of the injurious agent, tissue debris and removal of pro-inflammatory mediators to control inflammation will create an environment conducive to facilitate repair and regeneration, thereby limiting damage to the pulp (Eming *et al*. 2007; Buckley *et al*. 2013). The current evidence supports the notion that inflammation is a prerequisite for tissue repair (Eming *et al*. 2007), especially in dental pulp tissue (Goldberg *et al*. 2015). Recognition of bacteria by the odontoblast and fibroblast membrane receptors triggers an inflammatory response within the pulp tissue that modulates its repair process

(Goldberg *et al*. 2008). The production of cytokines in response to infection helps to eradicate microbes and create a balance between pro- and anti-inflammatory cytokines and facilitate a favourable environment for healing (O'Shea *et al*. 2002).

Severe inflammation will degrade the pulp tissues and may prevent regeneration, but a low-grade inflammation may be advantageous for the dental repair process (Cooper *et al*. 2010). The reaction of pulp to carious lesions and biomolecules suggests that the inflammatory reaction might be a prerequisite for the burst of progenitors implicated in pulp repair (Goldberg and Smith 2004). Repair by DC agents involves a cascade of four steps: a moderate inflammation, commitment of adult reserve stem cells, their proliferation and terminal differentiation (Schroder 1985; Goldberg *et al*. 2008). *In vivo* human study of pulp capping with $Ca(OH)_2$ or MTA has shown that they stimulate tertiary dentine formation but this is preceded by necrosis of a superficial layer of the pulp in contact with the capping agent and inflammation of the pulp due to high pH from the hydroxyl ions soon after placement of the capping agent (Nair *et al*. 2009). This study also showed milder inflammation with MTA leads to better barrier formation than $Ca(OH)_2$, supporting the fact that mild inflammation promotes repair and regeneration. There is evidence that necrotic cells release low levels of pro-inflammatory mediators (Acosta-Perez *et al*. 2008; Luheshi *et al*. 2009; Liu *et al*. 2015). Therefore, necrosis induced by $Ca(OH)_2$ or MTA induces a mild immune-inflammatory response which promotes repair (Aeinehchi *et al*. 2007). Expression of pro-inflammatory cytokines IL-1α, IL-1β, IL-2, IL-6 and IL-8 has been demonstrated by mineralising cells on application of MTA or bioceramic cements, supporting the role of inflammation in repair by cellular response to pulp capping agents (Chang *et al*. 2014; Chang *et al*. 2015). *In vivo* implantation of bioactive molecules into rat pulp showed that the first event was an inflammatory process within the first 3-7 days after implantation and resolution of inflammation within two weeks (Jegat *et al*. 2007). This supports the fact that inflammation is the initial step in the healing cascade (Goldberg *et al*. 2008).

TLR2 activation in human odontoblast-like cells selectively induces production of mediators that could positively or negatively influence inflammatory and immune responses in pathogen-challenged tissues. It has been suggested that cytokines may be responsible for regulating and fine-tuning the pulpal response to Gram-positive bacteria that enter dentine during the caries process (Farges *et al*. 2011). The p38 MAPK pathway discussed in the previous section, that is responsible for inflammatory cytokine production, was identified to

be active in primary dentinogenesis, down regulated in secondary dentinogenesis (Simon *et al*. 2009) and active in tertiary dentinogenesis (Simon *et al*. 2010). This suggests that a similar pathway is involved in inflammation during repair and regeneration.

EFAD diets when fed to rats showed increased cell density in the pulp (Vermelin *et al*. 1995). This was not due to mitotic cell division in the pulp, suggesting that the increase in cell population may be from arrested apoptosis (Nishikawa and Sasaki 1999). Essential fatty acids are transformed into prostaglandins and leukotrienes and the EFAD diet affected their production, thereby affecting the balance between normal cell death and renewal, suggesting a link between inflammation and cell renewal for pulp repair. A range of growth factors and cytokines are expressed by the odontoblast and DPCs and these have potential to signal cellular events during repair and regeneration. Dentine matrix has been shown to be a rich reservoir of cytokines that are sequestrated and fossilised in dentine, which are released during the slow progression of caries and certain restorative materials like MTA used in VPT, have a reparative and regenerative effect (Graham *et al*. 2007; Smith *et al*. 2012). The inter-relationship between immune and stem cells is apparent by the fact that C-X-C chemokine receptor 4 (CXCR4) is expressed on both cell types. The sharing of these receptors shows that immune and stem cells are recruited to facilitate defence and repair (About and Mitsiadis 2001). An *in vivo* assessment of pulp tissue from carious teeth without pulpal change has shown that IL-1β amplifies antimicrobial peptides through autocrine signalling in odontoblasts *in vitro* 100-fold more than LPS (Horst *et al*. 2011), thus supporting the role of inflammation in repair.

Dendritic cells have been found in close association with odontoblasts at the periphery of the pulp under the carious lesions and it is suggested that they may play a role in odontoblast differentiation and the dentine synthesis activity (Ohshima *et al*. 1995). The effect of dendritic cells on odontoblasts may occur through chemokine CCL20 produced by activated dendritic cells (Gafa *et al*. 2007; Marcet *et al*. 2007). Further, macrophage inflammatory protein-3 alpha (MIP-3alpha) and beta-defensin (BD)-2, which are CC-chemokine family proteins, enhanced the expression of the odontoblast marker DSPP gene in human mesenchymal stem cells, thus demonstrating the capacity of inflammatory cell products in facilitating pulp cell differentiation into odontoblasts through CC chemokine receptor (CCR) 6 (Shiba *et al*. 2003; Nakanishi *et al*. 2005; Iejima *et al*. 2007).

Sublingual implantation of extracted third molars after resection of the root and pulp floor demonstrated immunohistochemically that odontoblast differentiation can be stimulated by cytokines secreted by macrophages and dendritic cells (Saito *et al*. 2011). Furthermore, the complement system involved in inflammation promotes pulp progenitor cell migration to the site of injury to facilitate repair (Chmilewsky *et al*. 2013). The interaction between the inflammatory and repair process is confirmed by the fact that molecules C5a and stromal cell-derived factor (SDF-1)/CXCL12 are both upregulated in caries and this can signal recruitment of both immune and stem cells through their interaction with C5aR/CD88 and C-X-C chemokine receptor 4, respectively (Murdoch 2000; Jiang *et al*. 2008a; Miller *et al*. 2008). CXCR4 and its ligand/chemokine (SDF-1)/CXCL12 have been shown to be upregulated in inflamed pulps compared to healthy pulps (Jiang *et al*. 2008a,b).

Botero *et al*. (2010) reported that LPS is associated with recognition of TLR2 and TLR4, and it induces VEGF expression in dental pulp tissue via MAPK activation. VEGF is known to promote vascular tissue necessary for repair. Endogenous lipid mediators like lipoxins and resolvins produced during inflammation stimulate cellular and molecular events that cause resolution of inflammation and repair (Serhan and Petasis 2011). LBP, an acute-phase protein, can attenuate pro-inflammatory cytokines by binding to LPS, LTA, lipopeptide and peptidoglycan prevents its binding to host cells (Schumann 2011). LBP has been shown to be present in bacterially challenged inflamed pulps but is absent in healthy pulps (Carrouel *et al*. 2013). Thus, LBP neutralises the bacterial toxins and creates an environment for repair.

It has been demonstrated that in the early stages or resolution of inflammation the level of pro-inflammatory cytokines and reactive oxygen species (ROS) is low and at this concentration they upregulate p38 MAPK signalling stem/progenitor cell differentiation and mineralisation process and DSP and DPP expression (Cooper *et al*. 2014). *In vitro* stimulation of dental pulp cells with TNF-α causes differentiation of pulp cells towards an odontoblastic phenotype (Paula-Silva *et al*. 2009) and increases dentine mineralisation along with the expression of DPP, DPP and DMP-1 expression through the p38 MAPK pathway. This may aid the repair mechanisms involved in tertiary dentinogenesis. Soluble TNF receptors, IL-10, nitric oxide, regulatory T Lymphocytes (Tregs) have been reported as some of the anti-inflammatory mechanisms detected in the pulp (Eming *et al*. 2007; Blancou and Anegon 2010; Buckley *et al*. 2013). Odontoblasts have been shown to produce IL-10, which promotes

development of regulatory T (Treg) cells, which modulate the immune system (Chaudhry *et al*. 2011; Farges *et al*. 2011).

Adrenomedullin, a pleotropic cytokine identified in the pulp tissue (Musson *et al*. 2010), is upregulated during dental disease and has been shown to possess anti-inflammatory, (Nagata *et al*. 2017) antibacterial and immunomodulatory properties (Cooper *et al*. 2011). Adrenomedullin has been shown to be involved in development of the teeth (Montuenga *et al*. 1997) and in regulation of mineralised tissue repair through stimulation of osteoblast differentiation and mineralisation processes (Cornish *et al*. 1997; Musson *et al*. 2010). This once again confirms the interaction between inflammation and repair in pulp tissue.

In vitro studies have demonstrated that low levels of ROS produced during inflammation may stimulate stem/progenitor cell differentiation and mineralisation processes as demonstrated by increased alkaline phosphatase activity (Lee *et al*. 2006). Nitric oxide (NO), an anti-inflammatory free radicle (Connelly *et al*. 2001), is observed at higher concentration in bacterially-challenged inflamed dental pulp (Korkmaz *et al*. 2011; Farges *et al*. 2015). NO affects vascular tone, nociceptive input modulation and is implicated in dental pulp healing by promoting odontoblast differentiation and reparative dentine formation (Mei *et al*. 2007). Immunohistochemical analysis of odontoblasts from 30-month-old bovine teeth shows a link between the 38MAPK pathway and regulation of secretory activity by odontoblasts (Simon *et al*. 2009). There is evidence to show that NOD2 participates in odontoblast differentiation via down regulation of MAPKs (Keller *et al*. 2011; Lee *et al*. 2014). It has also been demonstrated that MAPKs activation is necessary in odontoblast differentiation and activation (Jaiswal *et al*. 2000).

Substance P and CGRP have trophic effect in pulpal fibroblasts *in vitro* (Trantor *et al*. 1995) and sectioning of inferior alveolar nerve and capsaicin treatment causes reduction of these neuropeptides, which causes reduction of secondary dentine deposition in rat teeth (Jacobsen and Heyeraas 1996). This suggests that pulpal nerves, through neuropeptides, modulate immune-inflammatory response of the pulp, healing and secondary dentin deposition. *In vitro* study of the effect of glial cell line-derived neurotrophic factor (GDNF) on dental pulp cells (DPCs) has shown that GDNF possess a cytoprotective effect by counteracting the inflammatory effect of TNF-α and promoting cell survival and proliferation of DPCs (Gale *et al*. 2011). It has been suggested that plasticity of inflammatory cells may

cause them to differentiate into an osteo/odonto progenitor lineage in inflammation (Kuwana *et al*. 2003). Further, inflammatory cytokines may cause the fibroblast-like pulpal cells to de-differentiate and trans-differentiate into reparative cells (Young and Black 2004).

In contrast to other bioactive materials, Emdogain gel did not increase the pH, cause chemical injury and any injury observed was limited (Fransson 2012). It was *in vivo* that when fibroblast growth factor 2 (FGF2) was applied to exposed pulp in germ free rats using a collagen sponge, it stimulated migration and proliferation of pulp cells and invasion of vessels into the dentine defect (Kikuchi *et al*. 2007). These findings suggest that a non-inflammatory process may be implicated in cell recruitment, proliferation and differentiation of pulp cells expressing phosphorylation/mineralisation protein of the extracellular matrix (Fransson 2012). The same study also suggested that bacteria may differentially affect the odontoblasts ability to repair the dentine barrier. These findings suggest that there may be two pathways in pulpal repair; one inflammatory and one non-inflammatory.

1.5 Current treatment options

Current endodontic treatment options aim to maintain the vitality of the pulp where possible through the eradication of bacterial infection and prevention of recurrent infection through VPT. Where it is not possible to maintain vitality, the pulp is removed and the canals, shaped, cleaned and filled to prevent recurrent infection. The current treatment options for infected and inflamed pulp tissue is based on pulpal and periapical diagnosis (AAE 2013, 2016).

1.5.1 Vital pulp therapy

VPT relies on regenerative potential of the dentine-pulp complex to maintain pulp vitality, thereby preventing the need for root canal treatment, which could be expensive and time consuming (Tziafas 2004; Trope 2008). Treatment modalities under VPT include indirect pulp capping (IPC) in deep carious lesions, direct pulp capping (DC) or pulpotomy in case of pulp exposure (Ghoddusi *et al*. 2014). Classical studies (Kakehashi *et al*. 1965) have proved the role of bacteria in pulpal infection, inflammation and necrosis. Fungi and viruses are also implicated in pulpal infections (Siqueira and Sen 2004). Therefore, VPT involves removal of the injurious agents by operative procedures, control of infection and application of bioactive agents that have dentine regenerative potential and finally sealing the cavity with a

restorative material to prevent bacterial microleakage (Tziafas *et al*. 2000). On the contrary, there is also evidence to show that dentine bridge can form without a bioactive dressing in pulp exposure of germ-free rats in response to sterile hair and food (Kakehashi *et al.* 1965) It is difficult to establish the severity of the inflammation of the pulp by clinical assessment using signs and symptoms presented by the patient and investigations using thermal, electric pulp testing and radiographic assessment. VPT has been shown to have a higher success rate in teeth with deep carious lesions or in teeth with symptoms of reversible pulpitis (Aguilar and Linsuwanont 2011; Coll *et al*. 2017). Encouraging results with favourable outcomes over five years have been observed in permanent teeth with symptoms of irreversible pulpits (Asgary *et al*. 2015). Therefore, it would be worthwhile considering VPT as an option of treatment before embarking on root canal treatment where possible.

1.5.2 Selective or stepwise caries excavation

When treating deep carious lesions there is a chance of exposing the pulp in the process of caries excavation. The procedure whereby the caries is not completely removed to prevent the risk of pulp exposure, covered with a cavity sealer or liner and then restored to provide a good seal, is termed as IPC (AAoPD 2016). One of the following two approaches are used in IPC. In the first approach, selective caries excavation-the residual caries is sealed into the cavity and no further intervention is made to remove the caries. In the second approach, the stepwise excavation technique, the caries is removed over two stages with a gap of several months to allow reactionary dentine formation.

The survival of vital pulp of teeth after IPC is shown to be >90% over a 40 months (Ghoddusi *et al*. 2014; Maltz *et al.* 2007). It has been shown that there is no difference in the signs and symptoms of pulpal disease in teeth treated with partial caries removal or complete caries removal. Furthermore, there was no difference between teeth treated by partial caries removal or stepwise excavation (Ricketts *et al*. 2006; Ricketts *et al*. 2013). This review also showed that both stepwise and partial excavation reduced the incidence of pulp exposure in symptomless vital carious teeth. On the contrary, another systematic review reported that the risk of pulp exposure was less for incomplete caries removal compared to the stepwise approach (Schwendicke *et al*. 2013). The success of indirect pulp capping depends on the placement of a well-sealed restoration (Thompson *et al*. 2008) and the lining material is less critical (Hilton 2009; Leye Benoist *et al*. 2012; Pereira *et al*. 2017). There is current evidence

to suggest that lining improves the outcome of indirect pulp therapy (Chauhan *et al*. 2018). This evidence is suggestive of the self-healing ability of the dentine-pulp complex when bacterial load is decreased and its nutritional source is cut off. Agents like MTA and biodentine have got antibacterial in addition to providing a good seal as will be discussed later. Triclosan incorporated into restorative material can also do this.

1.5.3 Direct pulp capping

Direct pulp capping is defined as the treatment in which mechanical or traumatic vital pulp exposure is covered with biomaterial placed directly on exposed pulp before placement of a restoration to facilitate formation of reparative dentine in order to maintain pulp vitality (AAE 2016). The primary odontoblasts are destroyed by the pulp exposure and inflammation of the pulp stimulates progenitor/stem cells in the underlying uninfected pulp to differentiate into odontoblasts-like cells and produce reparative dentine (Tziafas 2004).

Various factors affect the success of DC. Traumatic exposure of the pulp has a higher success (absence of peri-apical radiolucency or root canal treatment) rate (92%) with DC compared to carious exposure (33%) (Al-Hiyasat *et al*. 2006). This is because caries causes invasion of the pulp by bacteria creating inflammation of the pulp (Bergenholtz and Lindhe 1975; Watts and Paterson 1987) which compromises the healing response of the pulp compared to mechanical exposure where there is no or very little inflammation (Cooper *et al*. 2010). A more recent systematic review shows an 88% success rate over 2 to 3 years with maintenance of pulp vitality for DC in teeth with cariously exposed pulps (Aguilar and Linsuwanont 2011). Matsuo, *et al.* (1996) showed that age, gender, teeth, presence of spontaneous pain, size of exposure did not influence the success of DC, but the degree of bleeding did so. On the contrary, Dammaschke, *et al*. (2010) and co-workers reported that the success of DC was reduced by age, presence of spontaneous pain and type of restoration. Even though it has been shown that the pulp capping agent did not influence the success of DC (Vural *et al*. 2017), there is evidence that the use of MTA produces more favourable results with DC compared to other materials (da Rosa *et al*. 2017). Furthermore, studies have shown that placement of a well-sealing restoration at the time of DC is crucial for the success of DC (Pinto *et al*. 2006).

1.5.4 Pulpotomy

Pulpotomy is a procedure in which the inflamed pulp tissue beneath a pulp exposure is removed and a bioactive material is placed over the remaining pulp to facilitate reparative dentine formation (AAoPD 2016). It could be partial pulpotomy or full/complete pulpotomy depending on the amount of pulp tissue removed (AAE 2016). Partial or Cvek pulpotomy is the surgical removal of a small portion of the coronal pulp tissue to preserve the remaining coronal and radicular pulp (AAE 2016). The inflamed tissue is removed to the level of healthy coronal pulp tissue (to the level until bleeding can be controlled). Full/complete pulpotomy is the surgical removal of the entire coronal portion of the vital pulp to preserve the vitality of the remaining radicular pulp (AAE 2003).

In pulp exposed by caries there is conflicting evidence regarding the success of pulpotomy. It has been shown that in younger patients the superficial pulp is resistant to bacteria and partial pulpotomy has shown a 93% success over 4.5 years (Mejare and Cvek 1993). The logical explanation for this observation would be the surgical removal of the superficial infected and inflamed pulp (Guthrie *et al*. 1965) creating an environment conducive to healing in the remaining vital pulp. This could be a reason for the reported higher success rate of 99% in the literature for pulpotomy (Aguilar and Linsuwanont 2011; Asgary *et al*. 2015; Asgary *et al*. 2017). Furthermore, it has been shown that neither the maturation state of the root apex, capping material nor the restoration used in pulpotomy influenced the success rate (Alqaderi *et al*. 2016).

Where there is necrosis with or without apical periodontitis, root canal treatment needs to be initiated. This involves removal of the infected coronal and radicular pulp, disinfection of the pulp chamber and canal system and then sealing root canals to prevent infection, followed by restoration of teeth. Even though root canal treated teeth survival rate is as high as 87% over 10 years (Ng *et al*. 2010; Burry *et al*. 2016), it weakens young teeth with immature apices, in addition to the cost and time for treatment. Hence, where possible, preservation of pulp vitally is important.

1.6 Different agents used for vital pulp therapy

The type of material placed over the pulp in VPT is one of the factors that determine the outcome of VPT. An ideal pulp capping material should be biocompatible and bioactive

stimulating reparative dentine formation, maintain pulpal vitality, release fluoride to prevent secondary caries, should be bactericidal or bacteriostatic, nontoxic and non-cariogenic, adhere to dentine, adhere to restorative materials, resist forces during restoration placement and during the life of restoration, be sterile, be radiopaque and provide a seal against bacterial microleakage (Qureshi *et al*. 2014; da Rosa *et al*. 2017). No one material at present has been shown to possess all of these properties (Roberts *et al*. 2008). An understanding of the various materials used for VPT would be useful in order to make the best choice of material for clinical use and the development of new biomaterials for VPT to improve its outcome.

1.6.1 Calcium hydroxide

$Ca(OH)_2$ has the longest history as a pulp capping agent (Zander and Glass 2005). $Ca(OH)_2$ has been shown to create an environment that facilitates the formation of reparative dentine (Kiatwateeratana *et al*. 2009). As bacteria are the primary cause of pulpal necrosis, the antimicrobial property of $Ca(OH)_2$ (due to its high pH of 12.5) (Bystrom *et al*. 1985). Pulp capping with $Ca(OH)_2$ on contaminated pulps in primates over a two year follow-up period showed successful dentine bridge formation (Cox *et al*. 1985). $Ca(OH)_2$ has also been shown to release growth factors and bioactive dentine matrix components, which have been shown to promote dentine regeneration at the site of pulp exposure (Graham *et al*. 2006; Park *et al*. 2015a). *In vitro* studies have shown that Ca^{++} stimulate fibronectin synthesis by dental pulp cells which can induce differentiation of pulp progenitor cells into a cell phenotype that produces mineralised tissue (Mizuno and Banzai 2008; Park *et al*. 2015a). $Ca(OH)_2$ has also been shown to have the potential to activate stem cells (Adams *et al*. 2006) and fibroblasts (Lansdown 2002) to facilitate wound healing.

Even though $Ca(OH)_2$ has shown favourable characteristics, there is still controversy regarding this use. $Ca(OH)_2$ destroys pulp tissue due to its caustic effect from the high pH. The caustic action reduces the size of the pulp and the dentine bridge reduces the size of the pulp even further. There is contradictory evidence regarding the ultrastructure of the $Ca(OH)_2$-induced dentine bridge. Scanning electron microscopy and microradiographic techniques showed that the thickness of the dentine bridge increased with a longer post-treatment period (Franz *et al*. 1984, 1985). These studies were done on non-carious teeth with intentional pulp exposure. The presence of bacteria and inflammation in DC teeth

influenced the continuity of the dentine barrier (Cvek *et al*. 1987). Clinical retrospective observation studies have shown an 80% failure rate over 10 years with $Ca(OH)_2$ DC in carious exposed pulp, and placement of a definitive restoration within two days of exposure increased success (Barthel *et al*. 2000). However, other studies have reported porosity in the dentine bridge (called tunnel defects) after DC with $Ca(OH)_2$ (Kitasako *et al*. 2008; Chen *et al.* 2017). It has also been reported that bacterial migrate into the pulp through the tunnel defects from deterioration of the restoration and that the $Ca(OH)_2$ was washed away after 6 months (Cox *et al*. 1996).

1.6.2 Mineral trioxide aggregate

In recent years, MTA, which was used for corrective endodontic procedures errors and retrograde filling material in root-end resection surgery, has gained popularity as a direct pulp capping material. It is biocompatible with tissue and induces the formation of hard tissue and also possesses osteogenic potential (Washington *et al.* 2011). MTA contains $Ca(OH)_2$, calcium silicate, tetacalcium aluminoferrite, tricalcium aluminate, tricalcium silicate, dicalcium silicate and bismuth oxide (Camilleri *et al*. 2005; Khan *et al*. 2016). MTA has been shown to possess superior properties of biocompatibility and sealing ability (Torabinejad and Parirokh 2010), radiopacity, low solubility, long-term stability, prevention of bacterial infiltration and dentinogenic and osteogenic potential (Tawil *et al*. 2015). MTA-treated pulps showed no pulpal inflammation after a week compared to $Ca(OH)_2$, where inflammation persisted up to three months. The same study on healthy human teeth showed that MTA produced a more predictable hard tissue barrier compared to barriers with tunnel defects where $Ca(OH)_2$ was used (Nair *et al*. 2009). A similar result with MTA and $Ca(OH)_2$ in intentionally pulpotomised premolars was observed (Chacko and Kurikose 2006). It should be taken into consideration that both these studies were done on iatrogenically exposed pulps and not on infected or inflamed pulps. Further *in vitro* studies have shown that MTA has the ability to release dentine matrix components and thereby influence dentine repair and regeneration (Tomson *et al*. 2007). It has also been shown to stimulate expression of dentine sialoprotein by odontoblasts to facilitate dentinogenesis (Min *et al*. 2008). Some studies have reported antibacterial and antifungal properties of MTA (Parirokh and Torabinejad 2010a). MTA is not without disadvantages (Parirokh and Torabinejad 2010b): it has toxic elements such as arsenic (Monteiro Bramante *et al*. 2008), long setting time

(Torabinejad *et al*. 1995), high pH during setting (Santos *et al*. 2005; Camilleri 2008) potential of discolouring teeth (Parirokh and Torabinejad 2010a) and is expensive (Chin *et al*. 2016).

A randomised controlled trial has shown favourable results over two years in permanent molars with carioulsy exposed pulps treated with MTA and $Ca(OH)_2$. The size of exposure influenced the outcome (Chailertvanitkul *et al*. 2014). A similar success based on absence of radiographic abnormality, resolution of existing radiographic periapical lesions and absence of spontaneous pain and tenderness to percussion and palpation between MTA (92%) and $Ca(OH)_2$ (88%) pulpotomies in carious pulp exposure of permanent posterior teeth with closed apices has been observed (Alqaderi *et al*. 2016).

There is good evidence from clinical studies that show MTA VPT increases the success by 2.67 times compared to $Ca(OH)_2$. The study also showed MTA has a success rate of 80% compared to 59% for $Ca(OH)_2$ over 42 months (Mente *et al*. 2014) based on positive response to cold test, absence of clinical signs and symptoms, apical periodontitis, internal root resorption and no loss of function. Good success rates have been observed with MTA VPT in mechanically and cariously exposed human pulps (Bogen *et al*. 2008; Min *et al*. 2008; Sawicki *et al*. 2008). A systematic review concluded that MTA has a higher clinical success rate for direct pulp capping comparing to $Ca(OH)_2$, and might be a suitable replacement for $Ca(OH)_2$ (Zhu *et al*. 2015). A recent study has shown a 92.7% success rate based on absence of clinical signs and symptoms of pulpal pathosis, radiographic signs of pathosis and complete radiographic healing or reduction in periapical index score in mature permanent teeth with carious exposure and irreversible pulpitis treated with MTA pulpotomy (Taha *et al*. 2017). The drawback of the study was that there were no proper controls. Overall, there is sufficient evidence to support use of MTA in VPT over other materials.

In recent years newer materials called bioactive endodontic cements have become available, claiming properties similar to MTA but without its disadvantages. They generally contain calcium and silicate in their composition (Torabinejad *et al*. 2017). These materials are generally compared against MTA but clinical studies evaluating the bioactive endodontic cements are very limited and most studies have methodological shortcomings and a low level of evidence. Therefore, their use should be considered with caution.

1.6.3 Materials containing bioactive molecules

In recent years, increasing knowledge of the reparative and regenerative potential of the dentine-pulp complex has led to the exploration of a biological approaches in treating pulpal disease. Attempts are being made to use substances that mimic normal developmental processes through selective activation of non-collagenous proteins to potentiate the natural healing process of tertiary dentinogenesis.

BMP and TGF-β1 in particular have been targeted as non-collagenous proteins that may facilitate repair mechanisms (Smith 2003; Smith *et al*. 2012; Smith *et al*. 2016). An *in vivo* model involving pulp exposure in rat molars followed by application of bioactive molecules showed that BSP was effective in inducing a homogenous well-mineralised layer of reparative dentine and BMP-7 induced the formation of osteodentine coronally and a homogenous mineralised structure in the root canal. These two molecules proved superior to $Ca(OH)_2$ and the mineralisation process involved recruitment of cells and their differentiation into osteoblasts that produced mineralised extracellular matrix (Goldberg *et al*. 2001). The downside of this study is that the model used healthy pulps rather than inflamed pulps to evaluate the effect of these molecules, which is where they will find their use in a clinical situation. Experiments in ferret dental pulps with pulpal inflammation simulated with lipopolysaccharides did not exhibit reparative dentinogenesis when treated with BMP-7 (Rutherford and Gu 2000). Use of BMP-7 in dog canine teeth after pulpotomy resulted in dentine bridge formation (da Silva *et al*. 2008), through again, the underlying condition of the pulp was healthy. The role of sequestrated growth factors in signalling reparative events has been emphasised (Smith *et al*. 2001; Smith 2003).

Use of TGF-β1-loaded microspheres in a chitosan bilayer membrane for pulp capping in a canine model promoted 3-6 times more reparative dentine compared to $Ca(OH)_2$ (Li *et al*. 2014). Experiments on *ex vivo* tooth slice culture models reported that the odontoblasts responded to TGF-β1 and nerve growth factor by expression of receptors to these molecules, indicating the influence of bioactive molecules from dentine and pulp on odontoblasts during pulp repair (Magloire *et al*. 2001). Direct pulp capping with DPP in rats has shown rapid induction of reparative dentine formation and pulpal tissue response compared to $Ca(OH)_2$ (Koike *et al*. 2014). Amelogenins produced by pre-ameloblasts induce differentiation of odontoblasts from dental papilla cells during tooth development (Hu *et al* 2007; Uchida *et a*l

1991). Therefore, its healing potential on pulpal wounds was explored. There are contradicting outcomes for its use in VPT from different studies. *In vivo* VPT experiments with Emdogain (amelogenins) in swine premolars when examined histologically 2 and 4 weeks after treatment displayed large amounts of dentine-like tissue, which has at least twice that of the $Ca(OH)_2$ control (Nakamura *et al*. 2001). On the other hand, clinical trials evaluating Emdogain as a DPC agent in primary (Garrocho-Rangel *et al*. 2009) and permanent teeth (Kiatwateeratana *et al*. 2009) failed to demonstrate improvement in reparative dentine formation compared to $Ca(OH)_2$.

The repair of dental pulp with implantation of bioactive molecule involves a cascade of four events, namely: moderate inflammation; commitment of adult reserve cells; their proliferation; and their tertiary differentiation (Goldberg *et al*. 2003). The entire sequence of complex events in pulpal repair and regeneration, the involvement of each bioactive molecule, the time of their appearance in the repair process and the interaction between different molecules, is yet to be fully unravelled (da Rosa *et al*. 2017) to allow their optimal therapeutic use. These bioactive molecules are a promising treatment option, but more scientific and clinical research is needed before clinical use.

$Ca(OH)_2$ has been shown to be predictable in dentine bridge formation. The cost of MTA act as barriers for its use in general dental practice (Chin *et al*. 2016). Furthermore, the use of bioactive molecules for pulp healing is still in the early stages of experimentation and even if it becomes available for clinical use it will be expensive. Therefore, a search for a less expensive and easy to use material for VPT would be worthwhile exploring. Triclosan could be one such material.

1.7 Triclosan

Triclosan is a broad-spectrum antimicrobial which was discovered in 1960 by Swiss specialty chemical company Ciba-Geigy Co. (Basel, Switzerland) (ICIS 2008). Triclosan possesses antibiotic and antimycotic properties. It was first introduced 1972 as a surgical scrub and continues to be used in this application to the present day (Services 1994; Zafar *et al*. 1995). Guidelines for health care facilities recommend a 2% triclosan shower in surgical units as a regimen to decolonise the skin of patients who are carriers of methicillin-resistant *Staphylococcus aureus* (Coia *et al*. 2006). Following this, triclosan found its way into various

consumer products such as toothpaste, mouth washes, soaps, deodorants, shampoos, medical equipment, kitchen utensils, toys, socks and bedding. (Levy *et al*. 1999; Thompson *et al*. 2005; Alison 2014).

1.7.1 Physical and chemical properties of triclosan

Chemically, triclosan is a chlorinated aromatic compound (2,4,4'-trichloro-2'-hydroxydiphenyether) which falls under the category of a polychloro phenoxy phenol with a chemical formula of $C_{12}H_7Cl_3O_2$. It processes ether and phenol functional groups as in (**Figure 1.2**).

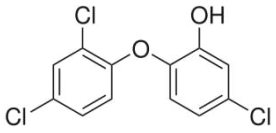


Figure 1.2: Chemical structure of triclosan

It has a molecular weight of 289.54 $g.mol^{-1}$. Physically, it is an off-white crystalline powder, tasteless with an aromatic phenolic odour. It is insoluble in water but readily soluble in non-polar organic solvents and strong basic solutions like DMSO, β cyclodextrin and acetone.

1.7.2 Mechanism of antimicrobial action

Triclosan is a non-ionic antibacterial agent with broad spectrum of activity against gram-positive and gram-negative organisms (Furia 1968; Blinkhorn *et al*. 2009). It is bacteriostatic at low concentrations and bactericidal at higher concentrations (McMurry *et al*. 1998; Suller and Russell 1999, 2000). Triclosan continues to be present in the oral mucosa for up to three hours after use of triclosan-containing dentifrice (Cummins 1991). It has been shown to have cumulative and persistent effects. Triclosan shows stability in plasma and oral bioavailability systemically in animal experiments (Rodricks *et al*. 2010). Therefore, suggesting its use as antibiotics and disinfectant.

Fatty acids are important for formation of cell membranes and are a source of energy for metabolism in bacteria and hence the viability of bacteria. The synthesis and breakdown of the fatty acid is up-regulated and down-regulated based on its availability. The bacteriostatic effect occurs by inhibiting fatty acid synthesis. Bacteria synthesise fatty acids through a Type

II fatty acid biosynthesis pathway which involves a group of enzymes called fatty acid synthases, of which enoyl-acyl carrier reductase (ENR), FabI or its homologue InhA are important (McMurry *et al*. 1999; Mendoza and Schujman 2009). Triclosan binds to ENR enzyme, thereby increasing its affinity to nicotinamide adenine dinucleotide (NAD^+), forming a stable complex ternary complex of ENR-NAD^+-triclosan, which cannot participate in fatty acid synthesis (McMurry *et al*. 1998; Kapoor *et al*. 2004; Sadowski *et al*. 2014). Triclosan alters the bacterial phospholipid membrane, destabilising it and affecting its functional integrity without causing cell lysis (Villalain *et al*. 2001). Intercalation of triclosan into bacterial cell membranes likely compromises the functional integrity of those membranes, thereby accounting for at least some of this compound's antibacterial effects (Guillen *et al*. 2004). Furthermore, amino acids, uracils and other nutrients uptake at the cytoplasmic membrane is affected by a low bacteriostatic concentration of triclosan (Regos and Hitz 1974).

Higher concentrations of triclosan are bactericidal by attacking membrane targets, causing leakage of intracellular contents such as K^+ (Suller and Russell 1999, 2000). Structural breakdown of the membrane and development of new adsorbing sites in gram-negative bacteria are contributed due to the Z pattern of triclosan adsorption (Denyer and Maillard 2002). Thus, it can be inferred that triclosan possesses more than one mechanism that differentiating its bacteriostatic and bactericidal actions. A recent randomised controlled clinical trial supports the antimicrobial action of triclosan where there was a reduction *of A. actinomycetemcomitans* and *P. gingivalis* in experimental gingivitis treated with triclosan-containing toothpaste (Pancer *et al*. 2016). Another randomised clinical study has demonstrated that brushing with triclosan toothpaste resulted in significantly reduced bacteraemia (Sreenivasan *et al*. 2017)**.** Therefore**,** its ability to prevent bacteraemia have an additional benefit in vital pulp therapy as SAG causes bacteraemia and abscesses. There is some suggestion that triclosan can affect hormone development but there is not strong evidence to substantiate it. It is considered to be a pollutant entering into the ecosystem suggesting the restriction of its use.

1.7.3 Anti-inflammatory effect and its mechanism

Microbes and trauma induce a harmful inflammatory response in a susceptible host. The microbes and microbial products induce cytokines and chemokines through innate immune receptors such as members of the TLR family. If chronic inflammation can be controlled along

with decreased bacterial load, then it would be possible to drive tissue responses towards repair rather than destruction (Blinkhorn *et al*. 2009). Control of infection along with modulation of inflammation to the right level could be helpful in controlling disease. *In vitro* studies using gingival fibroblasts have shown that the uptake and distribution of triclosan in the cytoplasm may affect the intracellular signal pathway involved in the production of inflammatory mediators (Mustafa *et al*. 2003). Triclosan has been shown to inhibit the effect of primary enzymes cyclooxygenase and lipoxygenase directly in the pathway of arachidonic acid metabolism and thereby reduce the formation of proinflammatory metabolites. This is likely the mechanism for the anti-inflammatory action of triclosan (Gaffar *et al*. 1995). Epithelial cells pre-treated with triclosan and stimulated with LPS showed inhibition of the expression of IL-8, IL-1α and TNF-α. In addition, they also inhibited the expression of IL-6, IL-12 and IFN-γ. The same study also showed that oral epithelial cells pre-treated with triclosan showed increased expression of the bioactive anti-microbial molecule β-defensin. It was also observed that LPS increased expression of TLR in epithelial cells and triclosan pre-treatment did not totally ablate the expression of TLR but markedly reduced it (Wallet *et al*. 2013). *Ex vivo* study using peripheral blood stimulated with LPS treated with triclosan showed down-regulation of TLR receptor signalling molecule and IL-1 and IL-6. The same study also showed up-regulation of growth factors related to BMP (Barros *et al*. 2010). Citrullination (citrullination is the post-translational conversion of peptidylarginine to peptidylcitrulline, a process catalysed by a family of calcium-dependent peptidylarginine deimnases. This type of protein modificationis linked to apoptosis and terminal differentiation of cells implicated in chronic diseases like periodontitis and rheumatoid arthritis) and carbamylation (carbamylation is post translational modification of proteins by enzyme -independent modification of lysine residues against which autoantibodies are subsequently induced) have significant roles in inflammatory periodontal disease. Treatment of macrophages containing citrullination and carbamylation with triclosan *in vitro* reduced the generation of these proteins (Bright *et al*. 2017). These findings show that triclosan has the potential to exert anti-inflammatory effects through different pathways and through different cells. Clinical studies have shown that triclosan has the potential to reduce inflammation. Triclosan is considered to be effective in the anti-inflammatory treatment of hidradenitis suppurativa in dermatology (Hessam *et al*. 2016) and chronic recurrent oral aphthous ulcers (Altenburg *et al*. 2014). A clinical double-blind study evaluating the efficacy of triclosan-containing toothpaste in reducing gingival inflammation and significant reduction in gingival bleeding, an indicator of inflammation, at 6 weeks and 12 weeks compared to toothpaste that did not

contain triclosan. The triclosan-containing toothpaste also contained agents such as fluoride and calcium glycerophosphate, but the effects these agents would have had on the gingival inflammation was not evaluated in this study (Peter *et al*. 2004). Another randomised clinical trial over 14 days also supported the effectiveness of triclosan in controlling gingival inflammation (Nandlal *et al*. 2016). A systematic review supports the efficacy of twice daily brushing with triclosan-containing toothpaste in the management of periodontitis (Blinkhorn *et al*. 2009). The antimicrobial and anti-inflammatory effects of triclosan complement each other, making it the choice of treatment for a number of oral diseases (Blinkhorn *et al*. 2009) such as gingivitis, periodontitis, halitosis, dentine hypersensitivity and aphthous ulcers.

1.7.4 Use of triclosan in dentistry

Triclosan-containing toothpaste has been shown to provide superior protection against plaque, gingivitis and caries. It exhibits superior stain removal and protects against the progression of periodontal disease (Panagakos *et al*. 2005). Use of triclosan over a four-week period in a randomised controlled trial demonstrated considerable reduction in plaque and gingival inflammation (Shim *et al*. 2012). Triclosan has been shown in clinical trials to possess anti-calculus potential (Svatun *et al*. 1993; Allen *et al*. 2002b; Sowinski *et al*. 2002). Dental caries has been shown to be controlled effectively by use of triclosan-containing toothpaste (Mann *et al*. 1996; Finney *et al*. 2003).

Halitosis (oral malodour) is a common oral condition affecting more than 50% of the general population (Aylikci and Colak 2013). A clinical trial demonstrated that a regimen comprising a triclosan toothpaste and cetylpyridinium chloride mouth rinse demonstrated significant oral malodour reductions two hours after use with a progressive reduction in oral malodour (Nandlal *et al*. 2016). Another review also supported the beneficial effect of triclosan in the management of halitosis (Panagakos *et al*. 2005).

Dentine hypersensitivity is a common complaint in patients attending for dental treatment, with a prevalence ranging from 3 to 98% (Splieth and Tachou 2013). In a single-blinded randomised clinical trial of twice daily brushing with a toothpaste containing 0.3% triclosan polyvinyl methyl ether and maleic acid copolymer, triclosan was shown to provide an effective level of plaque control and gingival health. The study showed that triclosan toothpaste did not have any effect on decreasing dentine hypersensitivity compared to conventional fluoride toothpaste (Al Habashneh *et al*. 2017).

The antimicrobial and anti-inflammatory properties of triclosan have been shown to be helpful in the treatment of oral aphthous ulcers (Altenburg *et al*. 2014). Triclosan mouthwash has been proved to be effective in the management of post-chemotherapy oral mucositis (Carneiro-Neto *et al*. 2017).

Triclosan was shown in one systematic review to be the only effective agent in the management of peri-implantitis (Ata-Ali *et al*. 2015), yet another concluded that this was yet to be established (Salvi and Ramseier 2015). Incorporation of triclosan in biodegradable sleeves in dental implants to prevent implant related infections have shown promising results in animal experiments (von Plocki *et al*. 2012).

1.8 Models used to study pulp inflammation, repair and regeneration

The understanding of pulpal disease is key to developing novel treatment modalities. Disturbance in homeostasis of the pulp either by infection or trauma causes the pulp to respond through its immuno-inflammatory processes. These immuno-inflammatory responses are a complex interplay of various innate pulp cell or immune cells recruited to the site through circulation as described earlier. In order to understand pulpal disease, processes in this complex network of events needs to be untangled. Different models have been used in the past to help unravel the mysteries of pulpal disease. Some of them are overly simple and some of them are overly complex. The following section describes some of the models that have been used in the past to study pulpal inflammation, repair and regeneration will be explored. The different models presently available are *in vitro, in vivo and ex vivo*.

1.8.1 *In vitro* models

These are the mostly commonly used models and are relatively inexpensive and rapid. They reproducibly allow determination of the effects of different stimulations and treatment modalities with reliability and consistency (Arenholt-Bindslev and Horsted-Bindslev 1989; Gomes-Filho *et al*. 2009; Gomes-Filho *et al*. 2016). There is an ability to minimise variability among experimental samples by application of standardised protocols to specific cell populations, thereby eliminating differences arising from genetic variation. Large amounts of homogenous population of cells can be generated easily. The drawback with the *in vitro* model is that it uses at the most, two types of cells. It is difficult to re-create the spatial

relationship between these cells; hence it lacks the ability to simulate *in vivo* tissue organisation. This can be quite limiting for studying responses that involve interaction between different cells in a tissue. Furthermore, when working with primary cells multiple passages reduces the sensitivity of cells to stimulation (Williams *et al*. 1980). Multiple sub-culturing of cells can cause senescence, which is a problem in long-term studies (Mueller *et al*. 1980).

Most *in vitro* models in dentistry use pulp cells that are usually pulp fibroblasts, dental pulp stem cells, odontoblasts-like cells or periodontal ligament fibroblasts. These cells are usually from human, mouse or rat pulp tissue obtained by enzyme digestion or outgrowth of the pulp tissue (Huang *et al*. 2006b). *In vitro* cell culture of periodontal ligament fibroblasts has been used for the study of biocompatibility of endodontic materials (Schwarze *et al*. 2002). Pulpal fibroblasts have been used to evaluate the toxicity of composite material on the dental pulp (Tadin *et al*. 2014). Immortalised human dental pulp cells were used to evaluate the ability of calcium silicate-based cement to induce odontoblastic differentiation, angiogenesis and the inflammatory response in human dental pulp cells (Chang *et al*. 2015). This study is a very good example of where different inflammatory responses and reparative ability of the pulp was evaluated *in vitro* using pulp cells. Dentine regeneration by dentine matrix components was evaluated using mouse and rat undifferentiated dental pulp cells *in vitro* (Salehi *et al*. 2016). Dental pulp cells and dental pulp stem cells were used to evaluate inflammatory responses when challenged with bacterial LPS and the ability of a static magnetic field to modulate this response (Hsieh *et al*. 2015). This study showed that static magnetic field rendered higher tolerance to LPS induced inflammatory responses in dental pulp cells. Even though the results of these studies are quite informative, pulp tissue is composed of immune cells, ectomesenchymal cells, fibroblasts, preodontoblasts, odontoblasts, and dental pulp stem cells, but these studies focus only on one or two types of cell, which may not reflect the true response of pulp tissue. Furthermore, it does not consider the vascular and neural interaction observed in the tissue.

Odontoblasts cannot be cultured, as they are terminally differentiated cells. They are difficult to harvest without damage as they have processes that extend into dentine. Furthermore, they lose their phenotype and become fibroblast-like if cultured without contact with dentine (Munksgaard *et al*. 1978; Heywood and Appleton 1984; Huang *et al*. 2006a). Odontoblast-like cells have been used *in vitro* to study the ability of Biodentine to reduce

TNF-α-induced transient receptor potential (TRP) ion channels that play a central role in nociception (El Karim *et al*. 2016). It has been shown that immortalised odontoblast cells resemble odontoblasts in many aspects but not entirely (Panagakos 1998). A three-dimensional model was developed to characterise programmed cell necrosis using pulpal fibroblasts *in vitro* as spheroids (Le Clerc *et al*. 2013), but this still had only contained pulpal fibroblasts and the spatial arrangement was not similar to pulp tissue.

1.8.2 *In vivo* models

Different *in vivo* models in both animals and humans have been used to simulate odontogenesis, pathological conditions and repair/regeneration. Effect of trauma on development of teeth was evaluated *in vivo* in seven-day old rats (Prasanth and Saraswathi 2012). *In vivo* periapical lesions were developed by creating mechanical pulp exposures to evaluate the cytokine-signalling pathway that controls the regulation of inflammatory response in the periapical tissues and the development of periapical lesions (Liu *et al*. 2014). Pulp tissue samples were collected from patients to evaluate the level of expression of osteocalcin in reversible and irreversible pulpitis (Abd-Elmeguid *et al*. 2013). Osteocalcin is important in reactionary and reparative dentinogenesis (Hirata *et al*. 2005). An *in vivo* pulpitis model was created in rats by mechanical injury and bacterial LPS stimulation (Ma *et al*. 2015). The ability of dental pulp stem cells to re-vascularise the entire dental pulp was assessed by placing dental pulp stem cells into the cleaned pulp cavities of rabbit molars using a nano-fibrous spongy microsphere carrier and subcutaneously implanting them in mice (Kuang *et al*. 2016). The ability of dentine matrix proteins to induce secretory activity of odontoblasts and thereby facilitate reparative dentinogenesis was evaluated *in vivo* in ferret teeth (Smith *et al*. 1994). Clinical randomised control trials have also been used to evaluate efficiency of different therapeutic agents *in vivo*, as was used in a trial to evaluate the efficiency of different pulpotomy agents (Rajasekharan *et al*. 2016).

Even though these *in vivo* models are close to real life clinical situations and can yield a lot of information, they do have disadvantages. *In vivo* models are very expensive in terms of the cost of the animals and maintaining them, and large numbers of animals are required. Ethical approval can be a long and difficult process. Furthermore, if the study is long, the drop out number could be high, and it might be difficult to have enough power if the sample number is low, especially where patients are involved. It could be a challenge to obtain clear data due

to the difficulty in isolating the systemic effects. sometimes the responses could be due to the result of acquired immunity rather than the response of the local tissue of interest. There could also be differences due to the type of animal used in the study. Even though they may yield realistic results, there could be difficulty in obtaining homogeneity across the samples.

1.8.3 *Ex vivo* models

In order to overcome the various difficulties posed by the *in vitro* and *in vivo* models and to study various developmental, physiological and pathological conditions and to evaluate drug activity, *ex vivo* models have been developed. These models have the advantage of maintaining spatial orientation of cells in the tissue as *in vivo* and without the systemic influence, which is a hindrance in *in vivo* models. When evaluating new therapeutic agents *in vivo* one animal has to be used for each of the different doses being assessed; however, when using an *ex vivo* model, one animal may provide enough samples for the different doses, including the control, thereby reducing the number of animals needed. They reduce the practical and ethical issues associated with current animal models. The production of multiple samples from the same animal increases homogeneity between the samples. Different inflammatory cells can be transplanted into the model and various cytokines can be added to the model, thereby providing the ability to control the microenvironment. This in turn reduces the need for development of knockout animals and *in vivo* assays. Thus, it may be possible to reduce, replace and refine parameters before expensive and time-consuming *in vivo* studies are conducted.

Various *ex vivo* models have been developed for use in dentistry. An *ex vivo* organ culture model where rat teeth were sectioned labio-lingually and the pulp tissue was sectioned and removed carefully under magnification leaving the odontoblasts attached to the dentine, was used to study the effects of fluorides on the synthesis of sulphated glycosaminoglycans (Embery and Smalley 1980) and proteoglycan (Waddington *et al*. 1993) by odontoblasts. The effects of growth factors on the expression of collagen and extracellular matrix-degrading enzymes and the effect of gelatinolytic enzymes on dentine matrix remodelling were evaluated using *ex vivo* odontoblast organ culture models (Tjaderhane *et al*. 1998; Tjaderhane *et al*. 2001). In this model, impacted third molars were extracted, the roots sectioned off to expose the pulp chamber and the pulp tissue removed, leaving the odontoblast in the crown crucible, which was filled with media to nourish the cells. An *ex vivo*

short term culture of new-born rat mandibular incisors was used to evaluate the effect of different culture conditions on matrix formation by odontoblasts in a pulp dentine slice *ex vivo* culture system (Hasegawa 1989). This study showed that large amount of oxygen may be required for maintaining the function of odontoblasts in the pulp-dentine slice culture system. Furthermore, most of these models were used for studies over a short period of time.

An *ex vivo* rat tooth slice model was developed to study dentinogenesis (Sloan *et al*. 1998). This model paved the way for the development of the *ex vivo* rodent mandible culture model used to study bone repair (Smith *et al*. 2010). The details of the *ex vivo* tooth slice model which is of interest in this work and its evolution into a pulp infection model will be discussed in detail in the introduction to chapter 2.

1.9 Aims and objectives

Aims:

The overall aims of this thesis were:

1. to characterise and validate the SAG *ex vivo* pulp infection model
2. to use this model to investigate the effect of bacterial proteins and for testing the efficacy of the antimicrobial and anti-inflammatory effect of triclosan in the treatment of pulpal infection.
3. to develop this into a poly-microbial infection model.

Objectives:

The validation and characterisation of the previously developed SAG *ex vivo* pulp infection model will be done through:

- evaluation of histomorphometric changes in the dental pulp from SAG infection.
- developing a more reliable and reproducible method to quantify viable counts of odontoblasts and dental pulp cells in the entre pulp.
- developing a method to quantitatively evaluate the area of colonisation of the dental pulp by SAG bacteria.
- quantitatively evaluating the expression of inflammatory markers within the *ex vivo* pulp infection model.

- comparing the inflammatory response of SAG infected *in vitro* dental pulp cells to the *ex vivo* model to assess the usefulness of the *ex vivo* model compared to the *in vitro* cell culture model.

The validated and characterised model will then be used to investigate the effect of bacterial proteins produced by SAG on the dental pulp tissue and identify protein-related virulence factors:

- observing histomorphometric changes in the pulp stimulated with bacterial proteins.
- evaluating their effect on cell viability through cell counts.
- evaluating the inflammatory response of the pulp to SAG bacterial proteins.
- observing differences in pulpal response to the two different strains of SAG used in the study.
- identifying the virulence-proteins using mass-spectrometry.

The usefulness of triclosan will then be evaluated as an antimicrobial and anti-inflammatory agent in treatment of pulpitis using the *ex vivo* SAG pulp infection model through:

- the ability of triclosan to reduce cell death caused by SAG infection.
- the ability of triclosan to reduce the colonisation of the pulp by SAG.
- the effect of triclosan to modulate inflammatory response of the pulp to SAG infection.
- the ability of triclosan to neutralise any SAG virulence proteins that were identified.

An effort will be made to develop a poly-microbial *ex vivo* pulp infection model by:

- investigating the effect of *S. anginosus* and *E. faecalis* on each other's growth kinetics when co-cultured.
- investigating the effect of poly-microbial infection on pulp cell viability and compare it to individual *S. anginosus* or *E. faecalis* infections.
- evaluating staining techniques to differentially identify the two bacteria in the poly-microbial infection, and to evaluate the difference in the ability of the two bacteria to colonise the pulp in the mixed infection.

The data presented in thesis will have application for use of the *ex vivo* model for further understanding of pulpal infection and inflammatory processes and for investigation of new therapeutic agents for the treatment of pulpal infection and management of pulpal

inflammation. If triclosan can control SAG infection and pulpal inflammation in the model, this could pave the way for further evaluation as an economic alternative to currently available therapeutic agents for management of pulpitis. Exploring the development of a polymicrobial pulpal infection model could pave the way for the possibility to mirror the clinical situation of pulpal infection more closely.

Chapter 2

2 Characterisation and validation of the *ex vivo Streptococcus anginosus* group bacterial model

2.1 Introduction

Deep carious lesions are one of the commonest causes of pulpal inflammation. As lesions progress closer to the pulp, they may cause various levels of inflammation in the tissue (pulpitis) (Trowbridge 1981). Pulpitis can be reversible or irreversible. It is important to realise the importance of differentiating between the two states. Pulps diagnosed with reversible inflammation may be able to return to normal after appropriate treatment, whether or not pulp tissue is exposed directly to the oral environment. Irreversible inflammation may involve all part of the pulp, and if not treated in a timely manner, will inevitably lead to total necrosis of the pulp. There is no reliable way to consistently correlate the histological state of the pulp to the clinical signs and symptoms of pulpitis (Seltzer *et al*. 1963; Dummer *et al*. 1980; Reit 2010; Trowbridge 2012) until recently. Pain is not a reliable parameter for the diagnosis of reversible/irreversible pulpitis as severe inflammation of the pulp can be present without pain (Mejare *et al*. 2012). Even though spontaneous pain is considered as a clinical indicator of irreversible pulpitis, histologically there can be no evidence of pulp exposure or necrosis to indicate an irreversible state of the pulp (Mejare *et al*. 2012). More recent evidence suggest high correlation between histological findings and clinical signs and symptoms (Ricucci *et al*. 2014) Further, there is no obvious association between clinical investigation of asymptomatic deep carious lesions and the state of the pulp (Mejare *et al*. 2012). Therefore, it can be challenging to make a decision to maintain the vitality of the pulp. New evidence is emerging that pulps that are reversibly inflamed are diagnosed as irreversibly inflamed (Walters *et al.* 2017). This has led to the emergence of a new diagnostic system with new terminologies grading inflammation of the pulp to highlight the healing potential of the pulp (Walters *et al.* 2017). This has new diagnosis system guides treatment based on the level of inflammation of the pulp.

Most commonly, pulps diagnosed clinically as having irreversible pulpitis are treated by extraction or pulp extirpation followed by root canal treatment (Levin *et al*. 2009). If the tooth is extracted it may require replacement by a denture, bridge or implant (Al-Quran *et al*. 2011). This has long term maintenance and financial implications for the patient (Hannahan and Eleazer 2008; Al-Quran *et al*. 2011) and society, in addition to the effects on quality of life due to the

loss of the natural tooth (Brennan *et al*. 2008). There are significant advantages, in attempting to retain teeth through the use of root canal treatment. However, root canal treatment is not always successful; success rates of primary and secondary root canal treatment in recent years still only achieve 80% and 75%, respectively (Ng *et al*. 2007; Ng *et al*. 2008) based on absence of apical periodontitis in plain radiographs. Moreover, root canal treatment is often not affordable in many developing economies. Teeth left untreated can lead to the development of periapical pathosis (Yu and Abbott 2007).

Maintaining the vitality of the pulp, if possible, should have high a priority, and has a high rate of success even in cases with symptoms suggesting irreversible pulpitis (Zhang and Yelick 2010). With the loss of pulp vitality, the tooth loses its ability to respond to environmental challenges such as caries progression. Loss of vital pulp tissue also removes the possibility of reparative and regenerative dentinogenesis and this is especially important in young permanent teeth where root formation has not been completed. This affects the crown root ratio and also the dentine wall of roots of these teeth are thin, predisposing them to cervical root fracture (Cvek 1992). Canal filling materials used for root canal treatment can cause discolouration of the tooth, creating aesthetic problems (Goto *et al*. 2009). Maintaining the vitality of the pulp prevents bacterial colonisation of the root canal space and their effect on the periapical region as vital pulp provides better seal than root canal filling material (Lin *et al*. 2006; Rocha *et al*. 2008). Furthermore, the pressure and swelling from oedema of the inflamed pulp (Heyeraas *et al*. 1994) and the non-yielding wall of the pulp chamber stimulates nerve endings, causing severe pain in the acute phase of inflammation (Hargreaves 2002; Yu and Abbott 2007; Estrela *et al*. 2011). Therefore, if pulpitis can be controlled and its most frequent cause (microbial infection) treated, then the cells of the pulp can help in repair by the formation of a calcific barrier of reparative dentine (Bjørndal *et al*. 2010; Colombo *et al*. 2014). This will prevent the pulp from losing vitality and the sequela of the spread of infection.

Understanding pulpal infection is very important in order to identify appropriate therapy. If infection can be controlled and the inflammation of the pulp modulated it can tip the balance towards regeneration and repair rather than pulpal necrosis (Colombo *et al*. 2014). The success rates of vital pulp therapy are affected by the degree of infection and inflammation of the pulp and the type of material used (Yamaguchi *et al*. 2004; Cooper *et al*. 2010; Cooper *et al*. 2014; Ghoddusi *et al*. 2014).

A suitable model for investigating pulpal infection and inflammation and for evaluating treatment modalities to treat pulp disease is needed. Furthermore, the model will help us in

understanding pulpal disease. The model used in this thesis uses SAG bacteria for infection of the pulp. SAG are part of the normal commensal flora in the oral cavity (Poole and Wilson 1979), but they can cause opportunistic infection in other parts of the body (Neumayr *et al*. 2010; Moazzam *et al*. 2015). They have also been isolated from dentoalveolar abscesses (Cachovan *et al*. 2014). SAG are seen in the oral cavity and are one of the early colonisers of the pulp. Streptococci have been implicated in facilitating colonisation of the pulp by other bacteria (Love and Jenkinson 2002) and as the infection progress and suitable condition develop they are frequently seen in dentoalveolar abscesses. SAG is one of the frequently identified bacteria from these abscesses (Lewis *et al*. 1990; Robertson and Smith 2009). Studies have shown that when subcutaneous infection in mice was created with dental plaque containing low levels of SAG, it resulted in formation of abscesses with increased levels of SAG (Thurnheer *et al*. 2001; Okayama *et al*. 2005). However, there is a wide gap in our understanding of the role of SAG in the pulpal disease process from a carious lesion to the development of a periapical abscess.

Modelling pulp infection

Due to the complexity, systemic influence, expenditure and ethical issues of the *in vivo* model, the over simplified nature of the *in vitro* model mentioned previously and the lack of interaction between different cells of the tissue, it is difficult to exactly mirror an *in vivo* response. An *ex vivo* model may be a fitting one to address issues associated with the *in vivo* and *in vitro* models. An organotypic *ex vivo* tooth slice culture system has been developed (Sloan *et al*. 1998) that revealed that the structure of the dentine-pulp complex, along with the phenotypic morphology and secretory activity that was maintained in culture for up 14 days. This model paved the way for the development of the *ex vivo* pulpal infection model (Roberts *et al*. 2013). The lack of a pulp infection model before this made it impossible to study the effect of SAG bacteria on the pulp, but the validation and characterisation of the model by Roberts *et al*. (2013) were less reproducible. Therefore, a highly reproducible method needs to be developed to characterise and validate the model for future use in this work.

Objectives of the investigations in this chapter were:

1. To investigate the influence of different media on the growth kinetics of SAG in order to identify the strains of SAG necessary for the pulp infection model.
2. To evaluate the histomorphometric changes in the dental pulp from SAG infection.
3. To assess viable cell-count in per mm^2 of pulp tissue in a more reliable and reproducible way without bias from the researcher.

4. To evaluate not only the ability of the SAG bacteria to colonise the pulp tissue but also to quantitatively evaluate the colonisation area in percentage terms relative to the total area of the pulp.
5. To quantitatively evaluate the expression of pro-inflammatory and anti-inflammatory markers within the *ex vivo* model.
6. To assess the inflammatory response of *in vitro* dental pulp cells and compare this to the *ex vivo* model.

2.2 Materials and Methods

2.2.1 Characterisation of bacteria

2.2.1.1 Confirmation of the bacterial strain to be used for the experiments

2.2.1.1.1 Gram stain

The bacteria used for the study were obtained from the Oral Microbiology laboratory at the School of Dentistry, Cardiff University, UK. The bacteria used were *Streptococcus anginosus* (*S. anginosus*) (Ref 39/2/14A; source unknown), *Streptococcus constellatus (S. constellatus*) (Ref 45386; source high vaginal swab) and *Streptococcus intermedius (S. intermedius*) (Ref HW13; clinical source dentoalveolar abscess). These strains were genotypically and phenotypically characterised previously (Roberts *et al*. 2013).

In order to confirm the streptococcal strain to be used in the experiment, the bacteria stored on beads at -80°C were plated on fastidious anaerobic agar (FAA) (Lab M™ International Diagnostic Group plc, Bury, UK) supplemented with 5% (v/v) defibrinated sheep blood (TCS Bioscience Ltd., Buckingham, UK) and incubated overnight in an anaerobic cabinet at 37°C.

A sample from the plate was smeared on a glass slide with a drop of phosphate-buffered saline (PBS). The slide was air-dried, heat fixed and then flooded with crystal violet stain for 30 s, washed under running water and then the smear was covered with iodine for 5 s. The slide was decolourised by rinsing with acetone before counterstaining with fuchsin for 30 s. The slide was then rinsed and air-dried before being viewed by oil-immersion microscopy at x100 magnification to evaluate the shape and type of gram stain of the bacteria.

2.2.1.1.2 Molecular analysis

Culture of bacteria for molecular analysis:
One colony from the fresh overnight culture of the three strains of *S. anginosus* group (SAG) on the FAA plate was inoculated into brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) and incubated overnight on a mechanical rocker (Grant Bio™ Sheperth, UK) in a CO_2 incubator (5% CO_2; Don Whitley Scientific Ltd., Shipley, UK) at 37°C.

DNA extraction:

DNA extraction from the overnight broth culture was achieved using a Gentra Puregene Yeast/Bact. Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. 500 µL of the culture was transferred to a 1.5 mL micro-centrifuge tube on ice and centrifuged for 5 s at 16,000 x g. The pellet was retained and the supernatant discarded. The pellet was then re-suspended in 300 µL of cell suspension solution, mixed well by pipetting up and down, and 1.5 µL of lytic enzyme solution was added and mixed by inverting the tube 25 times. This was then incubated at 37°C for 30 min. After incubation the suspension was centrifuged for 1 min at 16,000 x g to form a cell pellet. The supernatant was then discarded and 300 µL of cell lysis solution was added to the pellet and mixed well by pipetting up and down. To this was added 1.5 µL RNase A solution and mixed well by inverting the tube 25 times. The tube was then incubated for 60 min at 37°C and incubated on ice for 1 min to quickly cool the sample. To this was added 100 µL of protein precipitation solution and vortexed vigorously for 20 s at high speed and centrifuged for 3 min at 16,000 x g. Taking care not to dislodge the pellet, the supernatant was carefully poured into a clean 1.5 mL microcentrifuge tube containing 300 µL of isopropanol. This was then mixed well by inverting 50 times and then centrifuged for 1 min at 16,000 x g. The supernatant was carefully discarded and the tube was drained on a clean piece of absorbent paper, taking care that the pellet was retained in the tube. Then 300 µL of 70% ethanol was added to the pellet and inverted several times to wash the DNA pellet. This was centrifuged for 1 min at 16,000 x g, and the supernatant carefully discarded. The tube was drained on a clean absorbent paper, taking care that the pellet remained in the tube. The pellet was allowed to air dry for 5 min. To this was added 100 µL DNA hydration solution and the tube was vortexed for 5 s at medium speed. This was then incubated at 65 °C for 1 h to dissolve the DNA. This was followed by incubation at room temperature (15-25 °C) overnight with gentle shaking. The sample was finally centrifuged 15 s at 16,000 x g and transferred to a storage tube, and stored at 4 °C, prior to use as a template for PCR reaction.

PCR amplification:

Forward and reverse primers distinct for bacterial-derived DNA fragments, were used for amplification. Universal primers specific for the domain bacteria were used for the amplification of 16S rDNA for *S. anginosus* 39/2/14A, *S. constellatus* 45386 and *S. intermedius* (HW13). The sequence for the forward primer D88 (5'-GAG AGT TTG ATY MTG GCT CAG-3') and reverse primer E94 (5'-GAA GGA GGT GWT CCA RCC GCA-3') were selected (Paster et.al. 2001).

The DNA extracted from the bacteria as described above was amplified using the following freshly prepared PCR master mix. A reaction volume of 50 µL was made up using 0.2 mM each

of deoxynucleoside triphosphate (dNTP), 1 mM magnesium chloride, 0.5 µM each primer, 1X Taq buffer and 0.5U of Taq polymerase (Promega UK Ltd, Southampton, UK), and 2 µL of bacterial DNA template. Nuclease-free water was used as a substitute for DNA template in the negative control.

Bacterial DNA was amplified by subjecting the reaction mixtures to an initial denaturation at 95 °C for 5 min. This was followed by 35 cycles with denaturation time of 45 s at 95 °C, 1 min of primer annealing at 55°C and 1 min of extension at 72 °C for 45 s for each cycle. Finally, the amplification was completed with a final elongation step of 72 °C for 5 min. The PCR reaction products were stored at 4°C until they were subjected to gel electrophoresis to confirm amplification. The products were then subjected to purification and further analysis.

Gel electrophoresis:

In order to visualise the PCR amplification products, they were subjected to gel electrophoresis. This helped to confirm that a single PCR product had been obtained. One percent agarose gel was prepared in 70 mL 0.5X Tris-Borate-EDTA (TBE) buffer and 0.1% SafeView (Product code: NBS-SV1, NBS Biologicals, Huntingdon, UK). Ten µL of the PCR product was mixed with orange/blue loading dye (Promega) and added to the wells. A ladder 1KbP (Product code: G571A, (Promega) was used to estimate the size of the PCR products. The gels were run at 80 V/cm^2 for 40-60 min after which the gels were visualised under ultraviolet light in a gel hood.

Purification:

Before PCR products were sequenced, they were purified using the QIAquick PCR Purification Kit (Qiagen). PCR products 50 µL were each mixed with 250 µL Buffer PB. If the product was orange/violet in colour due to the presence of loading dye, 10 µL of 3M sodium acetate, pH 5.0 was added to it. The samples were added to QIA quick columns and centrifuged for one minute at 17,900 x g. The flow through was discarded, the remaining bound DNA in the column was washed by adding 750 µL Buffer PE and then centrifuged for 1 min at 17,900 x g. The column was placed into a clean 1.5 mL centrifuge tube and 50 µL Buffer EB added to the centre of the column and centrifuged for 1 min at 17,900 x g. The purified DNA collected in the centrifuge tube was analysed by gel electrophoresis prior to freezing at -20 °C until subjected to sequencing.

Sequencing:

The purified PCR products were sent with their corresponding forward primer to the Sequencing Core Unit at the School of Bioscience (Cardiff University) where they were subjected to automated sequencing using the BigDye® Terminator v3.1 (Life technologies). The platform used

was the 3730xl DNA Analyser (Eurofins, Ebersberg, Germany). Basic Alignment Tool from the National Centre for Biotechnology Information (NCIB) for microbes http://blast.ncbi.nlm.nih.gov/Blast.cgi was used to identify the sequence, 99% was considered a reliable level of identification.

2.2.2 Media preparation

2.2.2.1 Brain Heart infusion media

To Prepare BHI, 37g of the medium was dissolved in one litre of purified water and autoclaved at 121°C for 15 minutes.

2.2.2.2 Modified tissue culture media

The modified tissue culture media (MTCM) previously established to sustain both tooth slice and SAG (*S. anginosus* 39/2/14A, *S. constellatus* 45386) (Roberts *et al*. 2013) was prepared. It consisted of Dulbecco's Modified Eagles's Medium (DMEM) with phenol red (Sigma Aldrich, Gillingham, Dorset, UK), supplemented with 10% heat-inactivated foetal calf serum (FCS), 0.15 mg/mL vitamin C (Sigma Aldrich), 200 mmol/L L-glutamine (Invitrogen, Carlsbad, USA), and 10% brain-heart infusion (BHI).

2.2.2.3 Media for standard culture of rat tooth slice

Media were prepared for culture of tooth slices with the following composition: DMEM with phenol red (Sigma Aldrich), supplemented with 10% heat activated FCS (Invitrogen), 0.15 mg/mL of vitamin C (Sigma Aldrich), 200 mmol/L L-glutamine (Invitrogen) 1% antibiotics containing 1,000 U/mL penicillin G sodium 10 mg/mL streptomycin sulfate, and 25 mg/mL amphotericin B (Invitrogen).

2.2.3 Growth kinetics of *S. anginosus* group (SAG) bacteria in different media

2.2.3.1 Growth kinetics in brain heart infusion media

SAG was cultured in BHI overnight as described above and centrifuged at 2600 x g rpm for 10 min (IEC centrifuge, Thermo Electron Corporation, Cambridge, UK). The supernatant was discarded and the pellet re-suspended in 3 mL BHI. The suspension was subjected to 1:1 (double dilution) in a 96-well plate to create a series of dilutions (1:10) with BHI as standard. The

absorbance was measured at 570 nm using a spectrophotometer (FLUOstar OPTIMA, BMG LABTECH, Aylesbury, UK).

Each of these dilutions from the 1:1 dilution series was subjected to tenfold dilution (1:10) and plated on FAA plates and incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h. The CFU/mL for each of the dilutions in the series was measured by counting the colonies using the table provided by (Don Whitley Scientific Ltd. Shipley, West Yorkshire, UK). These were then plotted against the absorbance of each of the dilutions using Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA) to obtain a standard curve for *S. anginosus* 39/2/14A, *S. constellatus* 45386 and *S. intermedius* HW13. The experiment was performed in triplicate and the standard error was calculated and plotted on the standard curve.

An overnight SAG culture in BHI was used to prepare a suspension on 10^2 CFU/mL of SAG in BHI. Two hundred µL of this suspension was added to the well of a 96-well plate and incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h on a mechanical rocker. The absorbance of the culture was measured every 3 hours at 570 nm for 24 h using the standard curve generated previously for the three strains of SAG and the absorbance for each of the SAG strains every 3 hours the growth curve in BHI for *S. anginosus* 39/2/14A, *S. constellatus* 45386 and *S. intermedius* HW13 were generated. This experiment was repeated three times and the standard error was calculated.

2.2.3.2 Growth kinetics in modified tissue culture media

Growth curves for *S. anginosus* 39/2/14A, *S. constellatus* 45386 and *S. intermedius* HW13 in MTCM were generated as described in **section 2.2.3.1** except that MTCM was replaced BHI.

2.2.4 The e*x vivo* tooth slice model

2.2.4.1 Preparation of tooth slice

Twenty-eight-day old male Wistar rats (Charles River, Margate, UK) were used for all the experiments. The animals were sacrificed by CO_2 asphyxiation and maxillary and mandibular incisors were immediately extracted using a sterile scalpel. The incisors were immediately transferred to DMEM prepared for the standard culture of tooth slices as described in **section 2.2.2.3**. The teeth were then cut into 2 mm (for histology) or 4 mm (for PCR) thick transverse sections using a diamond edged rotary saw (TAAB, Aldermaston, UK). The equipment and the

blade were disinfected by soaking in 70% ethanol. Sterile PBS was used to keep the blade cool during cutting by adding it to the well at the base of the blade. The sections were then transferred to DMEM.

2.2.4.2 Culture of the tooth slice

Before preparation of the tooth sections, 2 mL of DMEM prepared as described in **section 2.2.2.3** was added to each well of a 12-well plate and incubated at 37^{o}C in 5% CO_2. The tooth slices prepared as described in **section 2.2.4.1** were then immediately transferred to the wells of a 12-well plate (one section per well) and incubated for 12 h at 37^{o}C in 5% CO_2. The medium was changed to antibiotic-free medium and incubated at 37^{o}Cin 5% CO_2 on a rocker overnight to remove any antibiotics in the tooth slice. Antibiotic-free DMEM was prepared by mixing DMEM with phenol red (Sigma Aldrich), 10% heat activated foetal calf serum (Invitrogen) and 0.15 mg/mL of ascorbic acid. The tooth slice was free of dead cells on the second day.

2.2.5 The *ex vivo* pulp infection model

The tooth slice cultured in antibiotic-free media as described in **section 2.2.4.2** was again washed in antibiotic-free DMEM by pipetting. This was repeated three times with fresh antibiotic-free DMEM. An overnight culture of SAG prepared in BHI as described in **section 2.2.1.1** was inoculated in MTCM and incubated at 37^{o}C in 5% CO_2 on a mechanical rocker to reach its exponential phase at approximately 7 to 8 h. These SAG planktonic suspensions were diluted in MTCM to produce suspensions of 10^2 CFU/mL *S. anginosus* 39/2/14A or *S. constellatus* 45386. Each well of a 12-well pate was filled with 2 mL of 10^2 CFU/mL *S. anginosus* 39/2/14A in MTCM and another 12-well plate was filled with 2 mL of 10^2 CFU/mL *S. constellatus* 45386 in MTCM. To each of these wells, one washed tooth slice as prepared above was added and incubated at 37^{o}C in 5% CO_2 over 24 h. The control tooth slices were incubated in MTCM only at 37^{o}C in 5% CO_2 for 24 h.

2.2.6 Processing of tooth slices for histology

Tooth slices that were incubated with bacteria or in media without bacteria were transferred to 10% (v/v) neutral-buffered formalin solution for a minimum of 24 h to fix the tissue. They were then transferred to 10% formic acid and allowed to demineralise for 48 h under constant agitation. The formic acid solution was changed after 24 h.

Tooth slices were then transferred from formic acid into biopsy cassettes and processed manually through graded chemicals in the following sequence:

1. 50% ethanol for 2 h.
2. 70% ethanol for 2 h.
3. 95% ethanol, overnight.
4. 100% ethanol for 2 h.
5. 100% ethanol for 2 h.
6. 100% ethanol for 2 h.
7. 100% ethanol, overnight.
8. 100% ethanol for 1 h.
9. Methyl salicylate for 1 h.
10. Methyl salicylate for 1 h.
11. 0.5% necloidine in methyl salicylate for 1 h.
12. 0.5% necloidine in methyl salicylate for 1 h.
13. 1% necloidine in methyl salicylate for 1 h.
14. 1% necloidine in methyl salicylate for 1 h.

All the above treatments were done under continuous mechanical agitation by placing it on a mechanical rocker.

15. Tooth slices were placed in molten wax at 60^{o}C in a slide drying oven (Binder, Tuttlingen, Germany) for 1 h.
16. The molten wax was changed after 1h and fresh wax was added and left at 60^{o}C for 1 h
17. Step 16 was repeated once again and left at 60^{o}C for 1 h.
18. After 1 h the wax was changed once again, and the tooth slices were left in molten wax at 60^{o}C overnight.

Following processing, the tooth slices was embedded in paraffin wax (Leica Biosystems, Milton Keynes, UK). Five µm thick sections of the wax embedded teeth were cut using a microtome (Leica). They were then floated on water at 40^{o}C in a water bath and mounted on a glass slide. The slides were dried in a slide drying oven at 60^{o}C overnight to improve adhesion of the section to the slide before staining.

The histological sections were stained using an automated tissue stainer (Thermofisher Scientific, Loughborough, UK). The stainer took the section through alcohol, xylene and water washes and then stained it with haematoxylin and eosin (H&E) stains. It was then taken through a series of alcohol washes and finally immersed in xylene. A coverslip was glued over the stained

section before viewing using an Olympus AX20 light microscope (Olympus Corporation, Southend-on-Sea, UK).

2.2.7 Quantification of intact cells on the tooth sections

In order to evaluate the count of intact cells, five tooth slices were used and after fixing, processing and H&E staining, one section from each of the five tooth slices were taken and sectional images of the entire tooth section were taken using an Olympus AX70 upright compound microscope (Olympus Corporation) and image analysis software PAX-it (PAX-it™ Villa Park, Illinois U.S.A*)* at 20X magnification. The sectional images were reconstructed to give the full image of the tooth section using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The numbers of odontoblast and pulp cell nuclei were calculated using the same software. The blue field from the image was extracted and the moments threshold was applied to separate cells. The nuclei that were close to each other were split using the watershed function. The number of particles in the range of 3 to 100 μm^2 were counted and the data was normalised to the pulp area and the standard error and mean were calculated. Then percentage change in cell count for each time point compared to 0 h was calculated.

2.2.8 *Ex vivo* pulpal infection model with stained bacteria

Bacterial staining

Fluorescein diacetate (FDA) (Sigma Aldrich) stain was prepared by adding one gram of FDA in 100 mL of acetone to make a 1% solution (w/v). Following the preparation of a SAG planktonic suspension of 10^2 CFU/mL **section 2.2.5** 20 µL of 1% FDA in acetone was added for every mL of the bacterial suspension and incubated at room temperature with the FDA for 30 min. It was then filtered using 0.2 µm polyvinylidene fluoride filters (PVDF; Elkay Laboratory Products Ltd, Basingstoke, UK) and the filtrate was discarded. The bacteria captured in the filter were re-suspended in MTCM to yield 10^2 CFU/mL of an FDA-stained planktonic suspension.

Culture of stained bacteria on tooth slice

The FDA-stained SAG planktonic suspension was used to infect the tooth slices prepared as described in **section 2.2.4**. These were incubated at 37^{o}C in 5% CO_2 for 24 h. The control tooth slices were incubated in sterile MTCM to which FDA was added and was subjected to the same filtration process as the FDA-stained SAG planktonic suspension.

2.2.9 Computing area of SAG bacteria colonisation

The tooth sections were fixed and processed as described in **section 2.2.6** and viewed under a fluorescent microscope with a FITC filter and the images captured using an Olympus AX70 upright compound microscope (Olympus Corporation) and image analysis software PAX-it (PAX-it™) at 10x magnification. The sections were then stained as described in **section 2.2.6** and images were captured of the same field at the same magnification. The experiments were performed with five tooth slices. ImageJ software (National institute of health) was used to quantify the area of the pulp. The green field of the fluorescent image was extracted, and this was converted to a binary form using the moments threshold method. The pulp area was manually selected and the total area of the pulp measured. The area covered by the bacteria was measured and calculated as a percentage area of the total area of the pulp.

2.2.10 Evaluation of inflammatory markers

2.2.10.1 *In vitro* co-culture of pulp cells and SAG bacteria

2.2.10.1.1 Isolation and culture of rat pulp cells

Preparation of a fibronectin plate was undertaken by reconstituting fibronectin (Sigma Aldrich) in PBS with Ca^{2+} and Mg^{2+} at 1 µg/µL. Fibronectin to PBS (+Ca and +Mg) at 1:100 or 120 µL in 11,880 µL for 12 wells was prepared. One mL of fibronectin in PBS was added to each well, the plate was wrapped in parafilm and stored at 4°C overnight. If the plate was to be stored for more than 24 h before use, it was stored at -20°C and then thawed at 4°C before use.

A working medium was prepared with 500 mL αMEM containing ribonucleosides, deoxyribonucleosides, 2mM L-glutamine (Invitrogen) and 1% antibiotics containing 1,000 U/mL penicillin G sodium, 10 mg/mL streptomycin sulphate and 25 mg/mL amphotericin B (Invitrogen). The culture medium was made by supplementing αMEM with 10% heat inactivated foetal calf serum, 0.15 mg/mL L-ascorbic acid 2-phosphate (Sigma Aldrich), and 1% antibiotics containing 1,000 U/mL penicillin G sodium, 10 mg/mL streptomycin sulfate and 25 mg/mL amphotericin B (Invitrogen).

The rat incisors were collected in 1:10 antibiotic-antimycotic/working medium. The dental pulp tissue was extracted into 1 mL working medium, spun at 400 g for 5 min and the supernatant removed. Dental pulp was chopped into pellets with a scalpel. Tissue was collected in a 5 mL bijou with 1 mL of collagenase/dispase. Tissue was incubated at 37°C for 45 min and was agitated

half way through the incubation. The resulting cell suspension was filtered using a 70 µm nylon strainer with 9 mL working medium in a 50 mL tube. This was then spun at 400g for 5 min, resuspended in 1 mL working medium and cell counts performed using a haemocytometer. The cell suspension was diluted in working media to give 4000 cells per cm^2 (38,000 cells per well in a 6 well plate). Fibronectin was removed and 1mL of cell suspension per well was added and incubated at 37°C for 20 min. Media was removed, which contained non-adherent cells and added to the well below. Following fibronectin removal, 1 mL of fresh working medium was added to the well containing adherent cells. A further 1 mL of culture media was added to each well for both adherent and non-adherent cells. Cells were incubated at 37°C in 5% CO_2 until confluent and were subsequently passaged using trypsin, suspended in freezing mixture and stored in liquid nitrogen until used.

2.2.10.1.2 Co-culture of rat dental pulp cells with SAG bacteria

Isolated dental pulp cells were seeded onto 6 well plates at 10^5 cells per well containing media prepared as described in **section 2.2.2.3** and incubated at 37°C in 5% CO_2 to allow attachment overnight. The wells were washed three times with antibiotic-free MTCM prepared as described in **section 2.2.4** and allowed to incubate for 12 h to remove any residual antibiotics. A standard suspension of 10^2 CFU/mL of *S. anginosus* 39/2/14A and *S. constellatus* 45386 were prepared in MTCM. The media in the wells containing the dental pulp cells were removed and replaced with media containing the bacterial suspension and incubated in a CO_2 incubator at 37°C for 24 h.

2.2.10.1.3 RNA extraction from *in vitro* pulp cells infected with SAG bacteria

Pulp cells were harvested at 0 h, 6 h, 12 h and 24 h. They were washed three times in sterile PBS. 500 µL of Trizol reagent (Sigma-Aldrich) was added to each well and allowed to lyse for 5 min at room temperature. The lysed cells from two wells were pooled together into a 1.5 mL centrifuge tube. To the 1000 µL of lysate, 173.3 µL of chloroform (Sigma-Aldrich) was added and vortexed for 30 s, followed by incubation at room temperature for 3 min. Heavy-phase lock gels (Scientific Laboratory Supplies, Nottingham, UK) were pelleted by centrifugation at 12,000 x g for 30 s. The RNA-containing extract from the 1.5 mL centrifuge tube was transferred to the phase lock gel tube and centrifuged at 12,000 x g for 10 min at 4°C. The aqueous phase was transferred to a fresh a 1.5 mL centrifuge tube and an equal volume of isopropanol (Sigma-Aldrich) was added before incubation at 20°C for 20 min. This was then centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was aspirated carefully and the pellet at the bottom of the tube was washed with 750 µL of 75% ice cold ethanol. The tube was centrifuged at 7,500 x g for 5 min at 4°C. The

supernatant was carefully aspirated and the RNA pellet was allowed to air dry. Once the RNA pellet was dry, 43 µL of nuclease free water was added and vortexed for 30 s. It was then placed in a water bath at 55 to 60°C for 10 to15 min to disperse the RNA in the water.

2.2.10.1.4 Purification of RNA

To the RNA suspension obtained above was added 1 µL Rnasin (Promega), 1 µL Dnase (Qiagen) and 5 µL of buffer (Qiagen) and this was incubated at 37^{o}C for 15 min.

2.2.10.1.5 RNA Quantification

Total RNA quantification was carried out using the Nanovue™ spectrophotometer (GE Healthcare Amersham, UK). The ratio of the absorbance at 260:280 nm for all samples was between 1.7 and 2.1, indicating acceptable levels of RNA purity. RNA was subsequently stored at -80 ^{o}C until PCR was performed. The experiment was performed in triplicate for each time point.

2.2.10.1.6 Production validation using PCR

Reverse transcription

Purified RNA, prepared as described in **section 2.2.10.1.3**, was used for reverse transcription. Five hundred ng of RNA was mixed with 1 µL of random primer (Promega) and the volume made up to 15 µL with nuclease-free water in a 0.25 mL PCR tube. This was then incubated at 70^{o}C for 5 min and immediately cooled down and stored in ice. Master mix was made with 5 µL of 5 x MMLV reaction buffer (Promega), 1.25 µL of dNTPSs (10 mM stock)(Promega), 0.6 µL RNasin (Promega), 1 µL MMLV enzyme (Promega) and 2.15 µL nuclease-free water (Promega), to give a total reaction volume of 10 µL. Ten µL of the master mix was added to 15 µL of RNA and the random primer mix prepared as described above to givea final volume of 25 µL and incubated at 37^{o}C for 1 h on a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd, Braintree, UK). For each reverse transcription reaction performed, a template control negative control was prepared by substituting sterile water for RNA. Negative controls were set up, and these excluded the reverse transcibed RNA. The resulting cDNA was cooled on ice and stored at -20^{o}C for use in PCR reactions.

Polymerase chain reaction

PCR reactions were carried out using reagents from Promega. The reagents were mixed in the following proportions for each reaction: five µL 5x Green GoTaq™ Flexi buffer, 0.5 µL 10 mM PCR

Nucleotide Mix, 1.25 µL forward primer (40 ng/µL stock), 1.25µL reverse primer (40ng/ µL stock), 1 µL $MgCl_2$ (25mM stock), 14.75 µL PCR grade water, 0.25 µL GoTaq™ DNA Polymerase and 1 µL of cDNA, being the final reaction volume to 25 µL. Mixtures were placed in the PCR machine and subjected to the following heat cycles: initial denaturation step at 94^{o}C for 2 min followed by 40 cycles of a 15 s at 94^{o}C (denaturation), 30 s at 62^{o}C (annealing) and 30 s at 72^{o}C (extension) for each cycle. The final step was at 72^{o}C for 5 min, on a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd, Braintree, UK). PCR products were stored at 4^{o}C until subjected to electrophoresis.

A list of all primer sequences used in the PCR reactions is shown in **Table 2.1**. A Blast search was run on each primer sequence in order to ensure specificity for the intended amplification targets. β-actin was used as a reference gene.

Table 2.1: Sequence for the forward and reverse primers used
Evaluation of inflammatory marker expression by dental pulp tissue infected with SAG bacteria was evaluated with the following primers

Primer	Sequence 5'-3'	Reference
β-actin	F:TACAACCTTCTTGCAGCTCCTCCGTCGC R:CATACCCACCATCACACCCTGGTGCCTA	Gatto *et al* (2008)
TNF-α	F:TCAGCCTCTTCTCATTCCTGCTCGTGGC R:TGGGCTACGGGCTTGTCACTCGAGTTTT	SigmaGenosys
IL-1α	F:CCGGGTGGTGGTGTCAGCAACATCAAAC R:CTGGAAGCTGTGAGGTGCTGATCTGGGT	SigmaGenosys
IL-1β	F:ATGCCTCGTGCTGTCTGACCCATGTGAG R:CCCAAGGCCACAGGGATTTTGTCGTTGC	SigmaGenosys
IL-10	F:CTGCGACGCTGTCATCGATTTCTCCCCT R:GCAGTCCAGTGATGCCGGGTGGTTCAA	SigmaGenosys
IL-18	F:TGGCTGCCATGTCAGAAGAAGGCTCTTG R:ACTGCGGTTGTACAGTGAAGTCTGCCAA	SigmaGenosys

Preparation of electrophoresis gels

PCR products were visualised on SafeView nucleic acid stain (NBS Biological, Cambridgeshire, UK) impregnated Tris/Borate/EDTA (TBE) agarose gels.0.5 x TBE buffer was used to make the agarose gels and as a running buffer. It contained 0.5 M tris-base (Sigma Aldrich), 0.5 M boric acid (Sigma Aldrich), 0.03M EDTA (Sigma Aldrich) and had a pH of 8. TBE buffer was diluted 10 times to a concentration of 0.5 x prior to use.

Agarose Gel Preparation

70 mL 2% agarose gels were prepared (Sigma Aldrich) using 0.5 x TBE buffer. Agarose (1.4 g) was dissolved into solution by heating in a microwave prior to the addition of 7 µL of SafeView (NBS Biological). The agarose solution was immediately poured into a casting tray with a well comb and left to cool.

Gel electrophoresis of PCR products

The prepared gel was submerged in 0.5 x running buffer and the comb removed. Ten µL of the sample was loaded to each well and 7 µL of 100 bp DNA ladder (Promega) was loaded into the first well of each gel. The gel was run at 120 mA for 45 min before visualisation. The gels were visualised using the Gel Doc ^{TM} scanner (Bio-Rad, Hemel Hempstead, UK) for visualization by UV light. The image was captured using quantity one image analysis software (Bio-Rad).

2.2.10.1.7 Real time quantification of gene expression in *in vitro* SAG-infected pulp cells

Real-time quantitative PCR (RT-qPCR) was carried out using Precision PLUS Master Mix premixed with ROX and SYBRgreen (Primer Design, Southampton, UK). A final reaction volume of 20 µL was made up with 10 µL of Master Mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 4 µL or nuclease free water and 25 ng of cDNA template in 5 µL. The reference gene used was β-actin. 20 µL of the final reaction mixture was loaded in each well of bright white real time PCR plastic 96 well plates (Primer Design) for real-time PCR from ABI machine. The samples were loaded in triplicate and sealed with an optical seal for bright white plastic (Primer Design). Plates were then loaded in an ABI real time qPCR machine and subjected to the following cycles: initial step cycle of 94^{o}C for 2 min followed by 40 cycles at 94^{o}C (denaturation) for 15s, a 62^{o}C (annealing) for 30s and a 72^{o}C (extension) for 30s. The final step was at 72^{o}C for 5 min. Relative expression of the inflammatory markers was calculated with β-actin as the reference gene and uninfected cells as the control using Livak's method (Livak and Schmittgen 2001).

2.2.10.2 E*x vivo* pulp infection model

2.2.10.2.1 RNA extraction from pulp tissue of *ex vivo* pulp infection model

Tooth slices of 4 mm length were prepared and cultured as described in **section 2.2.4** and *ex vivo* pulpal infection was established with 10^2 CFU/mL planktonic suspension of *S. anginosus*

39/2/14A or *S. constellatus* 45386, as described in **section 2.2.4.** The controls were tooth slices in MTCM without SAG infection. Tooth slices were incubated at 37^{o}C in 5% CO_2. One tooth slice was harvested from control, *S. anginosus* 39/2/14A co-culture and *S. constellatus* 45386 co-culture samples at 0 h, 6 h, 12 h and 24 h. The experiment was performed in triplicate.

Once harvested, the tooth slices were washed three times in sterile PBS. Then using a 1 mL syringe and a 25 gauge needle the pulp was flushed out of the tooth slice using PBS. Pulp tissue was transferred into 500 µL of Trizol reagent (Sigma-Aldrich) in a fume hood and homogenised using a homogeniser (Cole-Parmer UK, London, UK) at 30,000 rpm for 30 s. The homogeniser tip was washed on full power three times in PBS before homogenising the next sample. The homogenised sample was then left to lyse for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes.

The homogenate was centrifuged at 12,000 × g for 10 min at 4°C to remove the insoluble material (extracellular membrane, polysaccharides, and high molecular mass DNA). The supernatant contained RNA and protein, and was transferred to a fresh tube and subjected to the same process as described in section **2.2.10.1.3**.

2.2.10.2.2 Real-time quantification of gene expression in pulp tissue of the *ex vivo* pulp infection model

RNA was purified, quantified and reverse transcribed as described in **sections 2.2.10.1.4 - 2.2.10.1.5.1**. The resulting cDNA was stored at -20°C until used for RT-qPCR as described in **section 2.2.10.1.6** to evaluate the relative expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 and IL-10 as described in **section 2.2.10.1.7**.

2.2.11 Statistical analysis

The results were statistically analysed to detect significant differences between infected tooth slices and controls by Mann-Whitney U-test using Graph Pad statistical software (GraphPad Software, La Jolla, USA).

2.3 Results

2.3.1 Identification of bacteria by gram staining

The gram staining of *S. anginosus* (Ref 39/2/14A), *S. constellatus* (Ref 45386) and *S. intermedius* (Ref HW13) identified them as gram-positive cocci.

2.3.2 Molecular-based bacterial identification

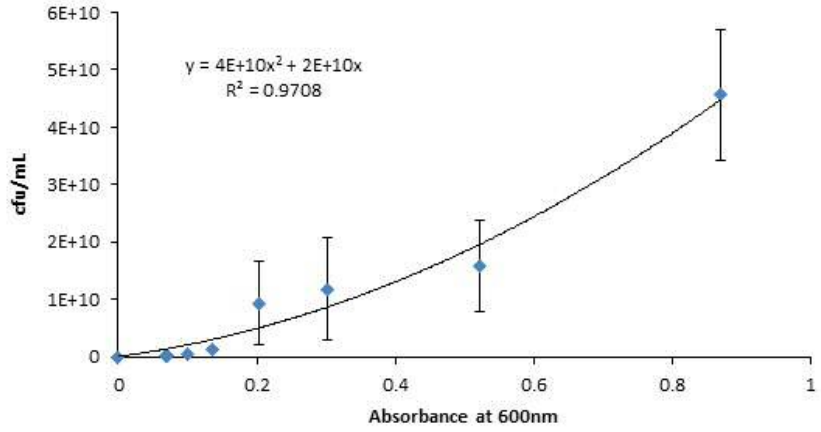
The isolates were identified by 16S rDNA gene sequencing. A 95% homology was considered as a reliable level of identification. **Table 2.2** shows the results of the molecular analysis.

Table 2.2: Molecular analysis results for identification of bacteria to be used in the experiments.

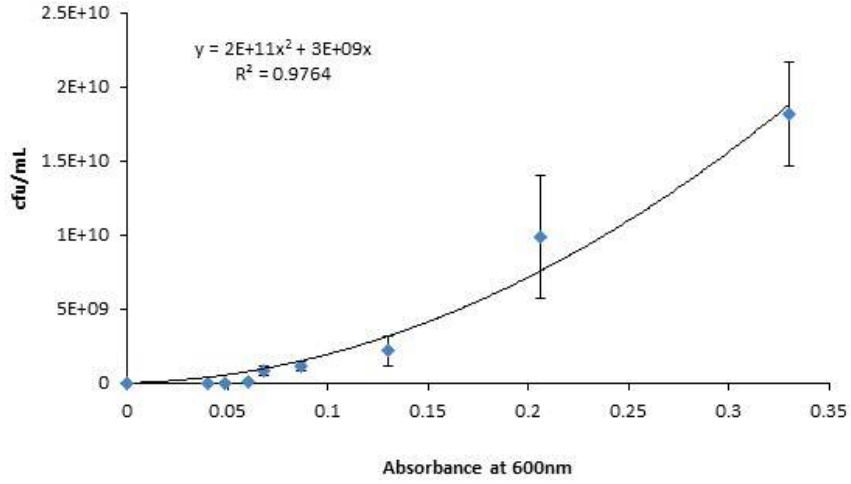
Strain number	Identification by 16s rDNA sequencing (BLAST)	Percentage homology to type strain
39/2/14A	*S. anginosus*	99.9%
45386	*S. constellatus*	99.1%
HW13	*S. intermedius*	99.9%

2.3.3 Standard curves for SAG bacteria

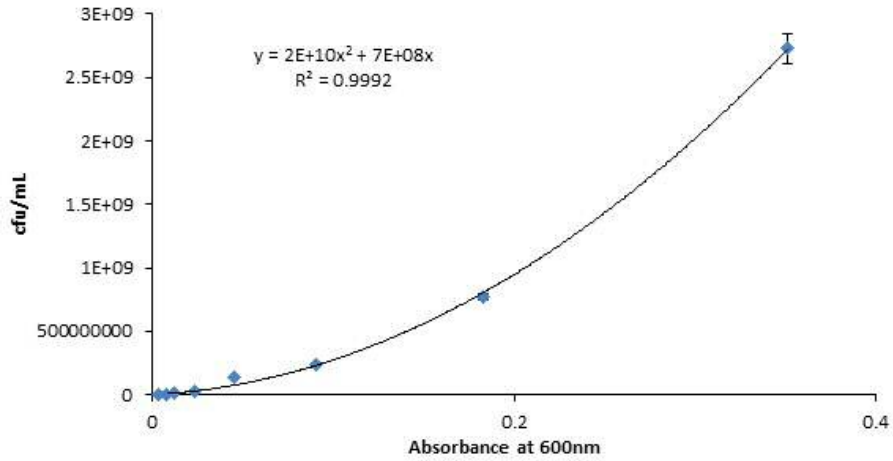
Standard curves were generated for a range of absorbance of *S. anginosus* (Ref 39/2/14A), *S. constellatus* (Ref 45386) and *S. intermedius* (Ref HW13) suspensions against their corresponding CFU/mL along with the equations obtained by linear regression as shown in **Figure 2.1a, b, c** respectively.


y = 4E+10x² + 2E+10x
R² = 0.9708
cfu/mL
6E+10
5E+10
4E+10
3E+10
2E+10
1E+10
0
0
0.2
0.4
0.6
0.8
1
Absorbance at 600nm

(a)


y = 2E+11x² + 3E+09x
R² = 0.9764
cfu/mL
2.5E+10
2E+10
1.5E+10
1E+10
5E+09
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
Absorbance at 600nm

(b)



(c)

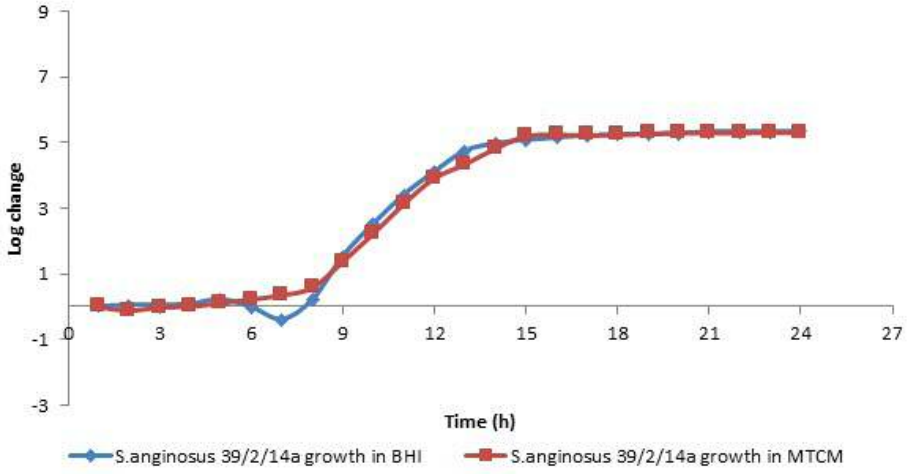
Figure 2.1: Standard curve generated for SAG

a) *S. anginosus* 39/2/14a absorbance and corresponding cfu/mL. The gradient of the line y=4E+10x+2E+10x allows calculation cfu/mL of *S. anginosus* 39/2/14a for a known absorbance of a suspension of *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 and their corresponding cfu/mL. The gradient of the line y=2E+11x2 +3E+09x allows calculation cfu/mL of *S. constellatus*; c) Standard curve generated from a series of absorbance of *S. intermedius* (HW13) and their corresponding cfu/mL. The gradient of the line $y=2E+10x^2+7E+08x$ allows calculation cfu/mL of *S. intermedius* (HW13) for a known absorbance of a suspension of *S. intermedius* (HW13). Error bars indicate SE for experiments were conducted in triplicate.

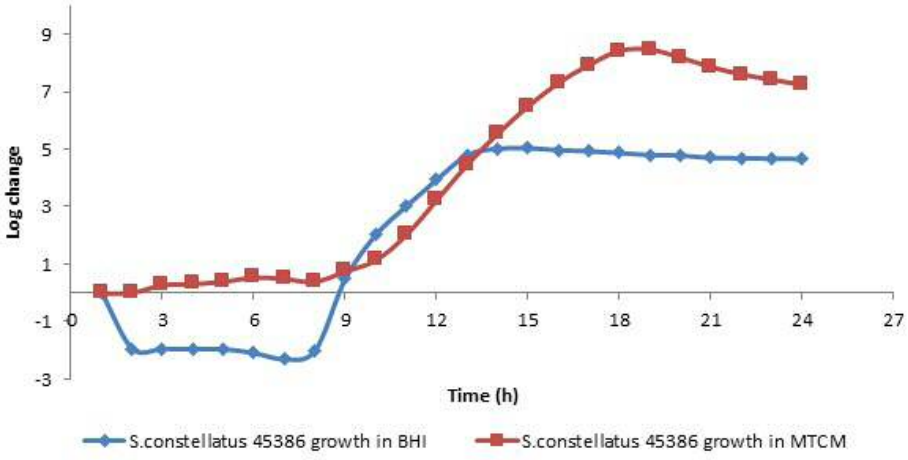
2.3.4 Comparison of growth kinetics of SAG in different media

The effect of BHI and MTCM on the growth of *S. anginosus* (Ref 39/2/14A), *S. constellatus* (Ref 45386) and *S. intermedius* (Ref HW13) was evaluated, normal bacterial growth characteristics were demonstrated by *S. anginosus* (Ref 39/2/14A) and *S. constellatus* (Ref 45386*)* strains in both media at 37^{o}C and 5% CO_2. *S. anginosus* (Ref 39/2/14A) had no difference in growth in either media throughout the different phases of growth as demonstrated in **Figure 2.2a**. In contrast, *S. constellatus* (Ref 45386) had a prolonged exponential phase of up to 18 h in MTCM compared to 12 h in BHI **Figure 2.2b**. The count also dropped in the lag phase when cultured in BHI, which could be due to change from sold medium to broth (Dagley *et a*l 1950), but it managed to reach same level as in MTCM at 9 h, at which exponential phase commenced in both media. For *S. intermedius* (Ref HW13) there was very little growth over 24 h in BHI and no growth was seen in MTCM media **Figure 2.2c**. This may be because *S. intermedius* (Ref HW13) may have special nutritional requirements. Therefore only *S. anginosus* (Ref 39/2/14A) and *S. constellatus* (Ref 45386) were used for further experiments. Furthermore, the log change was higher between 14 h and 18 h for *S. constellatus* (Ref 45386) in MTCM compared to BHI, where it had already reached its stationary phase **Figure 2.2b**. *S. intermedius* (Ref HW13) was eliminated as its growth kinetics in MTCM were not suitable to be used for the pulp infection model. As the nutritional needs of *S. intermedius* (Ref HW13) was different from the pulp tissue as MTCM did not support growth of *S. intermedius* (Ref HW13).

When *S. anginosus* (Ref 39/2/14A) and *S. constellatus* (Ref 45386) growth were compared against each other in BHI, there was no difference in growth between the two strains in their exponential phase **Figure 2.3a**. The growth in MTCM media was different for both strains in their exponential phase: *S. constellatus* (Ref 45386) exhibited a 2 h delay compared to *S. anginosus* (Ref 39/2/14A) in the start of the exponential phase. With a difference of $2.5x10^6$ CFU/mL between *S. constellatus* (Ref 45386) and *S. anginosus* over this period. (Ref 39/2/14A). Furthermore, the exponential phase was prolonged by another 4 h in *S. constellatus* (Ref 45386) compared to *S. anginosus* (Ref 39/2/14A) **Figure 2.3b**.


Log change
Time (h)
S.anginosus 39/2/14a growth in BHI
S.anginosus 39/2/14a growth in MTCM

(a)


Log change
Time (h)
S.constellatus 45386 growth in BHI
S.constellatus 45386 growth in MTCM

(b)

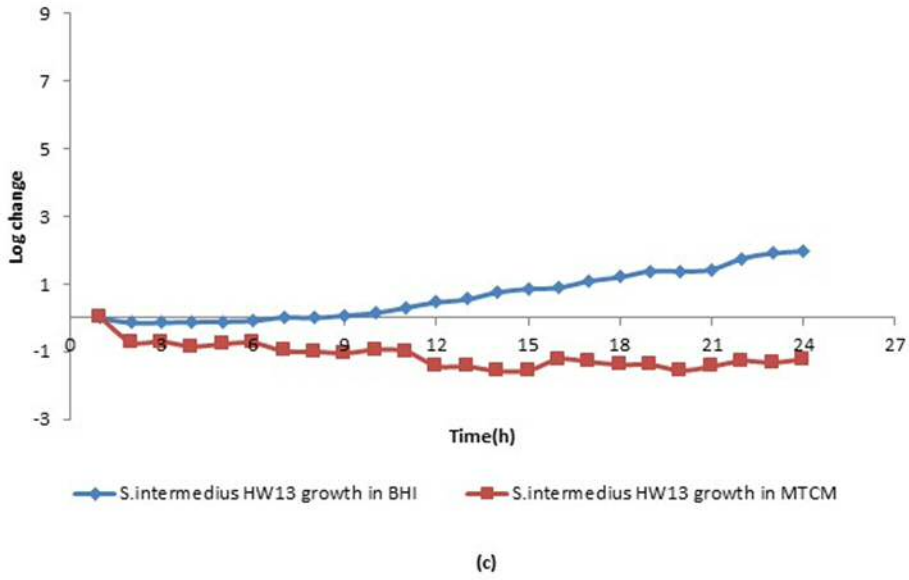
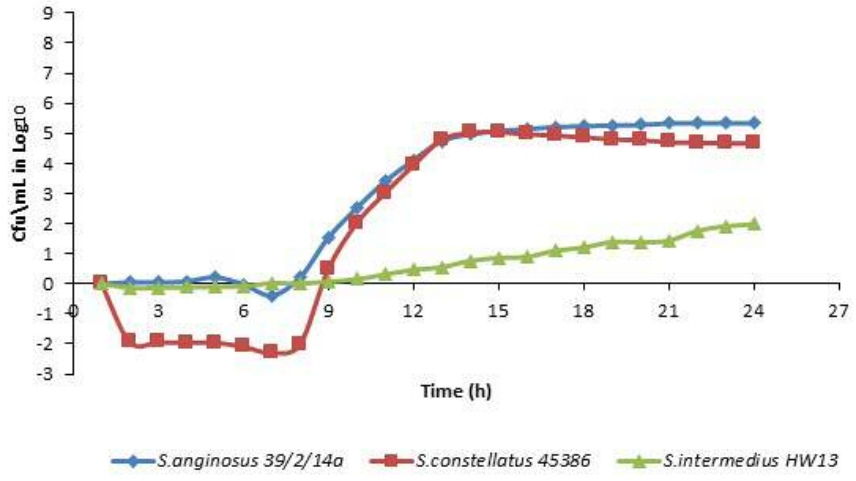
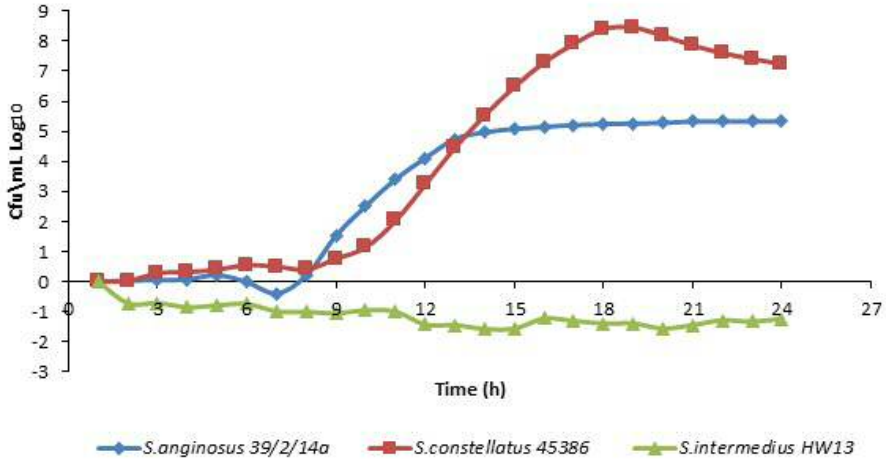


Figure 2.2: Comparison of the effect of BHI and MTCM on the growth of SAG
a) *S. anginosus* 39/2/14a over 24 h; b) *S. constellatus* 45386 over 24 h; c) S. intermedius (HW13) over 24 h. The experiments were conducted in triplicate.



(a)

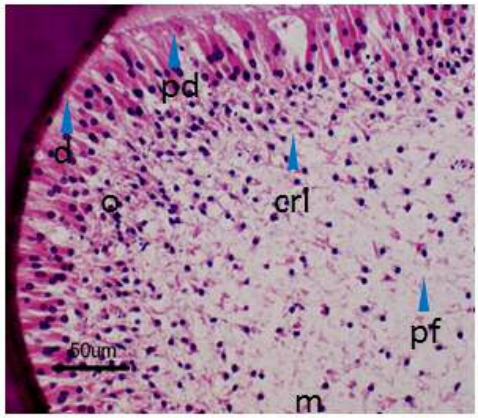


(b)

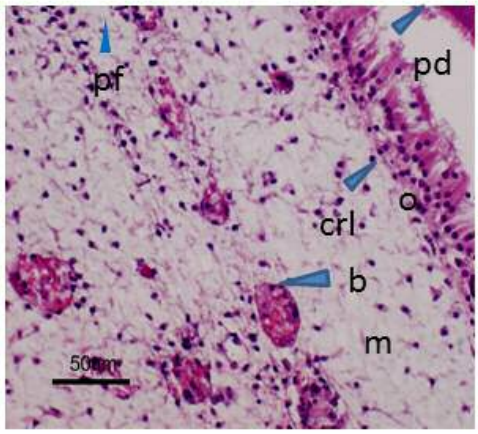
Figure 2.3: Comparison of the growth of SAG strains in different media over 24 h
a) *S. anginosus* 39/2/14a, *S. constellatus* 45386 and *S. intermedius* HW13 in BHI over 24 h, b) Comparison of the growth of *S. anginosus* 39/2/14a, *S. constellatus* 45386 and *S. intermedius* HW13 in MTCM over 24 h.

2.3.5 Histomorphometrics of the *ex vivo* pulp infection model.

When the H&E-stained, SAG-infected tooth slices were studied under the microscope and compared with un-infected tooth slices, defined areas of bacterial colonisation were observed in the pulp compared to that of the controls. The density of odontoblasts was higher in the controls when compared to the SAG-infected tooth slices. This difference in the cell density observed is due to cell death noted between the control tooth slices and SAG-infected tooth slice in the cell rich layer of Höhl, just below the odontoblast layer **Figures 2.4 & 2.5**. The pulp stroma cells also appeared to be sparsely distributed, indicating cell death in a degraded pulp matrix in SAG-infected tooth slices compared to control tooth slices. The loss of pulp stroma cells and breakdown of inter-cellular matrix was more prominent in *S. constellatus* (Ref 45386) infection compared to *S. anginosus* (Ref 39/2/14A) infection **Figures 2.4 & 2.5**. These observations demonstrate cell death and matrix degradation arising from SAG infection.

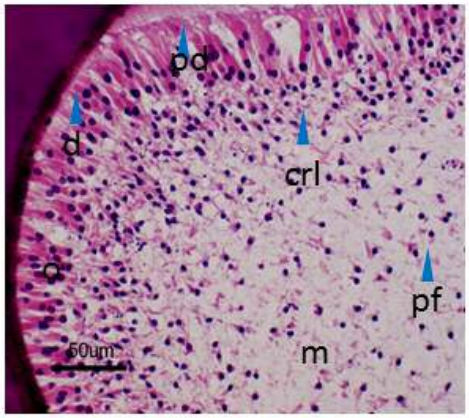


(a)

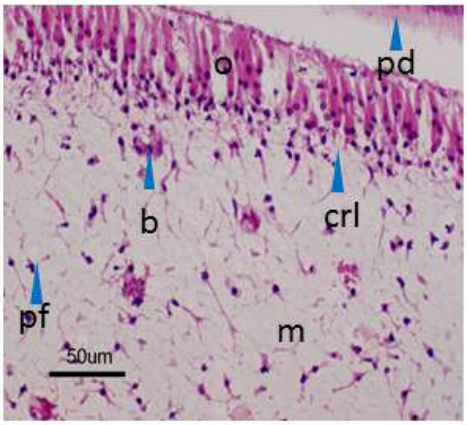


(b)

Figure 2.4: Histology of *S. anginosus* 39/2/14a co-culture with tooth slice over 24 h.
a) control X40 magnification, b) S. *anginosus* 39/2/14A co-culture on tooth slice X40 magnification*. o,* odontoblast; pd, predentine; crl, cell rich-layer of Höhl:, b, bacterial colonies; m, pulp matrix; pf, pulp stroma cells.
.



(a)



(b)

Figure 2.5: Histology of *S. constellatus* 45386 co-culture with tooth slice over 24 h.
a) control X40 magnification, b) *S. constellatus* 45386 co-culture on tooth slice X40magnification, o, odontoblast; pd, predentine; crl, cell-ich layer of Höhl:, b, bacterial colonies; m, pulp matrix; pf, pulp stroma cells.

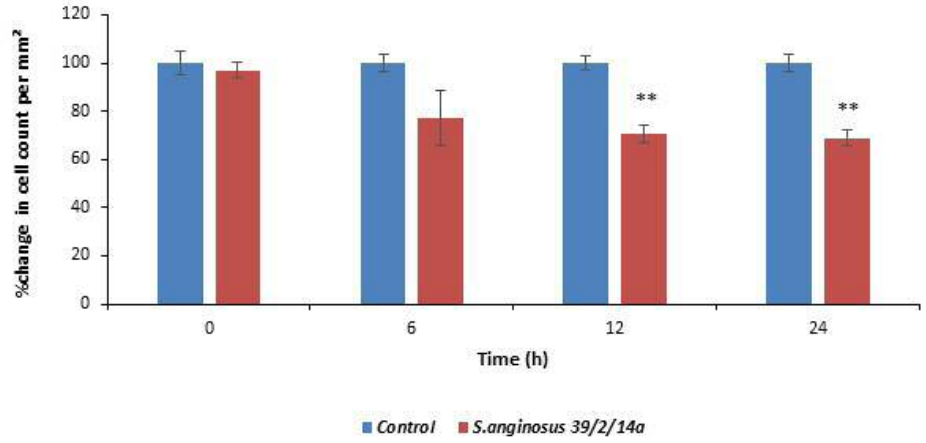
2.3.6 Comparison of difference in pulp cell viability between two strains of SAG

2.3.6.1 Odontoblasts

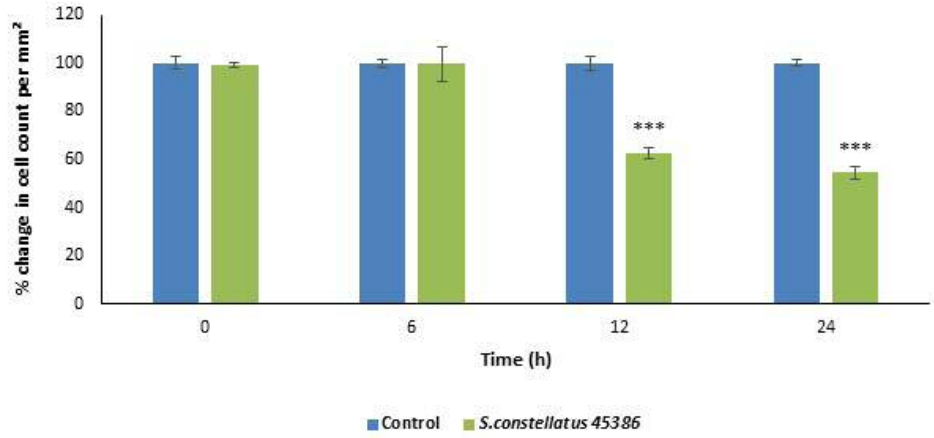
Histologically, there were reduction in the density and distribution of odontoblasts between infected and uninfected samples. There was a statistically significant reduction in odontoblast counts per mm² at 12 h (p=0.0028) and 24 h (p=0.0027) for *S. anginosus* (Ref 39/2/14A) and *S. constellatus* (Ref 45386) infection 12 h (p=0.006) and 24 h (p=0.001) compared to uninfected tooth slices **Figure 2.6a, b.** Furthermore, when the reduction was compared between the two strains there was a statistically significant reduction (p=0.0223) in odontoblast counts for *S. constellatus* (Ref 45386) infection compared to *S. anginosus* (Ref 39/2/14A) infection, when observed over a 24 h period **Figure 2.7**.

2.3.6.2 Viability of dental pulpal stromal cells

When evaluating viable dental pulp cells other than odontoblasts, there was a significant reduction at 12 h (p=0.0027) and 24 h (p=0.010) respectively, for *S. anginosus* (Ref 39/2/14A) infection; and for *S. constellatus* (Ref 45386) at 12 h (p=0.002) and 24 h (p=0.010), but a 20% greater reduction in viable dental pulp stromal cells was observed in *S. constellatus* (Ref 45386) infection compared to *S. anginosus* (Ref 39/2/14A) **Figure 2.8a,b**. When comparing *S. constellatus* (Ref 45386) against *S. anginosus* (Ref 39/2/14A), the former demonstrated a significant reduction at 12 h (p=0.028) and 24 h (p=0.040) compared to the latter **Figure 2.9**.



(a)



(b)

Figure 2.6: Effect of SAG infection on the odontoblast count over 24 h.
Odontoblast count was performed on transverse sections of tooth slices and estimated per mm² of pulp. There was a reduction in odontoblast count when infected with SAG at 12 h and 24 h compared to uninfected controls. Error bars indicate SE for n=5. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (**=p<0.01, ***=p<0.001)

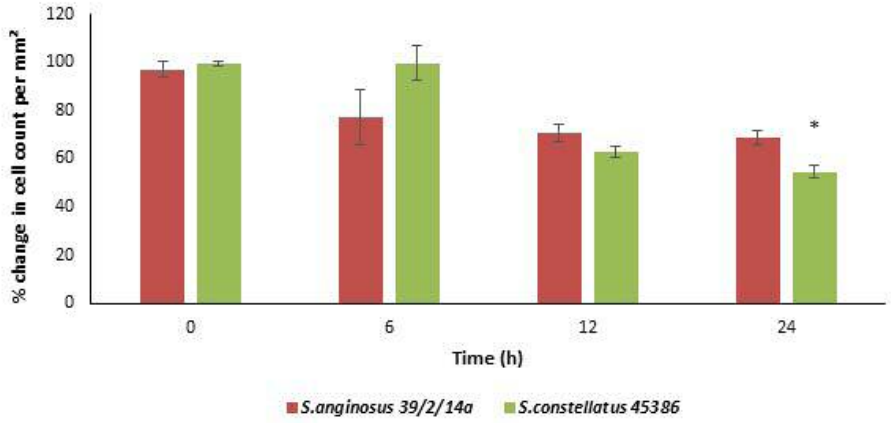
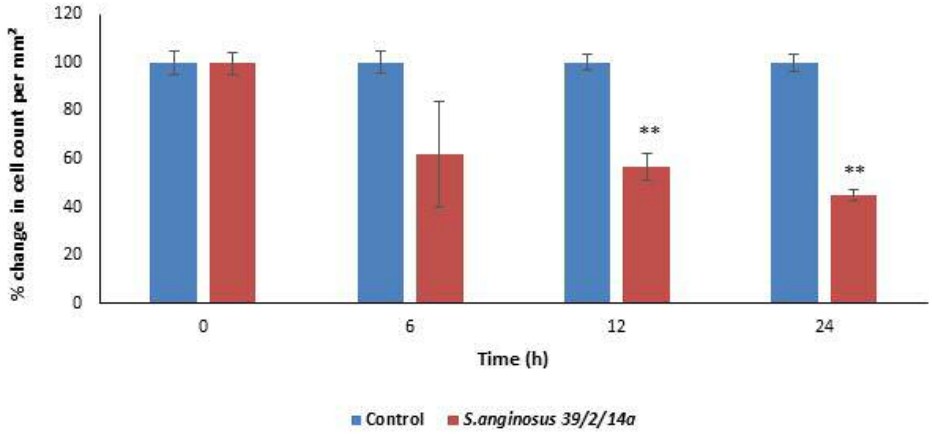
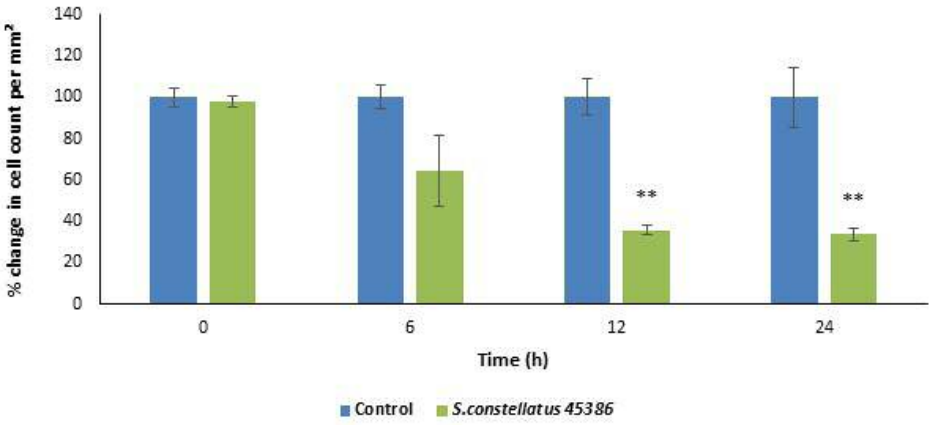


Figure 2.7: Comparison of the effect of the two SAG strains over 24 h on odontoblast counts. Odontoblast counts performed on transverse sections of tooth slices. Odontoblast counts were estimated per mm² of pulp. *S. constellatus* 45386 caused significant reduction in odontoblast counts compared to *S. anginosus* 39/2/14a at 24 h. Error bars indicate SE for n=5 (*=p<0.05).



(a)



(b)

Figure 2.8: Effect of SAG infection on the dental pulp stroma cell count over 24 h.
Dental pulp cell count performed on transverse sections of tooth slices estimated per mm² of pulp. There was a reduction in dental pulp stroma cell count when infected with SAG at 12 h and 24 h compared to uninfected controls. Error bars indicate SE for n=5. a) *S. anginosus* 39/2/14a, b) *S. constellatus* 45386 (**=p<0.01)

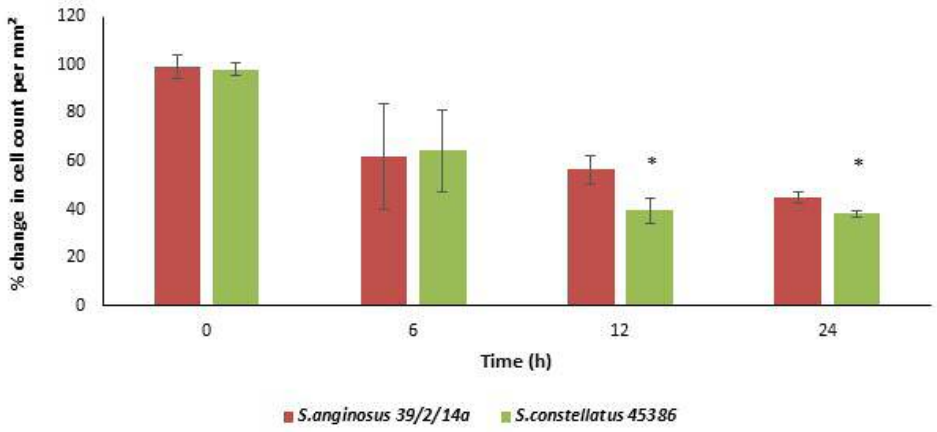


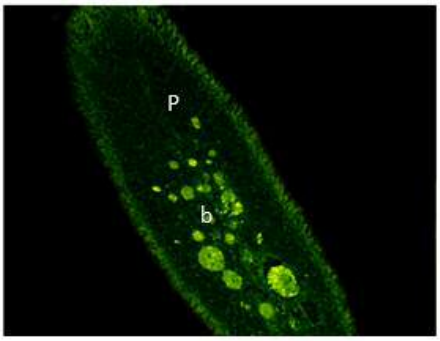
Figure 2.9: Comparison of the effect of the two SAG strains over 24 h on dental pulp stroma cells. Dental pulp stroma cell counts performed on transverse sections of tooth slices. *S. constellatus* 45386 caused a significant reduction in odontoblast count compared to *S. anginosus* 39/2/14a at 12h and 24 h. Dental pulp stroma cell counts were estimated per mm² of pulp. Error bars indicate SE for n=5. (*=$p<0.05$).

2.3.7 Quantification of area of colonisation of SAG in the tooth slice

FDA-stained bacteria were used to visualise pulp colonisation by SAG bacteria using a FITC filter. The sections were subsequently stained with H&E to re-visualise the same colonisation under light microscopy. The corresponding two images were taken as confirmation of the presence of bacterial colonies in the pulp tissue **Figure 2.10**. Once the presence of SAG was confirmed, the percentage area of colonisation of SAG compared to total area of the pulp was evaluated for both strains.

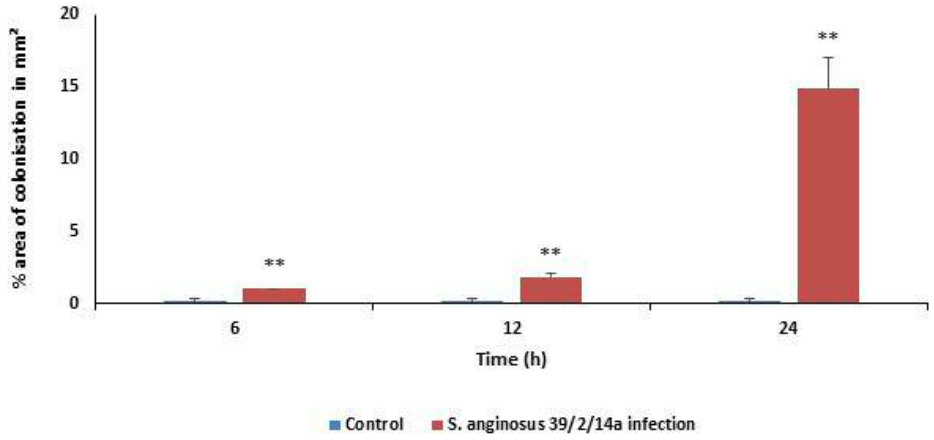
This was used to assess the ability of *S. anginosus* (Ref 39/2/14A) and *S. constellatus* (Ref 45386) to colonise the pulp. It will also be used elsewhere in this study to assess the ability of different treatment modalities to prevent bacterial colonisation. The percentage area of colonisation by *S. anginosus* (Ref 39/2/14A) increased over time compared to control tooth slices at 6 h ($p= 0.003$), 12 h ($p=0.005$) and 24 h ($p=0.002$) respectively. In *S. constellatus* (Ref 45386) infection the percentage area of colonisation also increased compared to controls and but only at 12 h ($p=0.004$) and 24 h ($p=0.017$); respectively, the observed increase was not significant at 6 h ($p=0.060$). *S. anginosus* (Ref 39/2/14A) showed colonisation at 6 h whilst significant colonisation was observed only at 12 h for *S. constellatus* (Ref 45386) infection **Figure 2.11a, b.**

(a)

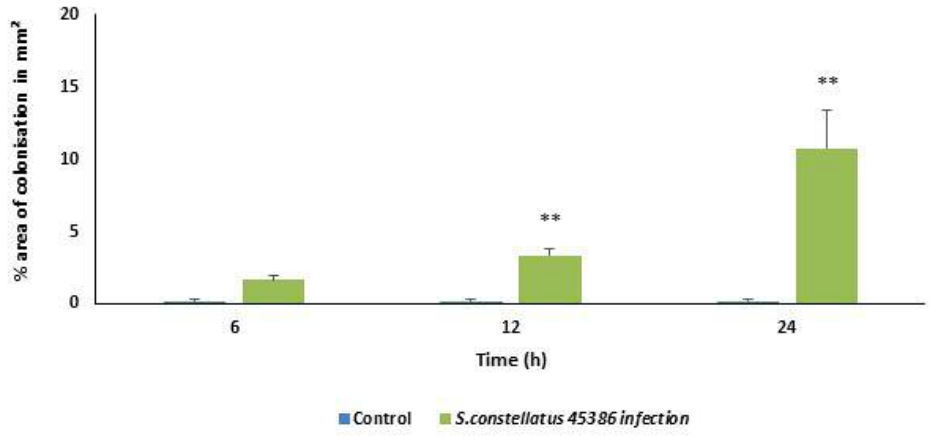


(b)

Figure 2.10: Tooth slices infected with FDA-stained SAG and corresponding H&E staining.
a) FDA-stained of cross-section at x20 magnification, b) H&E staining of the same cross-section at x20 magnification. o, odontoblast; p, pulp; b, FDA- and H&E- stained SAG bacteria.



(a)



(b)

Figure 2.11: Evaluation of the ability of SAG to colonise horizontal sections of pulp tissue over 24 h. The area of fluorescence from FDA-stained SAG measured under UV light relative to the total area of the pulp showed an increase in the area of SAG colonisation over time. Error bars indicate SE for n=5. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (Mann–Whitney U test; **=p<0.01).

2.3.8 Confirmation of expression of inflammatory markers

The expression of the housekeeping gene β-actin can be observed in **Figure 2.12b**. The agarose gel images (**Figure 2.12c, 2.13b, 2.14b, 2.15b &2.16b**) show the profile of the inflammatory markers expressed in the uninfected control dental pulp and *S. anginosus* 39/2/14A-infected dental pulp. The primers demonstrate a single band with the correct corresponding molecular weight. Both the uninfected and the SAG-infected pulp tissue demonstrate expression of the inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 and IL-10. No expression was seen in the negative control.

2.3.9 Relative expression of inflammatory markers by SAG infected pulp cells

In order to quantitatively evaluate the expression of the inflammatory markers, RT-qPCR was performed and the relative expression of the inflammatory markers by dental pulp cells with respect to the housekeeping gene β-actin was calculated. In the case of pro-inflammatory marker IL-1α, there was no significant difference in expression for SAG-infected dental pulp cells when compared to uninfected dental pulp cells as shown in **Tables 2.2 and 2.3** and **Figure 2.12a & 2.12d.** Pro-inflammatory markers IL-1β, TNF-α and IL-18 were up-regulated in a time dependent manner in *S. anginosus* (Ref 39/2/14A)-infected dental pulp cells compared to uninfected control cells and this difference was statistically significant **Table 2.3; Figures 2.13a, 2.14a & 2.15a**. In *S. constellatus* (Ref 45386) infection the difference was significant for the same markers only at 12 h and 15 h as shown in **Table 2.4** and **Figures 2.13c, 2.14c & 2.15c**. There was no expression of the anti-inflammatory marker IL-10 in *S. anginosus* (Ref 39/2/14A) or *S. constellatus* (Ref 45386)-infected dental pulp cells over 15 h and the same was true for the uninfected control dental pulp cells **Figure 2.16a,c**.

Table 2.3: Statistical difference in inflammatory marker expression between *S. anginosus* 39/2/14A-infected and uninfected pulp cells

p value for comparison of the level of expression of inflammatory marker by *S. anginosus* (Ref 39/2/14A) infected dental pulp cells compared to uninfected control dental pulp cells at 6 h, 12 h and 15 h using the Mann–Whitney U test.

Time (h)	**Inflammatory markers**				
	IL-1α	IL-1β	TNF-α	IL-18	IL-10
6h	p=0.391	p=0.005*	p=0.001*	p=0.032*	p=0.362
12h	p=0.554	p=0.002*	p=0.004*	p=0.028*	p=0.531
15h	p=0.493	p=0.001*	p=0.004*	p=0.005*	p=0.483

*Statistically significant

Table 2.4: Statistical difference in inflammatory marker expression between *S. constellatus* 45386 infected and uninfected pulp cells.

p value for comparison of the level of expression of inflammatory marker by *S. constellatus* 45386 infected dental pulp cells compared to uninfected control dental pulp cells at 6 h, 12 h and 15 h using the Mann–Whitney U test.

Time (h)	**Inflammatory markers**				
	IL-1α	IL-1β	TNF-α	IL-18	IL-10
6h	p=0.605	p=0.098	p=0.841	p=0.262	p=0.201
12h	p=0.845	p=0.002*	p=0.001*	p=0.002*	p=0.608
15h	p=0.674	p=0.013*	p=0.042*	p=0.003*	p=0.763

*Statistically significant

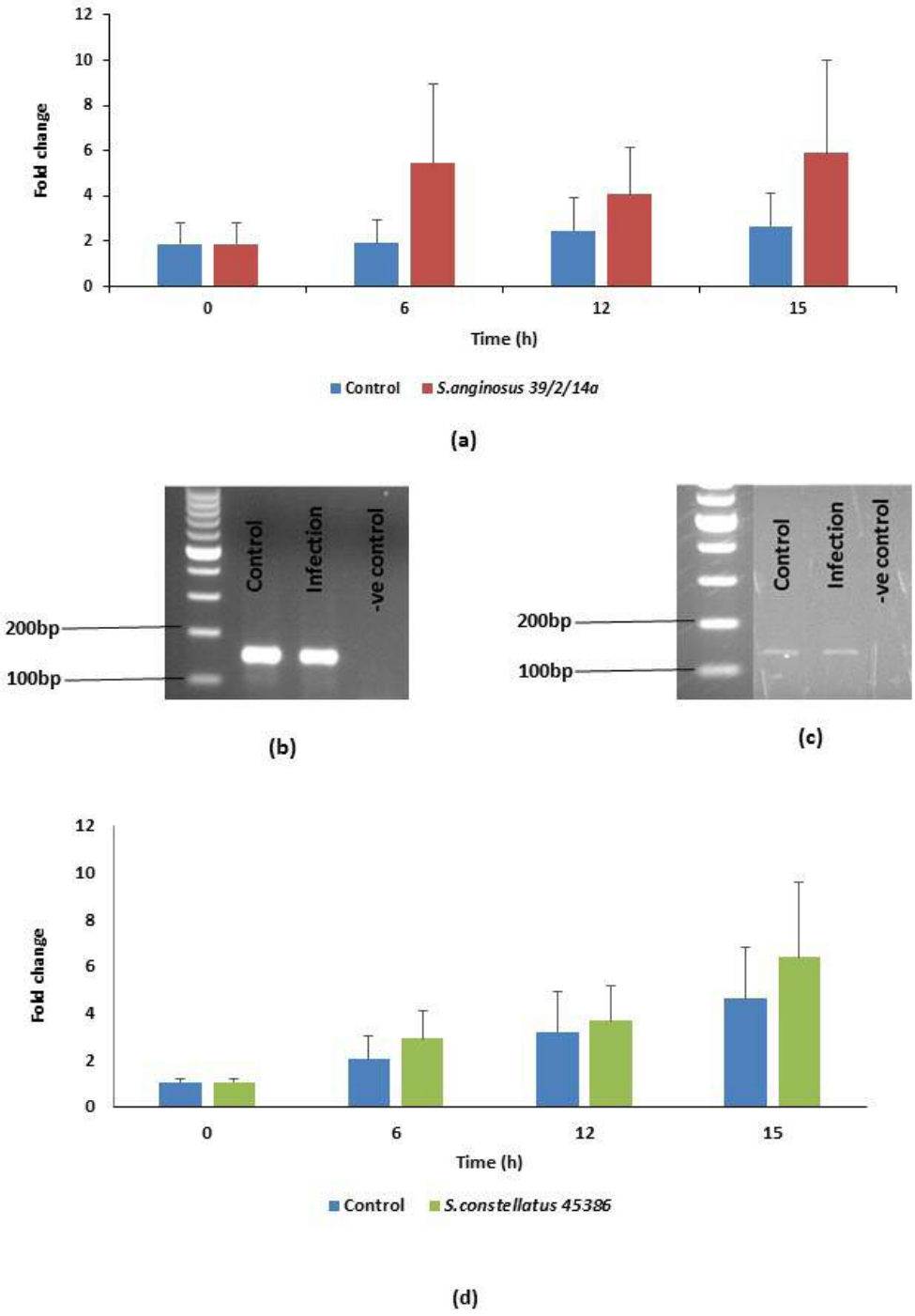


Figure 2.12: Effect of SAG infection on IL-1α expression in the dental pulp cells *in vitro.*
Expression of IL-1α in dental pulp cells infected with SAG bacteria compared with uninfected control relative to β-actin assessed by RT-qPCR over a period over 15 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiment conducted in triplicates. a) *S. anginosus* 39/2/14a; b) expression profile of β-actin, c) expression profile of IL-1α, d) *S. constellatus* 45386.

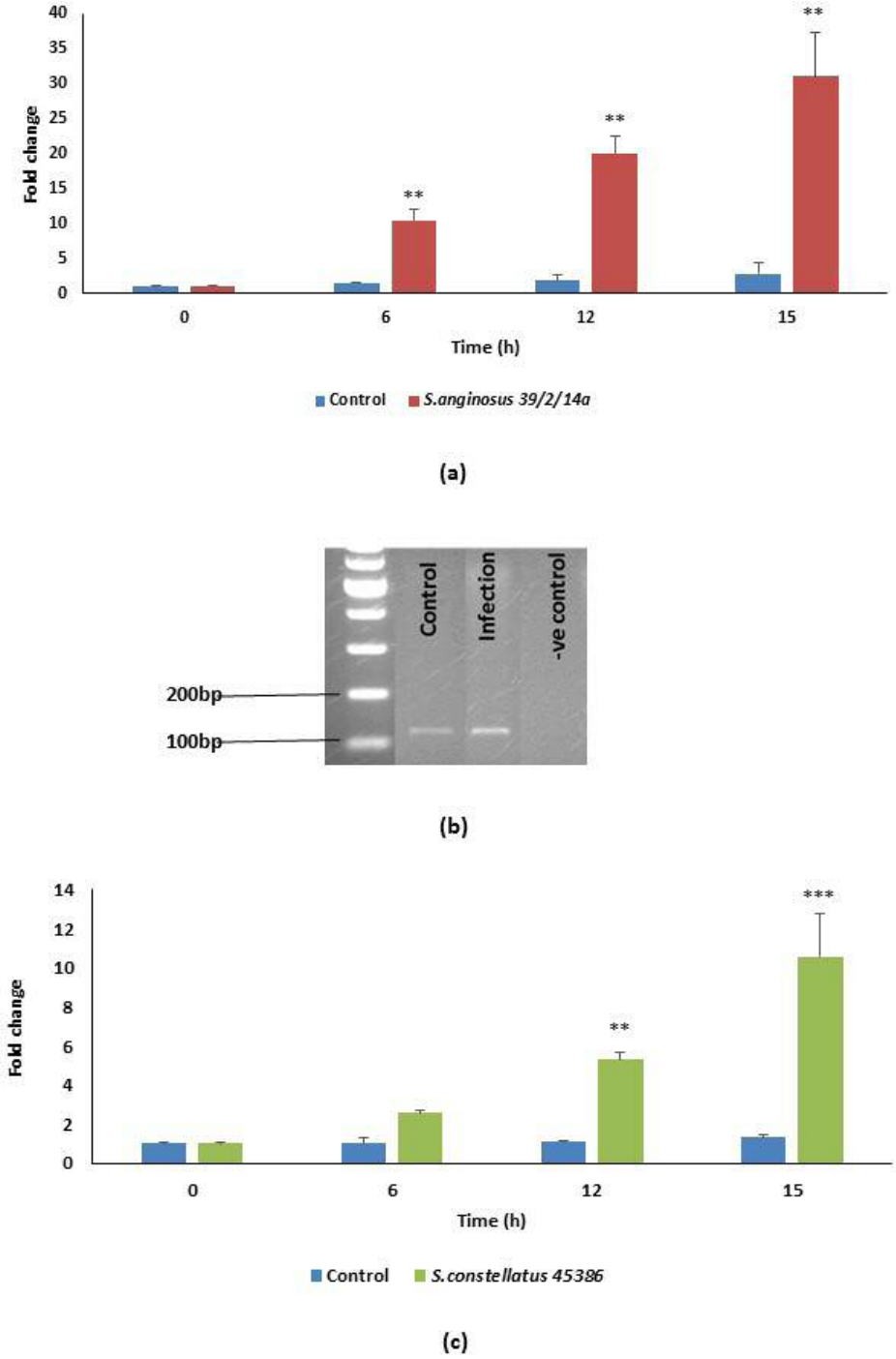


Figure 2.13: Effect of SAG infection on IL-1β expression in the dental pulp cells *in vitro.*
Expression of IL-1β in dental pulp cells infected with SAG bacteria compared with uninfected control relative to β-actin assed by RT-qPCR over a period over 15 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiment conducted in triplicates. a) *S. anginosus* 39/2/14a; b) expression profile of IL-1β; c) *S. constellatus* 45386 (**=p<0.01, ***=p<0.001).

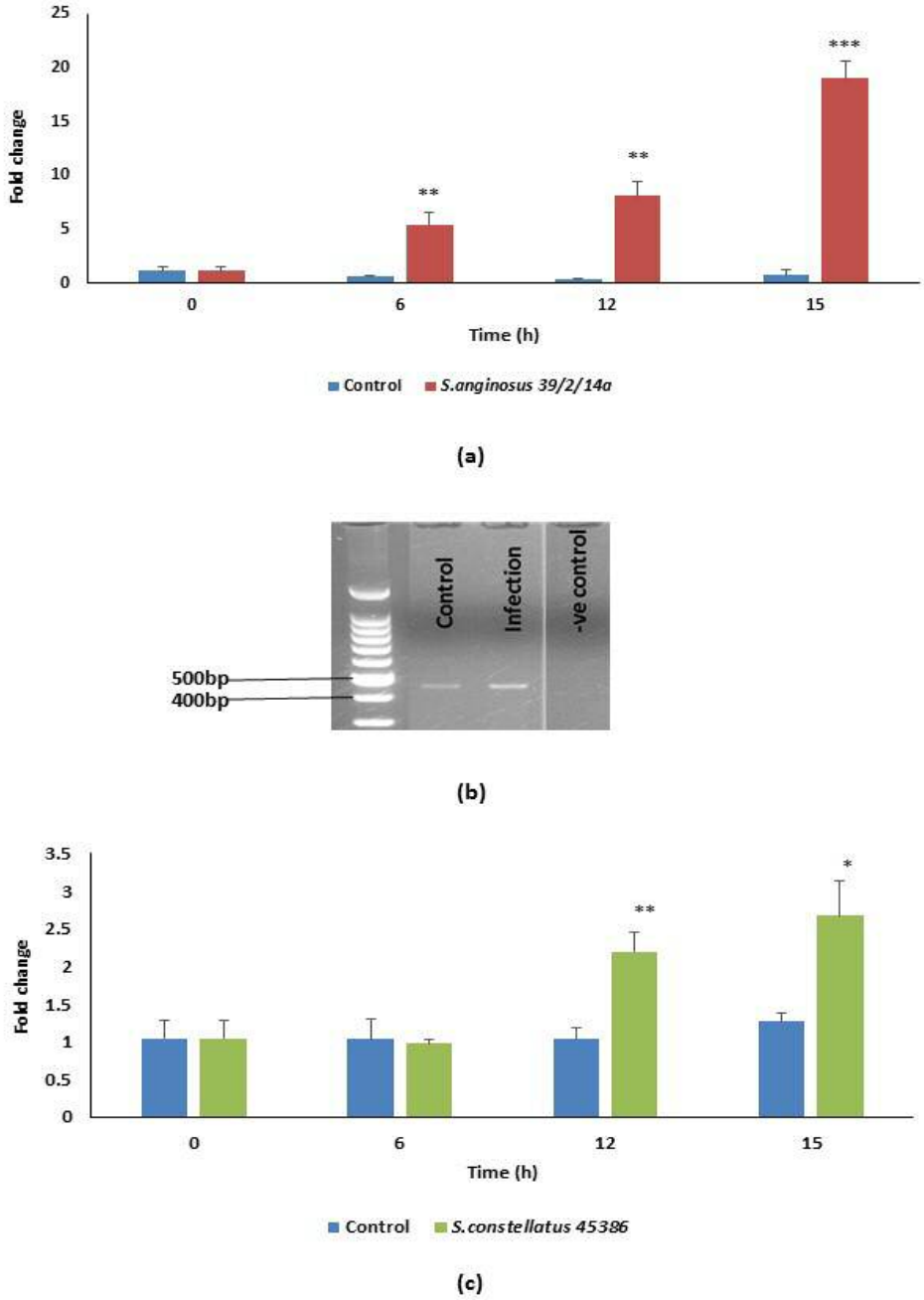
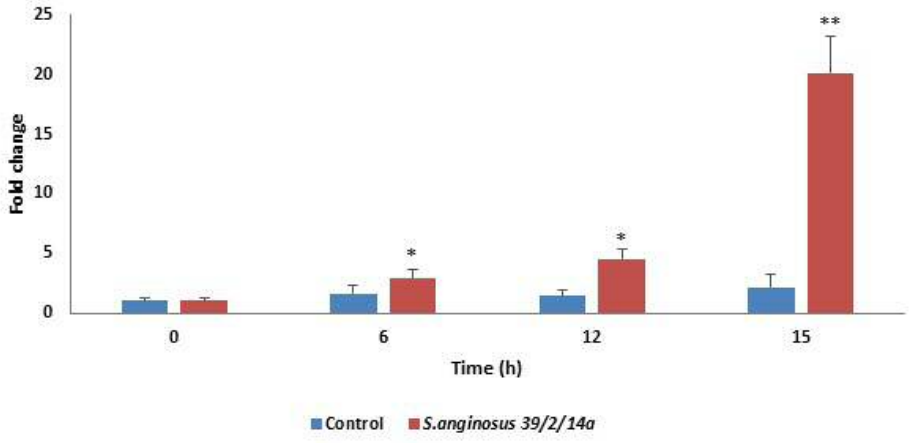
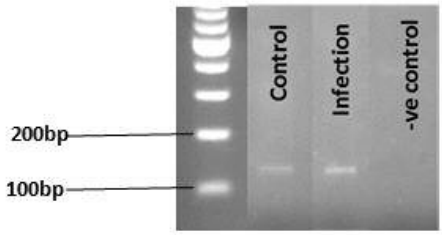


Figure 2.14: Effect of SAG infection on TNF-α expression in the dental pulp cells *in vitro.*
Expression of TNF-α in dental pulp cells infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 15 h. Each bar represents fold change compared to housekeeping gene β-actin. The error bars indicate SE for experiment conducted in triplicates. a) *S. anginosus* 39/2/14a; b) expression profile of TNF-α;, c) *S. constellatus* 45386 (*=p<0.05, **=p<0.01, ***=p<0.001).



(a)



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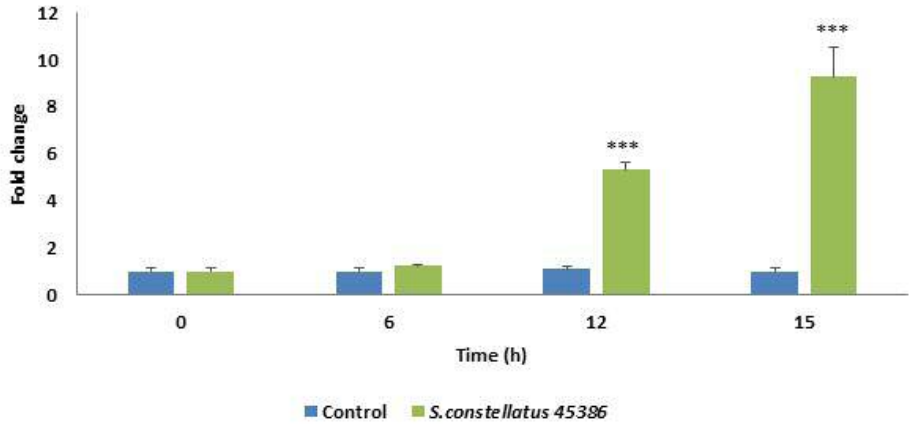


(c)

Figure 2.15: Effect of SAG infection on IL-18 expression in dental pulp cells *in vitro.*
Expression of IL-18 in dental pulp cells infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 15 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiment conducted in triplicates. a) *S. anginosus* 39/2/14a; b) expression profile of IL-18; c) *S. constellatus* 45386, (*=p<0.05, **=p<0.01, ***=p<0.001).

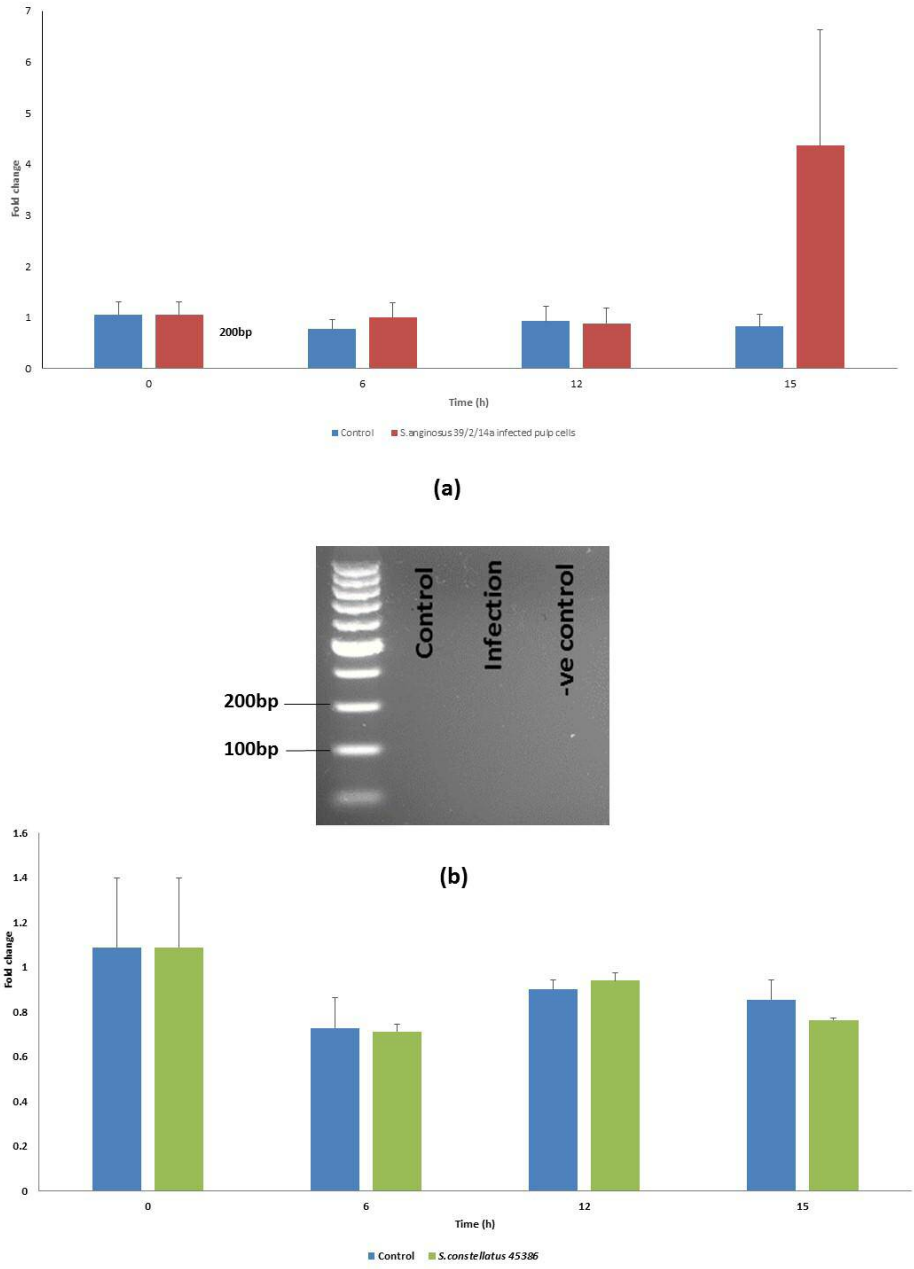


Figure 2.16: Effect of SAG infection on IL-10 expression in dental pulp cells *in vitro.*
Expression of IL-10 in dental pulp cells infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 15 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) expression profile of IL-10; c) *S. constellatus* 45386

2.3.10 Up-regulation of pro-inflammatory markers by SAG infection in pulp tissue

When pulp tissue was analysed by RT-qPCR for the expression of inflammatory markers, IL-1α expression did not show a statistically significant difference between the infected and control pulp tissue over 24 h, even though there was an increasing trend in the SAG-infected pulp **Tables 2.5 and 2.6**; **Figure 2.17**. However, the pro-inflammatory markers IL-1β and TNF-α were up-regulated by dental pulp tissue infected with SAG compared with uninfected pulp tissue at 12 h and 24 h **Tables 2.5 & 2.6**; **Figure 2.18 & 2.19**. IL-18 expression was first observed at 6 h in *S. constellatus* (Ref 45386) infected specimens compared to 12 h in *S. anginosus* (Ref 39/2/14a) **Figure 2.20**, and this increase in expression was statistically significant **Tables 2.5 & 2.6**. The SAG infection caused a time-dependent reduction in the expression of anti-inflammatory marker IL-10 compared to the control pulp tissue **Figure 2.21**.

Table 2.5: Statistical difference in inflammatory marker expression between *S. anginosus* 39/2/14A infected and uninfected pulp tissue

p value for comparison of the level of expression of inflammatory marker by *S. anginosus* 39/2/14A infected dental pulp cells compared to uninfected control dental pulp cells at 6 h, 12 h and 15 h using the Mann–Whitney U test.

Time (h)	**Inflammatory markers**				
	IL-1α	IL-1β	TNF-α	IL-10 ▲	IL-18
6 h	p=0.935	p=0.148	p=0.063	p=0.003*	p=0.371
12 h	p=0.863	p=0.044*	p=0.042*	p=0.003*	p=0.041*
24 h	p=0.774	p=0.016*	p=0.037*	p=0.002*	p=0.045*

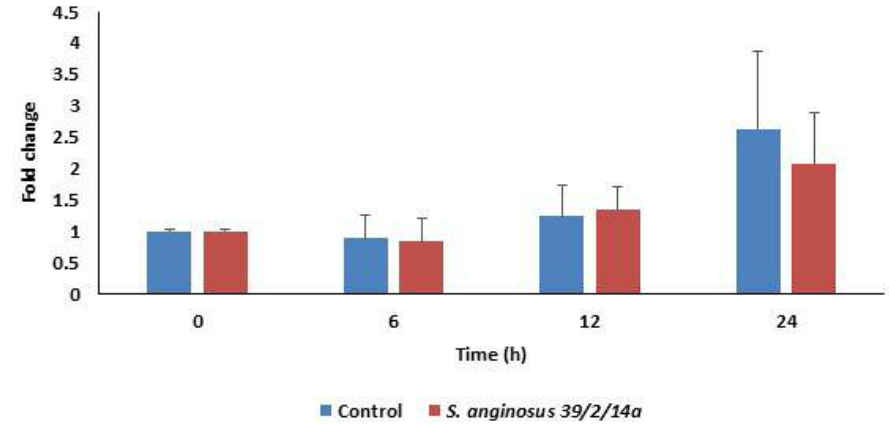
*Statistically significant ▲ decreased expression

Table 2.6: Statistical difference in inflammatory marker expression between *S. constellatus* 45386 infected and uninfected pulp tissue

p value for comparison of the level of expression of inflammatory marker by *S. constellatus* 45386 infected dental pulp cells compared to uninfected control dental pulp cells at 6 h, 12 h and 15 h using the Mann–Whitney U test.

Time (h)	**Inflammatory markers**				
	IL-1α	IL-1β	TNF-α	IL-10 ▲	IL-18
6h	p=0.178	p=0.182	p=0.353	p=0.048*	p=0.003*
12h	p=0.180	p=0.003*	p=0.045*	p=0.005*	p=0.041*
15h	p=0.092	p=0.001*	p=0.040*	p=0.043*	p=0.043*

*Statistically significant ▲ decreased expression



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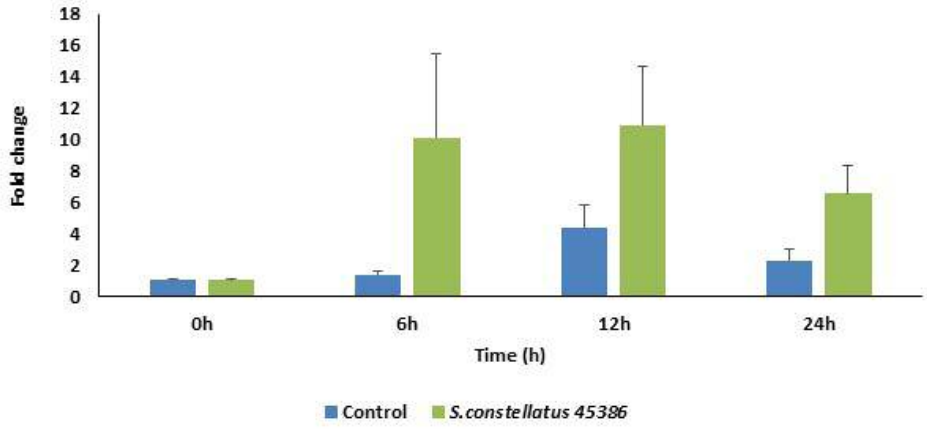
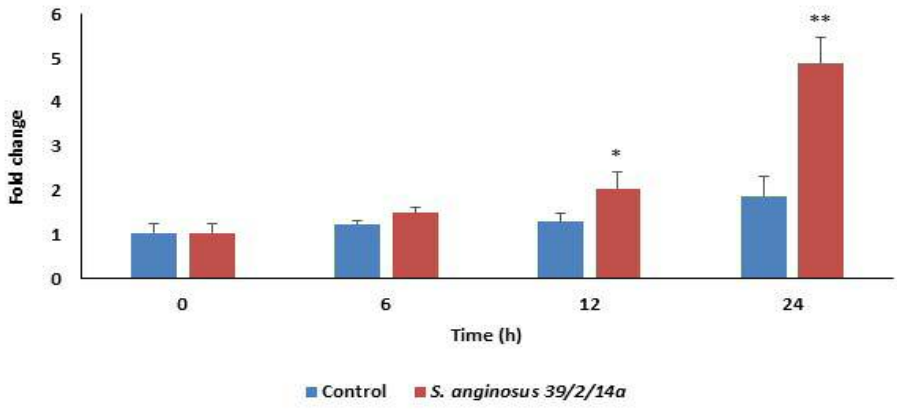


(b)

Figure 2.17: Effect of SAG infection on IL-1α expression in *ex vivo* dental pulp tissue.
Expression of IL-1α in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386.



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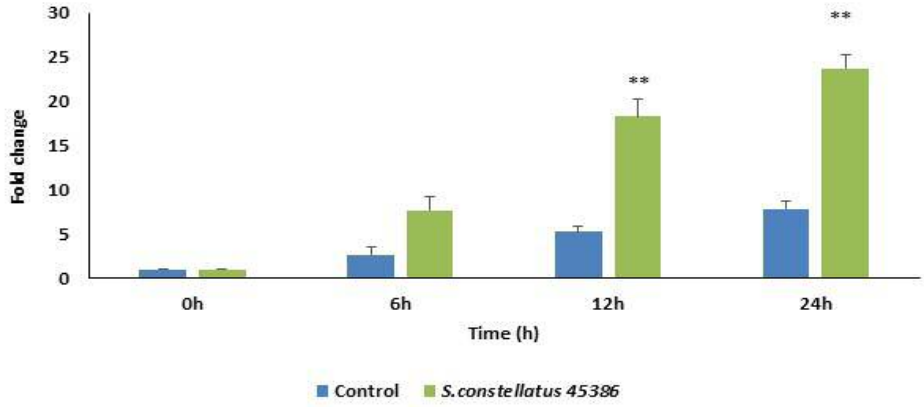
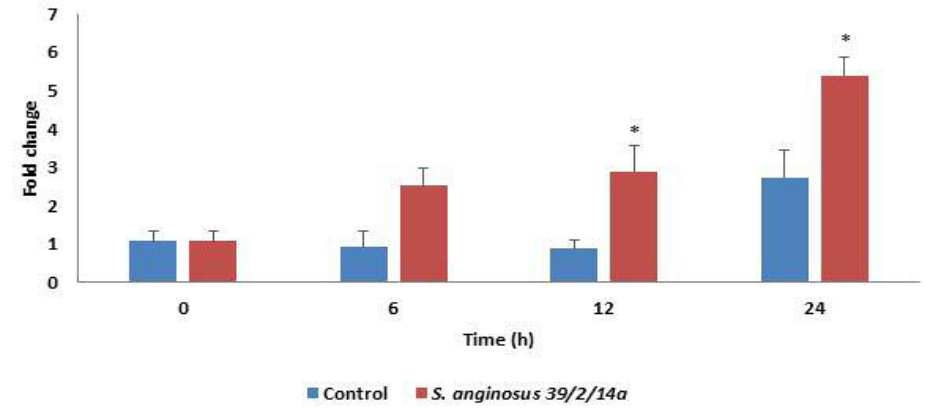


(b)

Figure 2.18: Effect of SAG infection on IL-1β expression in *ex vivo* dental pulp tissue.
Expression of IL-1β in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 24 hEach bar represents fold change compared to housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386(*=p<0.05, **=p<0.01).



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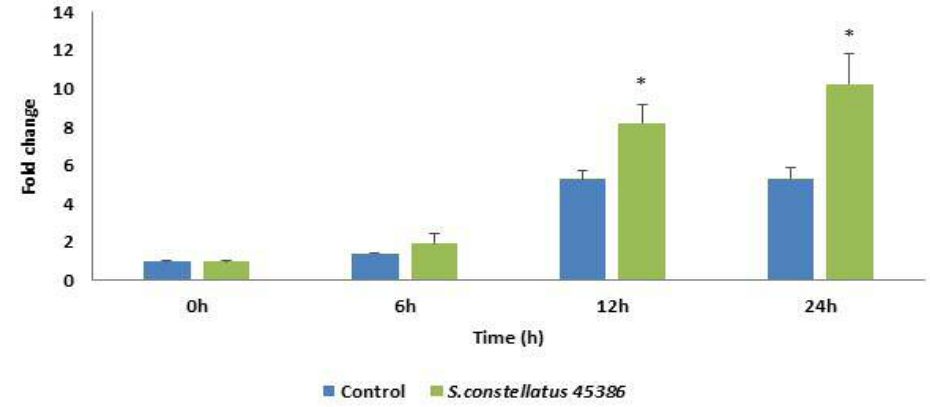
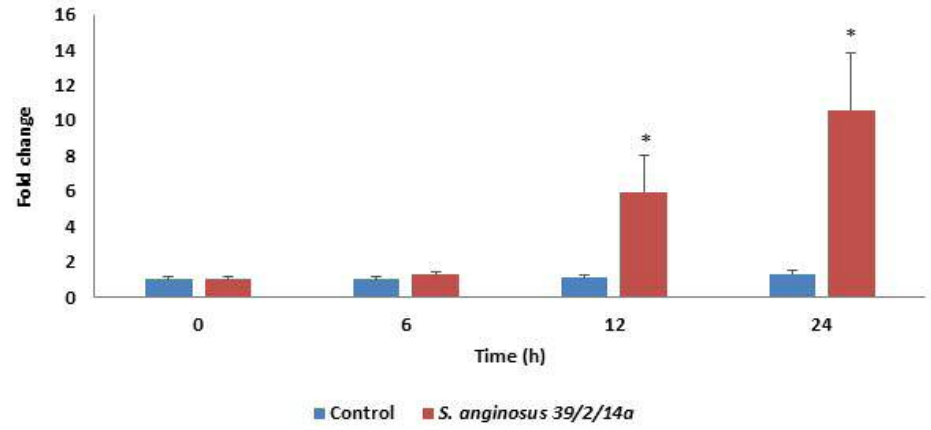


(b)

Figure 2.19: Effect of SAG infection on TNF-α expression in *ex vivo* dental pulp tissue.
Expression of TNF-α in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386(*=p<0.05, **=p<0.01).



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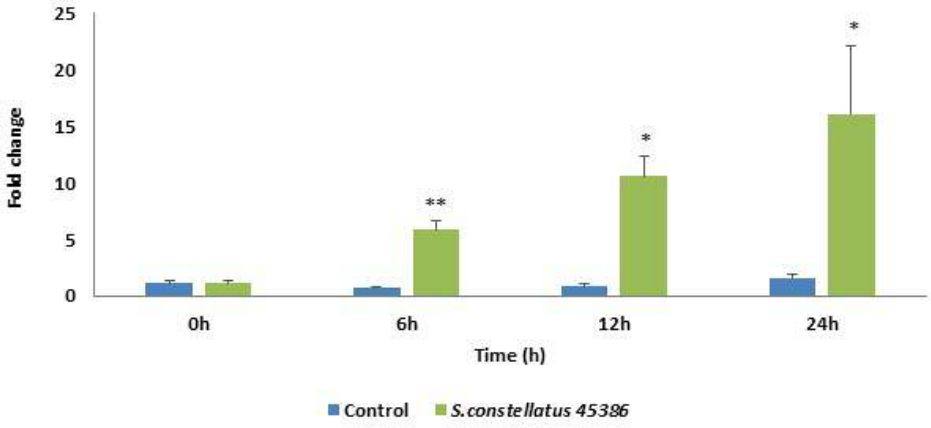
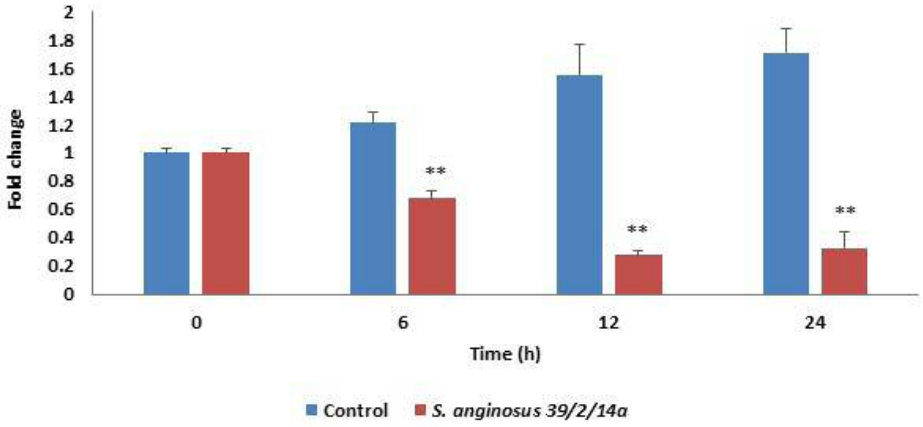


(b)

Figure 2.20: Effect of SAG infection on IL-18 expression in *ex vivo* dental pulp tissue.
Expression of IL-18 in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.01).



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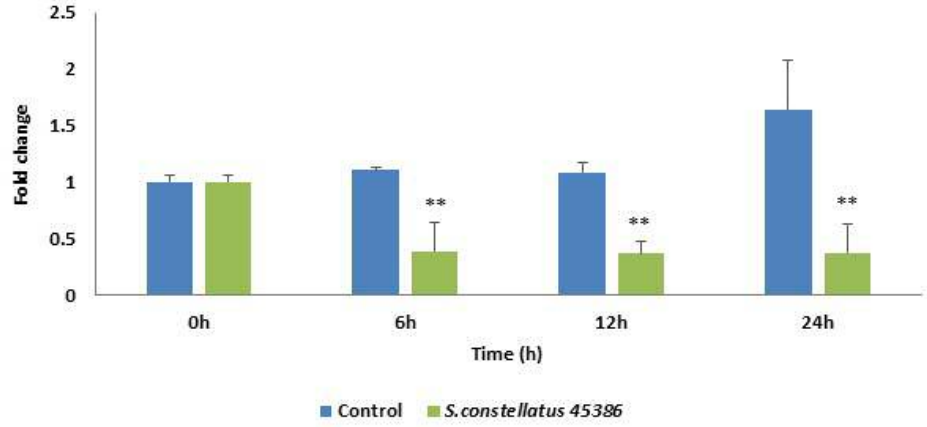


(b)

Figure 2.21: Effect of SAG infection on IL-10 expression in the *ex vivo* dental pulp tissue.
Expression of IL-10 in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b*) S. constellatus* 45386 (**=p<0.01).

2.4 Discussion

Through the evaluation of the growth kinetics of *S. anginosus* 39/2/14A and *S. constellatus* 45386 in BHI and MTCM, neither affected growth kinetics of SAG but growth in MTCM maintained a slightly prolonged exponential phase which could affect the way in which they infect and stimulate an inflammatory response in the pulp. It was interesting to note that *S. intermedius* (HW13) grew very slowly in BHI and no growth was observed in MTCM for the same period. This may be due to the difference in the nutritional requirements of *S. intermedius* (HW13) compared to *S. anginosus* 39/2/14A and *S. constellatus* 45386 and that of the pulp tissue. Therefore, it was unsuitable for further experiments and was excluded.

The *ex vivo* model histologically maintains the tissue architecture in the uninfected tooth slices as previously reported (Sloan *et al*. 1998). The odontoblasts were seen to be in contact with the dentine matrix which helped to maintain their phenotype and secretory activity (Heywood and Appleton 1984). The pulp tissue was intact and maintained the microenvironment of the cell in the pulp as compared to the other models which only maintained odontoblasts in contact with the dentine (Tjäderhane *et al*. 2001), thereby preventing any change of odontoblasts to fibroblast phenotype as observed in isolated odontoblast cultures (Sloan *et al*. 1998). Dentine matrix proteins influence reactionary dentinogenesis by stimulating existing odontoblasts and induction of a new generation of odontoblasts as demonstrated *in vivo* and *in vitro* (Smith *et al*. 1994; Smith *et al*. 1995), which is facilitated by the model as it maintains the intimate contact of odontoblasts with the dentine and the remainder of the pulp tissue. This contact with the whole pulp tissues may facilitate recruitment of dental pulp stem cells to differentiate into odontoblast like cells. The addition of SAG to this model will help assess the effect of infection on tertiary dentinogenesis by evaluating the tooth slices for expression of dentinogenic markers. Culturing of odontoblasts on dentine without the rest of the pulp tissue allowed evaluation of the odontoblasts in isolation and only for a short time span (Waddington *et al*. 1993), a limitation which is overcome by this model. In contrast, human tooth slices with the pulp were used to evaluate reparative dentinogenesis *in vitro* for up to one month (Magloire *et al*. 1996). As the SAG-infected tooth slice and control tooth slices can be obtained from the same tooth, it will be possible to make more comparable observations, specifically with observation of changes caused by the SAG infection being more robust.

The tooth slices infected with SAG showed areas of colonisation which were confirmed with FDA staining and this is supported by previous observations (Roberts *et al*. 2013). The area of colonisation of the pulp appeared to increase over time for both strains of SAG. This could be

due to co-aggregation to SAG already attached to the pulp. This was through the ability of streptococci to express adhesions that promote the attachment to other bacteria (Young *et al*. 1996; Jenkinson and Lamont 1997). Further SAG colonisation could also be facilitated by the expression of adhesins. Binding to extracellular matrix through adhesins is the most common pathological mechanism for streptococci (Allen and Hook 2002). Several adhesins expressed by streptococci which interact with host tissue through extracellular matrix proteins are collectively called Microbial Surface Components Recognising Adhesive Matrix Molecule bind to fibronectin, fibrinogen, collagen and laminin (Moschioni *et al*. 2010; Landrygan-Bakri *et al*. 2012; Asam and Spellerberg 2014). Some strains of SAG are known to produce hyaluronidase and chondroitinase which break down hyaluronic acid and chondroitin sulphate, components of extracellular matrix which may be a factor in promoting colonisation of the pulp by SAG (Whiley *et al*. 1990; Jacobs and Stobberingh 1995; Olson *et al*. 2013; Waddington *et al*. 2013; Sunwoo and Miller 2014). This is also supported by the presence of matrix breakdown observed in the histology of the *ex vivo* pulpal infection. Prolonged survival of the odontoblasts was observed in the *ex vivo* model compared to the *in vitro* dental pulp cell culture. This could be due to time needed for the SAG to produce these enzymes and for them to build up to a concentration sufficient to cause matrix break down, allowing bacteria to enter the tissue and eventually cause cell death an inflammatory response. In contrast, early cell death observed in the *in vitro* model may have been due to direct interaction of SAG with pulp cells due to the lack intercellular matrix. The previous model (Roberts *et al*. 2013), only confirmed the presence of the SAG in the pulp tissue, the model was further validated in this study by quantifying colonisation in terms of estimating the area of colonisation by SAG relative to the total area of the pulp tissue. This would help in quantitatively evaluating the ability of any antimicrobial agent in preventing pulp tissue colonisation when used in high-throughput experiments.

Matrix breakdown and bacterial invasion causes cell death of both odontoblasts and dental pulp cells. Both strains of SAG caused cell death of odontoblasts and dental pulp cells as shown in **section 2.3.6**. In contrast to a previous study using the same *ex vivo* pulp infection model (Roberts *et al*. 2013), *S. constellatus* 45386 caused greater loss of odontoblasts ($p<0.001$) and pulp cells ($p<0.01$) compared to *S. anginosus* 39/2/14a at 24 h. This observed difference could be due differences in the way the cell counts were assessed. The selection of certain areas of the pulp may not be a true representation of the whole pulp tissue as was done in the previous study (Roberts *et al*. 2013). The method of using digital image analysis to evaluate the entire area of the pulp (as done in this study), may have contributed to this difference in observation. The method used in this study is a more reliable and reproducible method and provides a true representation of the whole pulp rather than selecting areas of the pulp. Even though areas of

pulp were randomly selected previously (Roberts *et al*. 2013), unconscious bias may have influenced the data. Furthermore, the reduction became apparent for both cell types only at 12 and 24 h has also demonstrated by (Roberts *et al*. 2013). One of the reasons for the greater proportion of pulp cell death in *S. constellatus* 45386 compared to *S. anginosus* 39/2/14a could also be due to the difference in expression of virulence factors (Asam and Spellerberg 2014) between the two strains, which needs to be investigated further.

Evaluation of expression of various pro and anti-inflammatory markers in the dental pulp is necessary to understand the pulpal response to infection and to demonstrate a dynamic and responsive model system. Initial inflammation promotes reparative dentinogenesis through differentiation of progenitor cells (Sloan and Smith 2007; Goldberg *et al*. 2008; Paula-Silva *et al*. 2009). In contrast, prolonged chronic inflammation leads to tissue destruction and eventually pulpal necrosis (Bergenholtz 1977; Yamaguchi *et al*. 2004; Cooper *et al*. 2010; Cooper 2013). Therefore, modulating these responses could facilitate repair and regeneration of the pulp. IL-1α, IL-1β, TNF-α and IL-18 are common pro-inflammatory markers produced by the pulp in response to infection (Bletsa *et al*. 2006; Li *et al*. 2015; Olsen and Yilmaz 2016) IL-10, an anti-inflammatory marker which has a reciprocal relation with the pro-inflammatory markers, is also expressed by the pulp (Elsalhy *et al*. 2013; Yazid *et al*. 2014). As inflammatory mediators do not work in isolation, the balancing effect of pro- and anti-inflammatory markers is essential to maintain homeostasis (Elsalhy *et al*. 2013). Assessing the level of these markers will help evaluate the response the pulp produces when anti-inflammatory and anti-microbial agents are used for the treatment of pulpal infection.

Even though the expression of inflammatory markers was evaluated in the previous *ex vivo* model, it was only undertaken semi-quantitatively by measuring the intensity of the bands produced by the PCR products (Roberts *et al*. 2013). Furthermore, there was no comparison of *ex vivo* model response to *in vitro* infection of pulp cells. In order to improve the validity of the results, RT-qPCR was performed and the levels of expression were calculated relative to the housekeeping gene β-actin. In addition, the inflammatory response of *in vitro* dental pulp cells to SAG infection was also evaluated.

There were differences in the way the *in vitro* pulp cells responded to SAG infection compared to the response they produced in the *ex vivo* pulp infection model. The *in vitro* dental pulp cells infected with *S. anginosus* 39/2/14A produced a significantly increased expression of the pro-inflammatory markers IL-1β, TNF-α, IL-18 and no expression of anti-inflammatory marker IL-10 relative to uninfected control cells. There was no significant difference in the level of IL-1α

between infection and control even though there was a trend showing increase in expression by the infected cells. *S. constellatus* 45386 produced a similar response but the level of expression of the pro-inflammatory marker was less compared to that of *S. anginosus* 39/2/14a relative to uninfected cells. No expression of IL-10 in *S. constellatus* 45386 infection was noted. Furthermore, the increase in expression of inflammatory markers in *S. anginosus* 39/2/14A infected cells appeared at 6 h compared to 12 h in *S. constellatus* 45386 infected cells. This could be due to the slow and prolonged exponential phase of *S. constellatus* 45386 compared to *S. anginosus* 39/2/14a when grown in MTCM.

On the contrary, in the case of the *ex vivo* model, *S. constellatus* 45386 produced a higher regulation of the same pro-inflammatory markers compared to that produced by *S. anginosus* 39/2/14A, and compared to the uninfected tooth slices. The *in vitro* dental pulp cell culture when infected with SAG was viable only for 15 h but the tooth slice infection model survived up to 24 h. This prolonged survival time could be due to the organised tissue structure which requires a longer time for the bacteria to break down the tissue matrix in contrast to the *in vitro* culture where the cells are more vulnerable to bacterial attack.

This could also be the attributing factor for the delayed but significant up-regulation of IL-1β and TNF-α at 12 h in the pulp tissue when infected with SAG compared to uninfected pulp. No difference in the level of IL-1α was noted between the infected and control pulps. The down-regulation of IL-10 and up-regulation of IL-18 occurred as early as 6 h in the infected pulp. The expression of IL-10 in the uninfected pulp tissue increased over time but a down-regulation was observed in the SAG infected pulp over 24 h. *In vivo* stimulation of rat pulp with lipopolysaccharide has shown to increase the inflammatory and anti-inflammatory markers for 3 h and to reach baseline at 9 h (Renard *et al*. 2016). Other inflammatory markers such as IL-1β and TNF-α were also upregulated only for 3 h in the *in vivo* model. This difference could be due to the dynamic nature of the *in vivo,* model compared to the *ex vivo* model which lacks the influx of immune cells and systemic clearance of toxins. Furthermore, the continuous presence and growth of SAG in the *ex vivo* infection model could contribute to the upregulation of inflammatory markers, even at 24 h. This may be because the model is static and not dynamic due to the absence of continuous blood flow bringing in a constant supply of immune cells. The responses observed in the *ex vivo* model reproduced those observed in clinical pulp tissue samples and *in vivo* experiments (McLachlan *et al*. 2004; Bletsa *et al*. 2006; Li *et al*. 2015). Thus, the ability of the *ex vivo* model to mirror the response observed in *in vivo* infections makes it a potential model for evaluation of the efficacy of anti-inflammatories on pulpal infection. Furthermore, there are differences in the level of various inflammatory markers being expressed

by the pulp, the effect on cell viability and colonisation ability between the two strains of SAG. This may be due to the difference in the virulence factors produced by the two strains. A better understanding of the reasons for the differences in response produced by the different strains of SAG is needed in order to target specific virulence factors.

Chapter 3

3 Characterisation of pulpal response to *Streptococcus anginosus* group bacterial supernatant

3.1 Introduction

Inflammatory responses have been noted in the dental pulp to bacterial exotoxins and endotoxins even without evidence of bacterial invasion (Bergenholtz 1977). It could be inferred that bacteria do not actually need to colonise the pulp to produce a tissue response. There is substantial evidence for this. Non-cavitated enamel lesions with subjacent demineralised dentine have shown reductions in odontoblast numbers and alterations in sub-odontoblast layers due to an immune-inflammatory response by odontoblasts (Brannstrom and Lind 1965; Reeves and Stanley 1966; Hahn and Liewehr 2007b). Furthermore, non-cavitated active and slowly progressing caries show a reduction in the nuclear-cytoplasmic ratio of odontoblasts, with the sub-odontoblastic layer becoming indistinct in active lesions (Bjørndal *et al*. 1998).

Bacteria are not in contact with the dental pulp in the early stages of pulpitis caused by deep carious lesions. Rather, the toxins produced by the bacteria travel down the dentinal tubules, through the dentinal fluid and it is thought that this produces an inflammatory response in the pulp (Bergenholtz 1981; Hahn and Liewehr 2007a, b). Odontoblast-like cells in the pulp have been shown to express Toll-like receptors (TLRs) in response to bacterial toxins, which are capable of inducing inflammatory and immune responses in the pulp (Keller *et al*. 2010; Farges *et al*. 2011). There are a number of bacterial toxins which can provoke this inflammatory response.

Lipopolysaccharides (LPS), or endotoxin are cell wall components of gram-negative bacteria, and well documented initiators of inflammation, including pulpitis. LPS from *Porphyromonas gingivalis,* which can be seen in 48% of teeth with an endodontic infection, is shown to induce pulpitis (Siqueira *et al*. 2008). Lipoteichoic acid (LTA) and antigens from *Streptococcus mutans* (*S. mutans*) and *Enterococcus faecalis* (*E. faecalis*)*,* which are also common in endodontic infections, have been known to induce an immune-inflammatory response (Hahn *et al*. 2000; Kayaoglu and Ørstavik 2004; Baik *et al*. 2008; Hong *et al*. 2014). Enzymes such as hyaluronidase, collagenase, chondroitin sulphate and other components of bacteria including flagella,

peptidoglycans and nuclear material of bacteria have been implicated in tissue destruction through matrix degradation (Fujiyoshi *et al*. 2001; Akira *et al*. 2006).

The effect of these toxins on the pulp has been evaluated in the past on *in vitro* cell culture models using pulpal fibroblasts (Chmilewsky *et al*. 2015). To successfully regenerate the pulp, neutralising the adverse effects of toxins such as LPS and LTA is important and is one of the challenges that scientists and clinicians are facing (Yamagishi *et al*. 2011).

The *ex vivo* model has now been characterised and validated by the work in chapter 2 and, as SAG is found in deep carious lesions (Rocas *et al*. 2015), this model has the potential to be used to study the effect of SAG bacterial proteins on the pulp. It has been shown using the *ex vivo* model that SAG bacteria have the ability to colonise the pulp, causing cell death and provoking an inflammatory response. Once the bacteria establish themselves, both in deep carious lesions and in pulpitis, the bacterial community is protected from the host responses (Bjorndal and Mjor 2002) due to the structure of the dental hard tissue. The toxins therefore continue to leach out into the surrounding environment, causing further inflammatory responses and cell destruction in host tissue.

Mild irritations from these toxins in the early stages of pulpitis appear to stimulate reparative dentine formation. It has also been shown *in vitro* that LPS and LTA have the ability to recruit pulpal progenitor cells to facilitate regeneration (Chmilewsky *et al*. 2015). In order to study the immuno-inflammatory and regenerative response to the SAG proteins and simulate the *in vivo* situation, the *ex vivo* model was used in this study to apply known concentrations of bacterial proteins secreted by the SAG bacteria to evaluate the response.

It was also noted in chapter 2 that cell death and the inflammatory response was higher after exposure to *S. constellatus* 43386 compared to *S. anginosus* 39/2/14a. Furthermore, certain strains of SAG are more likely to cause infections than others and the virulence factors specific to the strain may be associated with the pathogenicity (Unsworth 1989; Jacobs and Stobberingh 1995).

Characterising the SAG bacterial supernatant will help identify its protein constituents. This will help in identifying the cause of the difference in cell death and inflammatory response between the two strains of SAG. This would then allow therapies to be developed to target specific putative virulence factors and thereby limit cell death and modulate the inflammatory response towards repair and regeneration.

Thus, the objectives of this chapter is to build on work described in the previous chapters by:

- Quantifying the protein concentration of the SAG bacterial supernatants using the BCA assay.
- Evaluating the effect of different concentrations of *S. anginosus* 39/2/14a and *S. constellatus* 45386 bacterial proteins over 24 h on the pulp using the *ex vivo* model by assessing viable cell counts of odontoblasts and dental pulp cells.
- Selecting a known concentration of SAG bacterial protein and measuring its effect on the histomorphometric changes of the pulp over 12 h and 24 h.
- Comparing the differences in immuno-inflammatory response of the dental pulp to a known concentration of *S. anginosus* 39/2/14a and *S. constellatus* 45386 bacterial proteins using the *ex vivo* model.
- Confirming the effect of SAG proteins on cell viability and immuno-inflammatory response by stimulating the pulp with heat-inactivated SAG proteins.
- SAG bacterial supernatant proteins will be separated using SDS-PAGE assay and identified using proteomics in order to target them.
- Identified protein/enzyme will be inactivated by heat treatment and applied to the pulp tissue and the effect of the protein or enzyme on the pulp tissue will be confirmed.

3.2 Materials and methods

3.2.1 Preparation of bacterial supernatant

Preparation of bacterial culture

SAG bacteria were cultured overnight in BHI (**section 2.2.1.1.2)** and 100 µL of this freshly prepared planktonic suspension was inoculated into 20 mL of fresh BHI and incubated in a 5% CO_2 cabinet at 37^{o}C for 6 h to reach its exponential growth phase. The planktonic suspension was then spun down at 2600 x g for 10 min, the supernatant discarded and the pellet re-suspended in 3 mL of BHI. This suspension was diluted to produce a suspension of *S. anginosus* 39/2/14A and *S. constellatus* 45386 containing 10^5 cfu/mL.

Preparation of bacterial culture for harvesting supernatant

Twelve aliquots of 250 mL BHI broth were prepared in tissue culture flasks. Six of the flasks were inoculated with 100 µL of a 10^5 cfu/mL suspension of *S. anginosus* 39/2/14A, each prepared as above. Another six flasks were inoculated with 100 µL of 10^5 cfu/mL suspension of *S. constellatus* 45386. They were then incubated at 37^{o}C in a 5% CO_2 cabinet. One flask of the six was harvested at 4, 6, 8, 12, 16 and 24 h for each strain.

Preparation of supernatant

The supernatants were prepared by two methods

1. Centrifugation
2. Filtration

250 mL of bacterial suspension prepared above was then divided into two parts of 125 mL each and one half was subjected to filtration and the other half was subjected to centrifugation.

Centrifugation

125 mL of bacterial suspension from each time point mentioned above was centrifuged at 2600 x g for 10 min using an IEC CLIO centrifuge (Thermo Electron Corporation, Rugby, UK) and the supernatant was collected and centrifuged again at 9000 x g for 10 min in a Heraeus IICO 17 centrifuge (Thermo Scientific, Loughborough, UK) and the supernatant collected and frozen at -20°C in 1 mL aliquots.

Filtration

The bacterial suspensions from each of the time points were filtered using a 0.2 µm Millipore filter (Elkay Laboratory Products Ltd., Basingstoke, UK) and the filtrate was frozen at -20°C in 1mL aliquots. The control for the supernatants was sterile BHI.

3.2.2 Quantification of the proteins in the SAG bacterial supernatant

Bicinchoninic acid assay (BCA assay)

The concentration of proteins in the supernatant and control BHI was quantified in order to standardise the amount of SAG supernatant protein used to stimulate the tooth sections and evaluate the response. The protein assay was undertaken using the Bicinchoninic Acid Protein Assay Kit (Sigma-Aldrich, UK).

The Protein assay is performed in a 96-well plate and a standard curve with BSA (1 mg/mL) was used. The BSA standard used had concentrations ranging from 1 mg/mL to 0.015 mg/mL. The standards were set up in triplicate on the plate and the samples in duplicate. 25 µL of SAG bacterial supernatant *(S. anginosus* 39/2/14A and *S. constellatus* 45386) collected at 4, 8, 12, 16, 20 and 24 h time points were added to the plate in duplicate. 25 µL of BHI was also added to the plate. 200 µL of BCA regent was added to all the standard and sample wells.

BCA working reagent was prepared by mixing 50 parts of sodium bicinchoninate solution (reagent A) with 1 part of cupric sulphate solution (reagent B). 200 µL of diluted BCA working reagent was added to each well. The plate was covered with a plastic seal and incubated for 30 min at 37°C. The absorbance at 570 nm was measured on a Microplate™ reader (BioTek Instruments Ltd., Swindon, UK). A standard curve was obtained by plotting the concentrations of BSA standard against absorbance at 570 nm. A line of best fit was created and the equation for the best-fit line was generated. Taking the average absorbance of the samples in duplicate and applying it to the equation for the line of best fit of the standard curve calculated the protein concentration of samples.

3.2.3 Stimulation of tooth sections with bacterial supernatants

Maxillary and mandibular incisors from 28-day male Wistar rats were extracted and cut into 2 mm thick sections and cultured as described **section 2.2.4**. Tooth slices were washed overnight

with antibiotic-free DMEM with no BHI to remove any residual antibiotics. MTCM containing 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL of 24 h filtered bacterial supernatant protein was prepared using *S. anginosus* 39/2/14A proteins and *S. constellatus* 45386 proteins. This MTCM, containing the different concentration of bacterial supernatant proteins, was transferred to a 12-well plate with each well containing 2 mL of MTCM with bacterial proteins. Three tooth sections were inoculated into media containing *S. anginosus* 39/2/14A proteins for each concentration of bacterial supernatant protein. Similarly, tooth slices were inoculated in the well containing MTCM with *S. constellatus* 45386 supernatant proteins. The control tooth slices were placed in MTCM only and were incubated for 24 h in a CO_2 incubator at 37°C. At the end of 24 h incubation the specimens were harvested, fixed in formalin and processed as outlined in **section 2.2.6**. The viable odontoblasts and fibroblasts were counted as outlined in **section 2.2.7** and compared to evaluate the effect of the different concentrations of the bacterial proteins and against the control.

3.2.4 Stimulation of the tooth sections with heat-inactivated bacterial supernatants

The heat-inactivated supernatant was prepared by heating 80°C for 20 min on a heating block. Upper and lower incisors from 28-day male Wistar rats were extracted and cut into 2 mm thick sections and cultured as previously described in **section 2.2.4**. Tooth slices were washed over night with antibiotic-free DMEM with no BHI to remove any residual antibiotics. MTCM containing 100 µg/mL heat-inactivated, 24 h filtered bacterial supernatant protein, was prepared using *S. anginosus* 39/2/14A and *S. constellatus* 45386. Three tooth slices were stimulated with *S. anginosus* 39/2/14A heat-inactivated proteins and three tooth slices were stimulated with *S. constellatus* 45386 heat-inactivated proteins and incubated for 24 h in a 5% CO_2 incubator at 37°C. The control tooth slices were incubated in MTCM.

At the end of 24 h incubation the specimens were harvested and fixed in formalin and processed as previously described **section 2.2.6**. The viable odontoblasts and fibroblasts were counted as described in **section 2.2.7** and compared to evaluate the effect of the heat-inactivated SAG supernatant proteins and against the control.

3.2.5 Quantification of inflammatory marker expression by quantitative polymerase chain reaction

Tooth slice stimulation with SAG denatured supernatant protein

Tooth slices were cultured as described in **section 2.2.4** and stimulated with 100 µg/mL of 24 h heat-inactivated SAG supernatant proteins in MTCM and incubated in a 5% CO_2 incubator at 37°C for 24 h. Control tooth slices were incubated in MTCM. Pulp tissue was harvested at 24 h and RNA was extracted as described in **section 2.2.10.2.1**. RNA was then used to quantify the relative expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-10 and IL-18 as described in **section 2.2.10.2.2**.

Tooth slices stimulated with SAG supernatant

Tooth slices 4 mm in length were prepared as outlined previously **section 2.2.4** and these were stimulated with 100 µg/mL of *S. anginosus* 39/2/14A and *S. constellatus* 45386 24 h bacterial supernatant proteins in MTCM and incubated in a 5% CO_2 incubator at 37°C for 24 h. Tooth slices were harvested at 0, 6, 12 and 24 h and RNA was extracted as described previously **section 2.2.10.2.1**. RNA was then used to quantify the relative expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-10 and IL-18 as described in **section 2.2.10.2.2**.

3.2.6 Separation of proteins in the SAG supernatant

3.2.6.1 SDS-PAGE

SDS-PAGE was performed using the NuPAGE Novex Bis-Tris Mini Gels (Life Technologies Ltd-Invitrogen, Paisley, UK). The protocol for the Bis-Tris Gel provided by the manufacturer was followed. The precast NuPAGE Bis-Tris Mini Gels with a gradient of 4-12% were used to separate different proteins of the supernatant and to identify changes in the proteins of the supernatants collected at different time points. Thirteen µL of the supernatant was mixed with 5 µL of NuPAGE LDS sample buffer 2 µL of NuPAGE reducing agent and heated at 70^{o}C on a heating block for 10 min. The Bis-Tris Mini Gel was loaded into the XCell Sure Lock mini-cell. The anode and cathode chambers were filled with running buffer. The outer chamber was filled with 600 mL 1X NuPAGE SDS running Buffer. The inner chamber was filled with 200 mL 1X NuPAGE SDS running buffer containing 500µL of NuPAGE antioxidant. Ten µL of the prepared sample was

then loaded onto the gel along with sterile BHI as control and standard molecular weight ladders. The gel was run at 200 volts for 50 min.

3.2.6.2 Staining gel to visualise protein bands

3.2.6.2.1 Silver staining

The SilverQuest™ Silver Staining Kit (Invitrogen Loughborough, UK) was used to stain the SDS-PAGE gel to visualise the protein in the SAG supernatant. The gel was first fixed for 20 min using the fixing solution made up of 40 mL ethanol and 10 mL acetic acid in 100 mL of water. Then the gel was washed for 10 min in wash solution made of 30 mL of ethanol and 100 mL of water. This was followed by sensitisation of the gel for 10min with sensitiser containing 30 mL ethanol and 10 mL sensitiser in 100 mL of water. The gel was then washed with 100 mL of wash solution made up of 30 mL of ethanol in 100 mL water, the second wash was for 10 min with 100 mL of water. The gel was then stained for 15 min with 100 mL of staining solution containing 1mL of stainer in 100 mL of water. This was followed by a wash for 1 min in 100 mL of water. Then the gel was developed for 4-8 min in 100 mL of developer made up of 10 mL of developer, and 1 drop of enhancer in 100 mL of water followed by retardation of the developer by adding 10 mL of stop solution to the developing solution and leaving the gel in it for 10min. Finally, the gel was washed with 100 mL of water for 10 min.

3.2.6.2.2 Colloidal Coomassie blue staining

SDS-PAGE was performed as in **section 3.2.6.1** and the gel was subjected to Colloidal Coomassie Blue Staining using the staining kit (Invitrogen). The process involved fixing the gel followed by staining. The fixing solution was made up by adding 50 mL of 100% methanol and 10 mL of 100% acetic acid to 40 mL of deionised water. Staining solution was made by adding 20 mL of methanol and 20 mL of stain A from the kit to 55 mL of deionised water. The gel was first put in the fixing solution and left on a shaker for 10 min at room temperature. The fixing solution was then replaced by the staining solution and allowed to shake for 10 min at room temperature. Five mL of stain B from the staining kit was then added to the staining solution and allowed to shake for 6 h. The staining solution was replaced by deionised water and left on the shaker for 7 h to remove any background staining and make the visibility of the bands clearer. The gel was then stored in 1% acetic acid until the bands were excised for mass spectrometry (MS) analysis to identify the protein in the bands of interest.

3.2.6.2.3 Concentrating the supernatant

As the bands on the gel were not very intense, the supernatant was concentrated using the Microcon centrifugal filter device from (Millipore Corporation, Billerica, USA). Filters were first washed with water by spinning at 9000 x g for 10 min then residual water in the filter was discarded and 500 µL supernatant sample was added and spun at 9000 x g for 30 min. The filter containing concentrated supernatant was turned upside down into fresh 1.5 mL centrifuge tubes and spun at 1000 x g for 3 min to yield 50 µL of concentrated supernatant. This filtrate was 12 times more concentrated than the original supernatant. Filtrates, obtained as above, were pooled and then concentrated again to give with 50 µL of supernatant, which is roughly 45 times more concentrated that the original sample. BHI was used as a control and was subjected to the same concentration process as the supernatants.

3.2.6.2.4 Gel visualisation

The gels were visualised using the Gel Doc ^{TM} scanner (Bio-Rad, Hemel Hempstead, UK) by white light. The image was captured using Quantity One image analysis software (Bio-Rad) and band intensity was measured using Pro-plus version 6.0.0.260 image analysis software (Media Cybernetics Inc. Bethesda, MD). Gels were scanned using the SOP Scanning one- or two-dimensional electrophoresis gels using the Image Scanner III (CBS/SOP/PPF/EQ/10VERSION 1).

3.2.7 Identification of the protein constituents of the SAG bacterial supernatant

3.2.7.1 Mass spectrometry (MS)

Gels that were stored in 1% acetic acid after staining with colloidal Coomassie blue were taken and gel plugs (1.5 mm diameter) of the protein bands of interest were manually excised and,placed in a 96-well plate. Peptides of the proteins in the gel plug were recovered by trypsin digestion using a slightly modified version of a previously described method (Shevchenko *et al*. 2006). Sequencing-grade modified trypsin (Promega) was used at 6.25 ng/µL in 25 mM NH_4HCO_3 and incubated at 37°C for 3 h. Finally, the dried peptides were re-suspended in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA; 5 µL) for MS analysis and an aliquot corresponding to 10% of the material (0.5 µL) was spotted onto a 384-well MS plate. Samples were allowed to dry and then overlaid with α-cyano-4-hydroxycinnamic acid (CHCA; Sigma, Dorset, UK); 0.5 µL prepared by mixing 5 mg matrix with 1 mL of 50% (v/v) acetonitrile in 0.1% (v/v) TFA).

MS was performed using a MALDI TOF/TOF mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyzer; Foster City, CA, USA) with a 200Hz solid-state laser operating at a wavelength of 355nm (Medzihradszky *et al*. 2000; Bienvenut *et al*. 2002; Brennan and Jefferies 2009). MALDI mass spectra and subsequent MS/Ms spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolutions of each other were excluded from the selection and the peaks were analysed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/Ms positive ion mode 4000 spectra were averaged with 1kV collision energy (collision gas was air at a pressure of 1.6 x 10^{-6} Torr) and default calibration.

Combined PMF and MS/MS queries were performed using the MASCOT database search engine v2.1 (Matrix Science Ltd, London, UK) (Perkins *et al*. 1999) embedded into Global Proteome Server (GPS) Explorer software v3.6 (Applied Biosystems) on the Swiss-Prot database or the TrEMBL database. Searches were restricted to *S. anginosus* and *S. constellatus* with trypsin specificity (one missed cleavage allowed), the tolerance set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/Ms. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identification confirmed if there was high quality tandem MS (good y-ion) data for ≥2 peptides (E value $p< 0.05$ for each peptide; overall $p< 0.0025$ or one peptide (only if E value was $p< 0.0001$).

3.2.8 Identification of hyaluronidase activity of SAG bacterial supernatant

Smith and Willett's method (Smith and Willett 1968) was used to evaluate the production of hyaluronidase by SAG. One hundred mL of BHI was prepared with 1.5 g of agar, autoclaved at 121°C for 15 min and cooled to 46°C. Six mg of hyaluronic acid sodium salt from bovine vitreous humor (Sigma) and 100 mg of 5% bovine albumin fraction V (Sigma) was dissolved in BHI by vortexing and filter sterilised using a 0.20 µm filter. This was then added to 10 mL of BHI with agar prepared above and mixed quickly to yield a final concentration of 400 µg/mL hyaluronic acid and 1% of bovine albumin fraction. This was poured onto a plate and allowed to solidify. Ten µL of hyaluronidase from *S. pyogenes* (Sigma), 10 µL of 10^5 CFU/mL *S. anginosus* 39/2/14a, 10 µL of 10^5 CFU/mL *S. constellatus* 45386, 10 µL of heat-inactivated *S. constellatus* 45386 bacterial supernatant was placed on the plate and incubated for 24 hours at 37°C, 5% CO_2. The plate was then flooded with 2N acetic acid for 10 min which binds hyaluronic acid and albumin

and forms a white precipitate. Hyaluronidase production was considered to have been present if a zone of clearing was observed.

3.2.9 Statistical analysis

Statistical analysis was performed on cell counts from histological staining of tooth slices using Graph Pad statistical software. Cell counts were performed as previously described in **section 2.2.7**. The Mann–Whitney U test was performed to determine the relative significance of the difference between experimental cell counts and inflammatory marker expression to that of the controls. This allowed comparison of the mean cell counts and inflammatory marker expression between tooth slices exposed to supernatants from different bacterial species, in addition to comparison with the control.

3.3 Results

3.3.1 Difference in protein concentration between SAG bacterial supernatants

The protein concentration for the supernatants from different strains of SAG collected at 4, 8, 12, 16, and 24 h were calculated using the standard curve and the equation as in **Figure 3.1** and were in the range of 5.8 ± 0.2µg/mL for *S. anginosus* 39/2/14a and 5.3±0.5 µg/mL for *S. consellatus* 45386 from 4 h to 20 h. The highest concentration of protein 5.9 ± 0.17 µg/mL was detected at 24 hours for *S. anginosus* 39/2/14a and 5.9 ± 0.3 µg/mL for *S. consellatus* 45386 at 24 h.

3.3.2 Histomorphometric changes due to the effect of SAG bacterial supernatants.

Tooth slices that were stimulated for 24 h with 100 µg/mL of 12 h SAG bacterial supernatant proteins demonstrated a reduction in density of odontoblasts compared to the unstimulated controls **Figure 3.2**. As observed in the SAG bacterial infection described in chapter 2, the cell rich layer of Höhl (just below the odontoblast layer) in tooth slices stimulated with SAG bacterial supernatant proteins also showed a sparse distribution of cells compared to the control. This reduction in density was more apparent in *S. constellatus* 45386-treated tooth slices **Figure 3.2c** compared to those treated with *S. anginosus* 39/2/14a **Figures 3.2b**. A similar appearance in the odontoblast and cell rich layer of Höhl was observed in tooth slices stimulated 100µg/mL with 24 h SAG bacterial supernatant proteins, but the difference compared to 12 h supernatant stimulation was that there was an even greater loss of cells **Figures.3.3**.

There was a greater cell death of pulpal fibroblasts in tooth slices stimulated with 12 h and 24 h SAG bacterial supernatant proteins. There was reduced density of pulpal fibroblasts and breakdown of intercellular matrix in supernatant stimulated tooth slices compared to unstimulated controls for both 12 h and 24 h supernatants. These changes were more extensive in *S. constellatus* 45386 compared to *S. anginosus* 39/2/14A for both 12 h and 24 h supernatant proteins **Figure 3.2 and 3.3**. These changes were more pronounced in 24 h supernatant proteins compared to 12 h supernatant proteins **Figure 3.2c &3.3 c**.

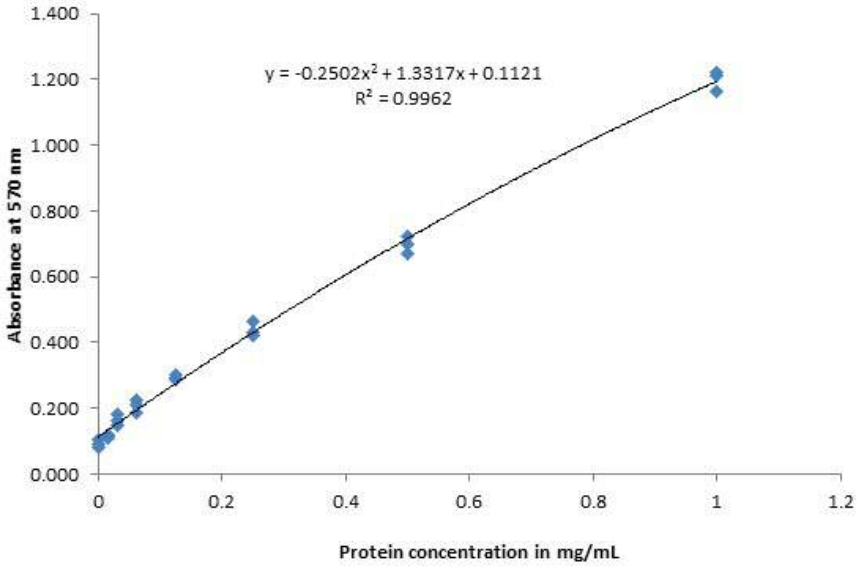
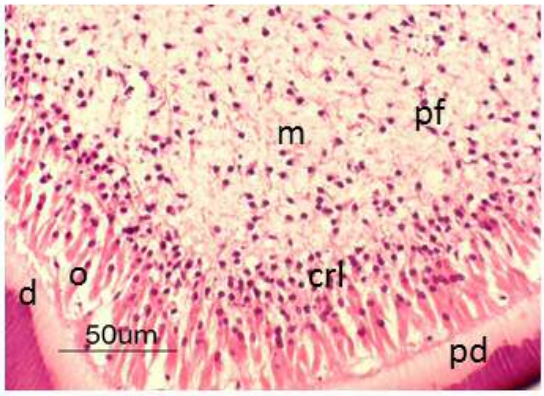
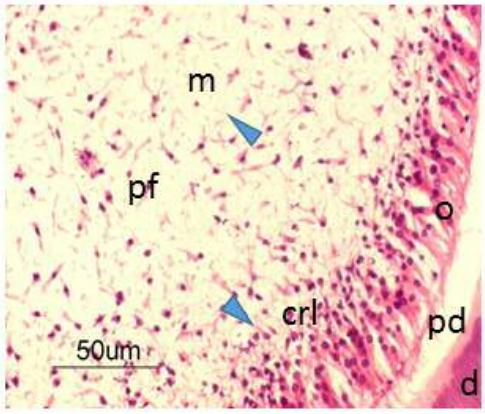
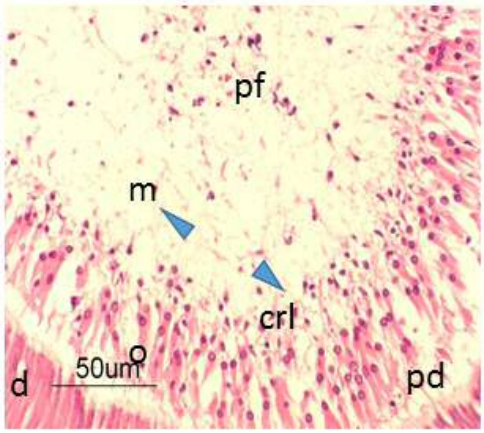


Figure 3.1: Standard curve generated from BCA assay for SAG supernatants.
The graph relates protein concentration to absorbance. The equation y= $0.2502x^2+1.3317x+0.1121$ µ/mL allows calculation of protein concentration in the supernatant of known absorbance.



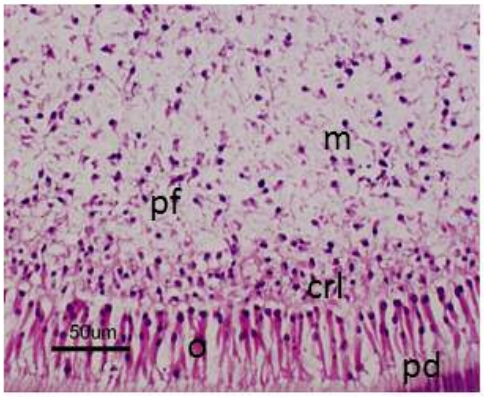
(a)



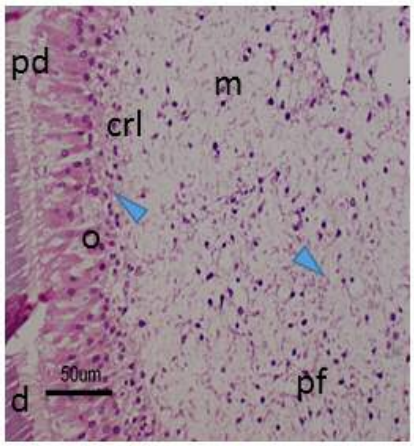


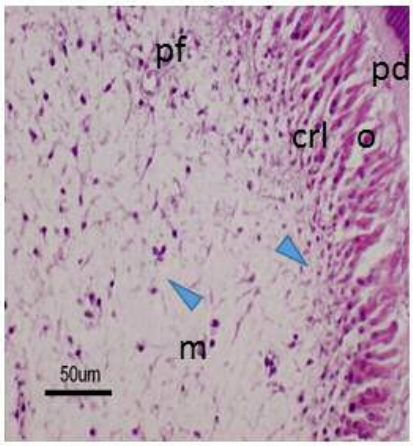
(b) (c)

Figure 3.2: Histology of tooth slices stimulated with 100µg/mL of 12 h SAG supernatant over 24 h. Blue arrows indicate the breakdown of intercellular matrix and reduction of odontoblasts and cells of the cell rich layer of Höhl. a) control X40 magnification, b) *S. anginosus* 39/2/14A supernatant stimulated tooth slice X40 magnification c) *S. constellatus* 45386 supernatant stimulated tooth slice X40 magnification. Key: o, Odontoblast; pd, predentine; crl, cell rich layer of Höhl: m, pulp matrix; pf, pulp fibroblasts.



(a)





(b) (c)

Figure 3.3: Histology of tooth slices stimulated with 100µg/mL of 24 h SAG supernatant over 24 h. Blue arrows indicate the breakdown of intercellular matrix and reduction of odontoblasts and cells of the cell rich layer of Höhl. a) control X40 magnification, b) *S. anginosus* 39/2/14A supernatant-stimulated tooth slice X40 magnification c) *S. constellatus* 45386 supernatant-stimulated tooth slice X40 magnification. Key: o, Odontoblast; pd, predentine; crl, cell rich layer of Höhl: m, pulp matrix; pf, pulp fibroblasts.

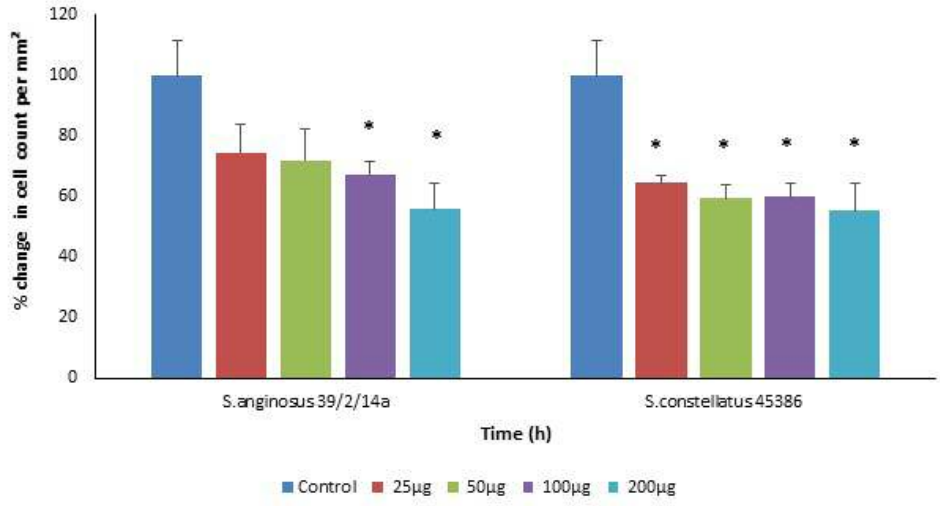
3.3.3 Dose-related variation in pulp cell viability on treatment with SAG supernatants

There was a reduction in odontoblast count when the tooth slices were stimulated with SAG bacterial supernatant proteins for all the different concentrations compared to the unstimulated controls. Compared to controls, this was a statistically significant relative reduction in odontoblast count to 67%/mm^2 and 56%/mm^2 for 100 µg (p=0.04) and 200 µg (p=0.03) (Mann–Whitney U test) of *S. anginosus* 39/2/14a proteins, respectively **Figure 3.4a**. *S. constellatus* 45386 caused a statistically significant relative reduction in odontoblast count to 64%/mm^2, 59%/mm^2, 59%/mm^2 and 55%/mm^2 (p=0.03) (Mann–Whitney U test) for protein concentrations 25 µg, 50 µg, 100 µg and 200 µg/mL respectively **Figure 3.4a**.

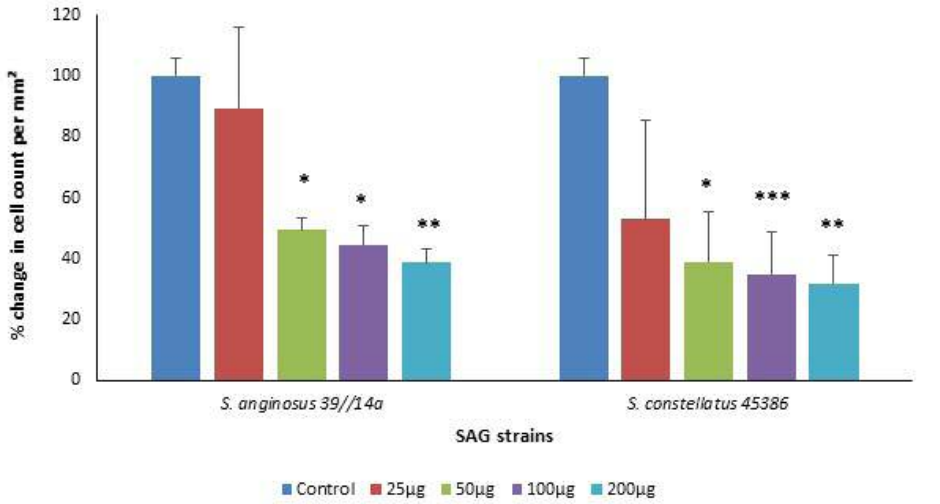
When the dental pulp cell count was assessed, there was a statistically significant, dose-dependent reduction in cell count for both SAG strain supernatant proteins compared to unstimulated control. For *S. anginosus* 39/2/14a the significance reduction in dental pulp cell count was 49%/mm^2 (p=0.04), 44%/mm^2 (p=0.02) and 38%/mm^2 (p=0.005) (Mann–Whitney U test) for 50 µg, 100 µg and 200 µg, respectively, compared to unstimulated control **Figure 3.4b**. *S. constellatus* 45386 exhibited a similar dose dependent reduction in dental pulp cell count but was significantly higher by 10%/mm^2 (p=0.02), 10% (p=0.001) and 8% (p=0.006) (Mann–Whitney U test) than that of *S. anginosus* 39/2/14a for 50 µg, 100 µg and 200 µg, respectively, for the same concentration of supernatant proteins **Figure 3.4b**. This confirms the findings of the histological observations **Figures 3.2 & 3.3**.

3.3.4 Confirmation of the effect of SAG bacterial supernatant proteins on cell viability with heat-inactivated SAG bacterial supernatant proteins.

When tooth slices were stimulated for 24 h with 100 µg of 24 h heat-inactivated SAG bacterial supernatants there was no significant reduction for *S. anginosus* 39/2/14a (p=0.22) and *S. constellatus* 45386 (p=0.25), respectively, for odontoblast counts **Figure 3.5a**. Similar observation were noted for dental pulp cell counts for *S. anginosus* 39/2/14a (p= 0.85) and *S. constellatus* 45386 (p= 0.75) **Figure 3.5b**.



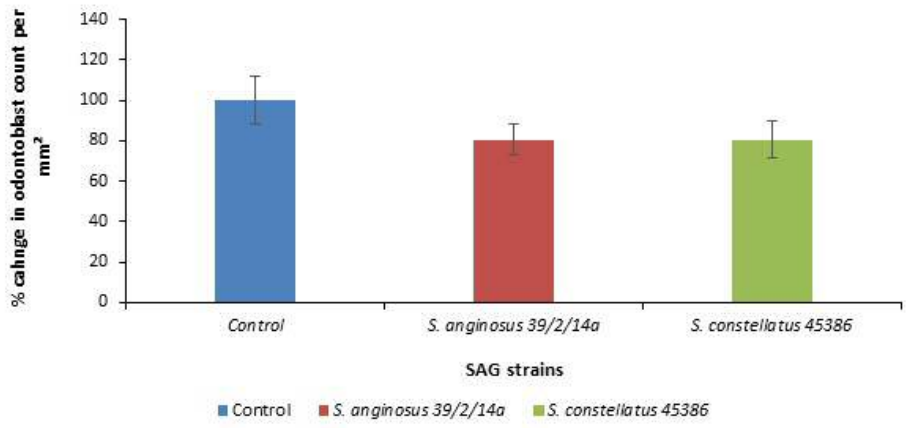
(a)



(b)

Figure 3.4: Effect of 25, 50, 100 & 200 µg/mL SAG supernatant proteins on the odontoblast and dental pulp cell count over 24 h.

Odontoblast and dental pulp cell count performed on transverse section of tooth slice and expressed as a percentage change in cell count per mm² relative to unstimulated control. The error bars indicate SE for experiments conducted in triplicate. a) Odontoblast count; b) dental pulp cell count (*=p<0.05 **=p<0.01, ***=p<0.01) from Mann–Whitney *U* test.



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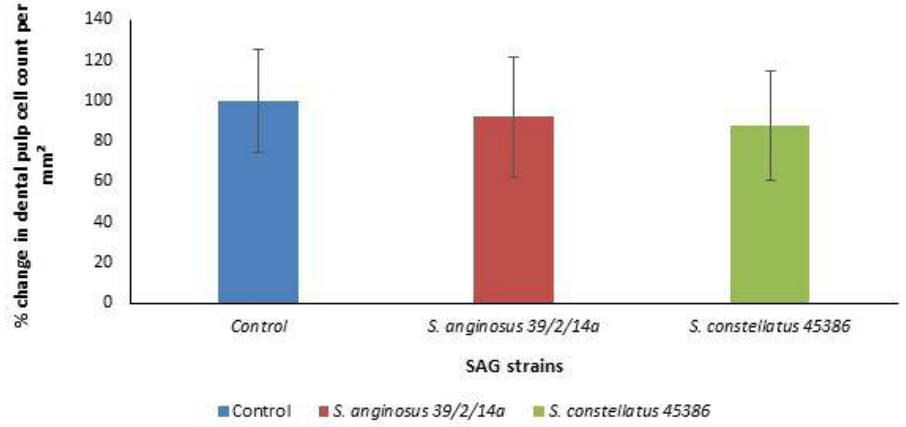


(b)

Figure 3.5: Effect of 200 µg/mL SAG denatured supernatant proteins on the odontoblast and dental pulp cell count over 24 h.

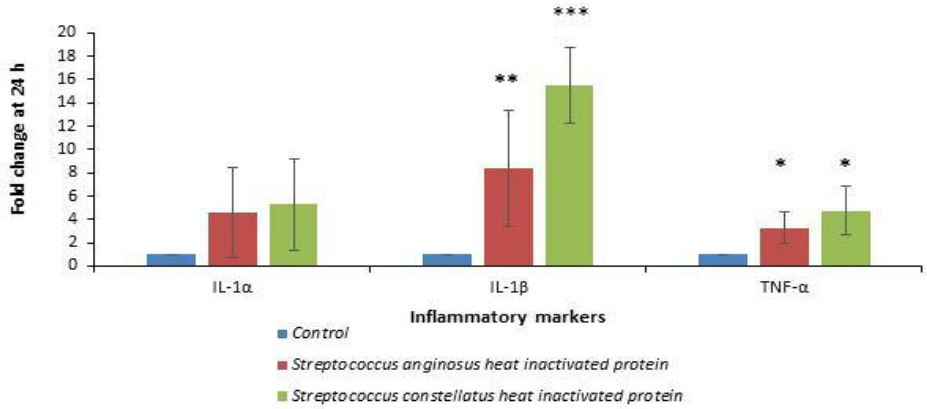
Odontoblast and dental pulp cell count performed on transverse section of tooth slices and expressed as a percentage change in cell count per mm² relative to unstimulated control. The error bars indicate SE for experiments conducted in triplicates. a) Odontoblast count; b) dental pulp cell count. (*=$p<0.05$ was considered significant from Mann–Whitney *U* test).

3.3.5 Inflammatory response of pulp tissue to heat-inactivated SAG bacterial supernatant proteins

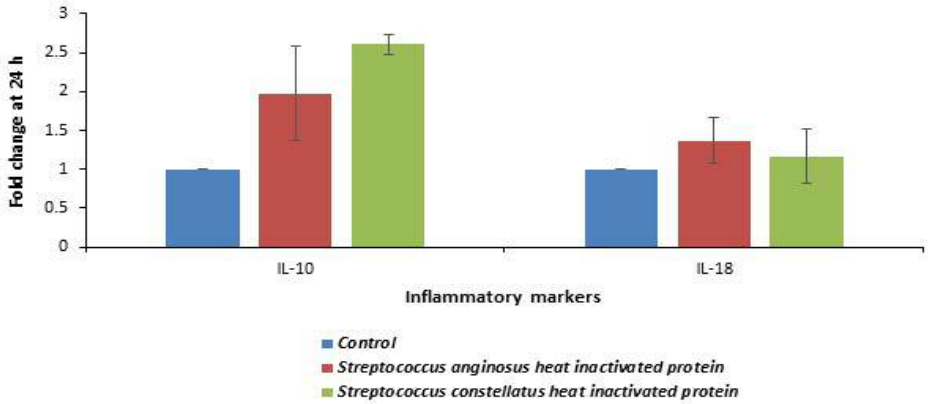
When pulp tissue was stimulated with heat-inactivated SAG bacterial supernatant proteins over 24 h and the expression of the pro- and anti-inflammatory markers assessed, the pulp stimulated with SAG heat-inactivated bacterial supernatant proteins showed an increasing trend in expression of the pro- inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 and the anti-inflammatory marker IL-10 **Figure 3.6**. Statistically significant difference was observed only for IL-1β with increase of 7-fold (p= 0.01) and 13-fold (p=0.001), and for TNF-α 3-fold (p=0.03) and 4-fold (p=0.04), for *S. anginosus* 39/2/14a and *S. constellatus* 45386, respectively, compared to unstimulated control slices. The heat-inactivated SAG bacterial supernatant was used in order to confirm whether the effect observed was definitely from that of SAG supernatant proteins. As heat inactivation denatures proteins and reduces their bioavalibility.

3.3.6 Inflammatory response of the pulp to SAG bacterial supernatant proteins

When the level of inflammatory marker expression was assessed, an increase in IL-1α levels in pulp stimulated with 100 µg/mL of SAG supernatant proteins was observed **Figure 3.7**, but this was not statistically significant compared to unstimulated controls **Table. 3.1**. IL-1β exhibited a time-dependent increase in expression **Figure 3.8** with a statistically significant increased expression of 3 fold and 11 fold at 12 h, and 8 fold and 12 fold at 24 h for for *S. anginosus* 39/2/14a and *S. constellatus* 45386, respectively, compared to unstimulated control. **Table 3.1**. TNF-α also exhibited a time-dependent increase in expression for both SAG strains **Figure 3.9**, this was statistically significant for *S. anginosus* 39/2/14a only at 24h with 5 fold increase, whereas for *S. constellatus* 45386 it was significant at both 12 h and 24h with 7 fold and 6 fold increases, respectively, compared to unstimulated control **Table 3.1**. IL-18 showed an increase of 2.5 fold and 2 fold at 12 h, and 7 fold and 12 fold at 24 h for *S. anginosus* 39/2/14a and *S. constellatus* 45386 supernatant proteins, respectively, **Figure 3.10**; this was significant compared to unstimulated control pulp **Table 3.1**. In the case of the anti-inflammatory marker IL-10, there was a statistically significant **Table 3.1** time-dependent decrease in expression by 8 fold and 6 fold at 12h, and 7 fold and 7 fold at 24 h **Figure 3.11** for *S. anginosus* 39/2/14a and *S. constellatus* 45386 supernatant proteins, respectively, compared to unstimulated control.



(a)



(b)

Figure 3.6: Inflammatory marker expression by SAG bacteria supernatant stimulated pulp compared to unstimulated pulp.

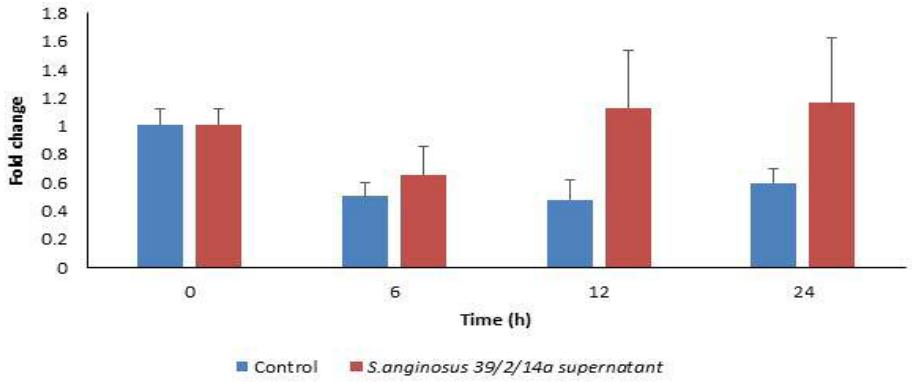
Expression of inflammatory markers in dental pulp tissue stimulated with inactivated SAG bacteria supernatant proteins compared with unstimulated control pulp tissue relative to β-actin, assessed by RT-qPCR over a period of 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) IL-1α; IL-1β; TNF-α b) IL-18 and IL-10 (Mann–Whitney U test; *p>0.05, **p>0.01, ***p<0.001).

Table 3.1: Statistical difference in inflammatory marker expression between SAG supernatant-stimulated and unstimulated tooth slices.

p values comparing the level of significance (Mann–Whitney U test) in inflammatory markers expressed by pulp tissue stimulated with SAG bacterial proteins to unstimulated controls.

Inflammatory markers	***S. anginosus* 39/2/14a**			***S. constellatus* 45386**		
	6h	12h	24h	6h	12h	24h
IL-α	p=0.53	p=0.21	p=0.02	p=0.48	p=0.66	p=0.95
IL-1β	p=0.42	p=0.03*	p=0.04*	p=0.43	p=0.03*	p=0.04*
TNF-α	p=0.19	p=0.32	p=0.03*	p=0.15	p=0.02*	p=0.01**
IL-18	p=0.36	p=0.03*	p=0.05*	p=0.31	p=0.04*	p=0.03*
IL-10	p=0.07	p=0.05*	p=0.01**	p=0.29	p=0.04*	p=0.04*

*Statistically significant * ($p<0.05$) and ** ($p<0.01$)



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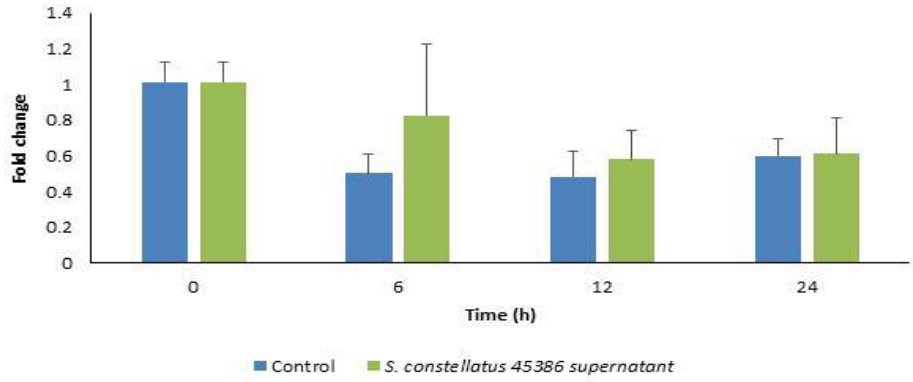
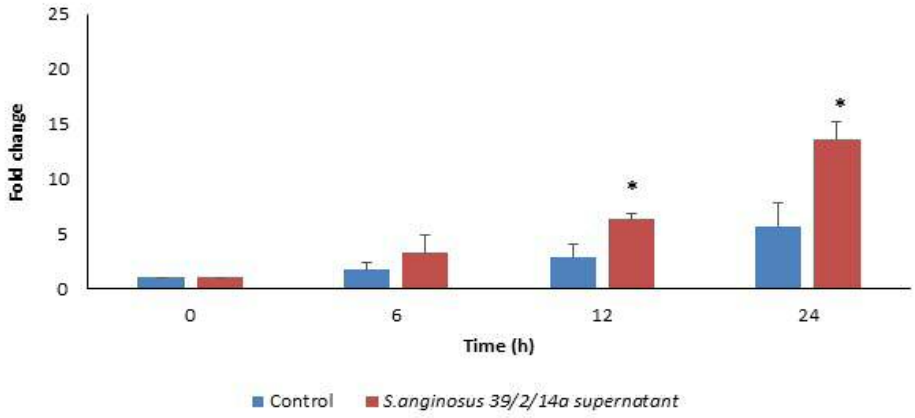
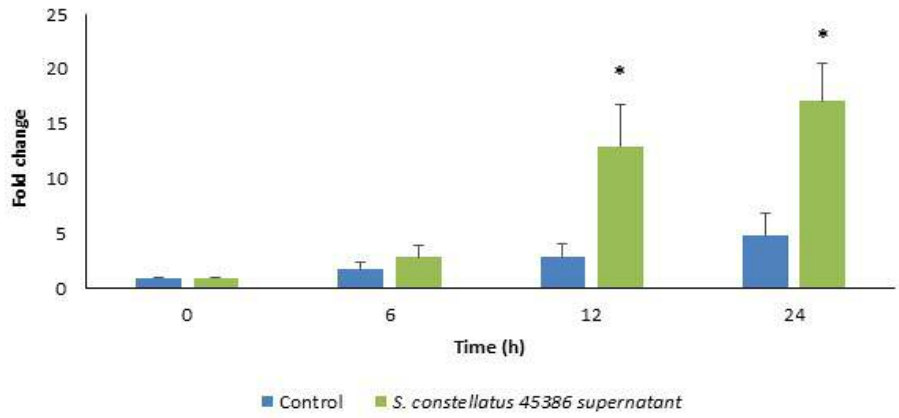


(b)

Figure 3.7: Effect of SAG supernatant proteins on IL-1α expression in *ex vivo* pulp tissue.
Expression of IL-1α in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period of 24 h. Each bar represents fold change compared to housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (Mann–Whitney U test; $p>0.05$).

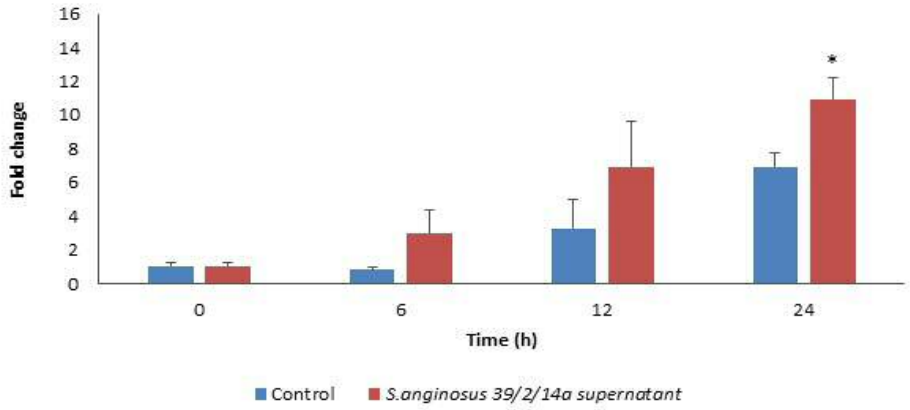


(a)



(b)

Figure 3.8: Effect of SAG supernatant proteins on IL-1β expression in *ex vivo* pulp tissue.
Expression of IL-1β in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period of 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (Mann–Whitney U test; *=p<0.05).



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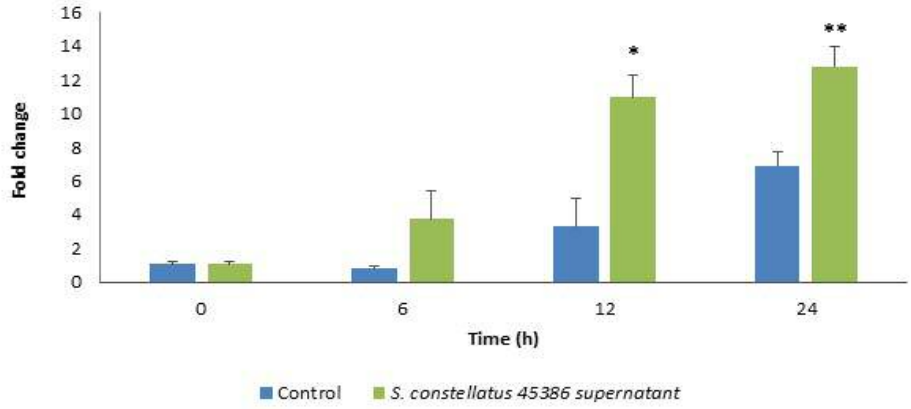
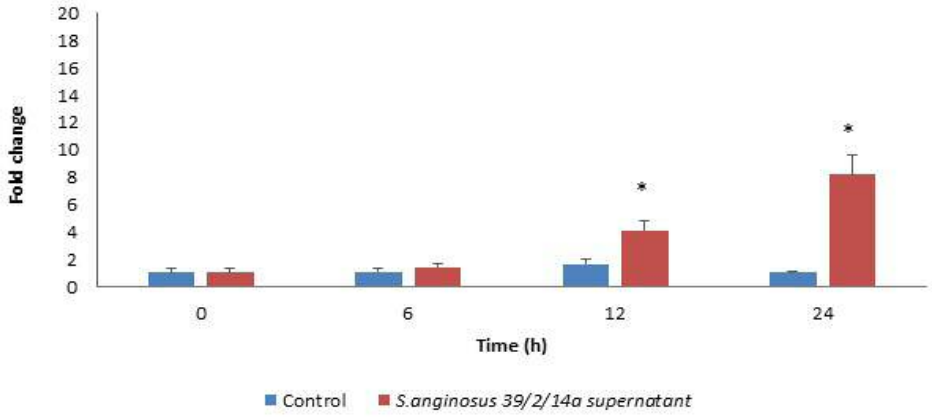
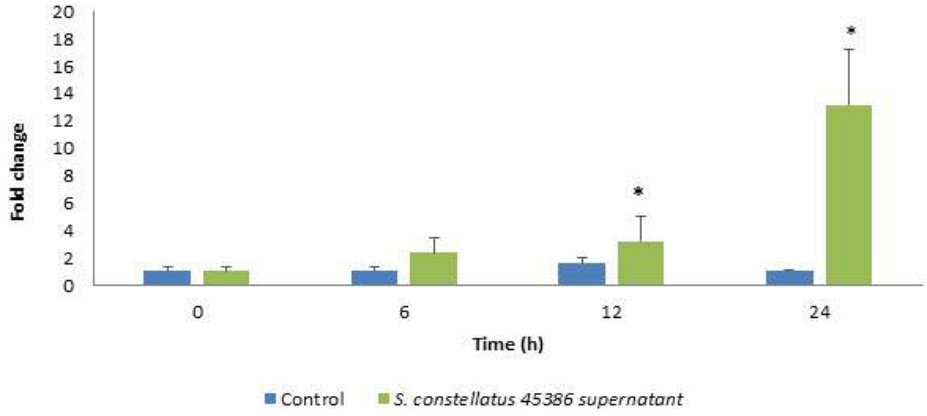


(b)

Figure 3.9: Effect of SAG supernatant proteins on TNF-α expression in *ex vivo* pulp tissue.
Expression of TNF-α in dental pulp tissue infected with SAG bacteria compared with uninfected relative to β-actin control, assessed by RT-qPCT over a period of 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a, b) *S. constellatus* 45386 (Mann–Whitney U test; *=p<0.05, **=p<0.01).

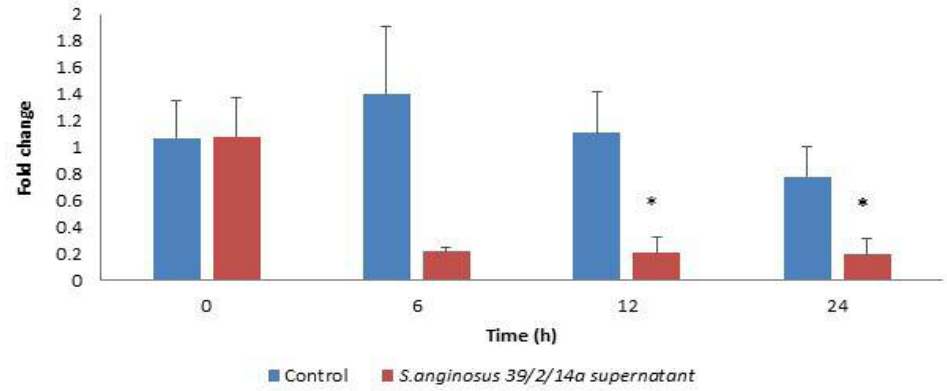


(a)



(b)

Figure 3.10: Effect of SAG supernatant proteins on IL-18 expression in *ex vivo* pulp tissue.
Expression of IL-18 in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period of 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicates. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386(*=p<0.05).



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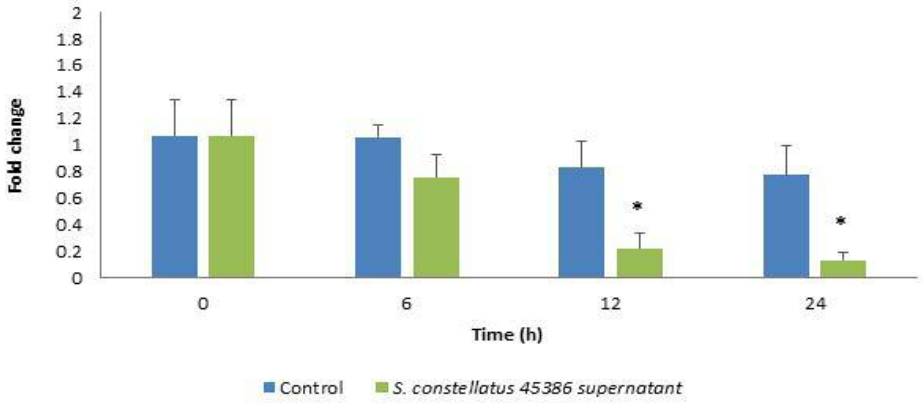


(b)

Figure 3.11: Effect of SAG supernatant proteins on IL-10 expression in the *ex vivo* pulp tissue. Expression of IL-10 in dental pulp tissue infected with SAG bacteria compared with uninfected control relative to β-actin, assessed by RT-qPCR over a period of 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (Mann–Whitney U test; **=p<0.01).

3.3.7 Comparison of supernatant protein composition prepared by different separation methods

SAG supernatants were prepared from a 24 h culture by centrifugation or filtration using 0.2 µm filters. Images of silver stained SDS-PAGE gels for both strains of SAG showed that there was no difference in protein composition of the supernatant prepared by gravity separation or filtration **Figure 3.12a&b**. The *S. anginosus* 39/2/14A supernatant had fewer distinct protein bands when compared to that of the *S. constellatus* 45386 supernatant **Figures 3.13 & 3.14**. When the neat SAG supernatants from different time points were subjected to SDS-PAGE and silver stained, bands became apparent, but when the same supernatants were stained with Coomassie blue, these bands were very faint **Figures 3.13b and 3.14b**. Therefore, supernatants were concentrated to 12 times their initial concentration and subjected to SDS-PAGE and Coomassie staining. This enabled better visualisation of bands **Figures 3.13d & 3.14d**. When supernatants were concentrated 45-fold subjected to SDS-PAGE and stained with Coomassie blue, *S. anginosus* 39/2/14a and *S. constellatus* 45386 showed 9 bands that were similar between the 2 strains in **Figure 3.15**.

The band of molecular weight 116.3 kDa was intense for *S. constellatus* 45386 compared to *S. anginosus* 39/2/14a. Other bands were approximately 100 kDa, 91 kDa and 84 kDa in size with the band at 84 kDa being more strongly expressed in *S. anginosus* 39/2/14a. The bands at approximately 48 kDa and 40 kDa appear to have the same intensity in both strains. The band corresponding to a molecular weight of 36.5 kDa were slightly more intense in *S. constellatus* 45386. The band corresponding to a molecular weight of 32.5 kDa showed stronger expression in *S. constellatus* 45386. The band at approximately 26kDa showed equal intensity in both strains. The two strains also showed bands that were different from each other **Figure 3.15**.

3.3.8 Effect of culture time on composition of SAG supernatant

When the supernatants from the two strains of SAG were collected at 4 h intervals over 24 h, subjected to SDS-PAGE and stained with silver stain, the majority of the proteins started to appear at 12 h in *S. anginosus* 39/2/14a but in *S. constellatus* 45386 they were not seen until 16 h **Figures 3.12c&d, 3.13 & 3.14**. The intensity of the bands increased over time **Figures 3.12c&d, 3.13 & 3.14** as concentration of the proteins in the supernatants increased **Figures 3.13c & 3.14c**. *S. anginosus* 39/2/14a expressed two additional proteins only at 24 h. The first one is approximately 52.4 kDa and the second one is approximately 20 kDa.

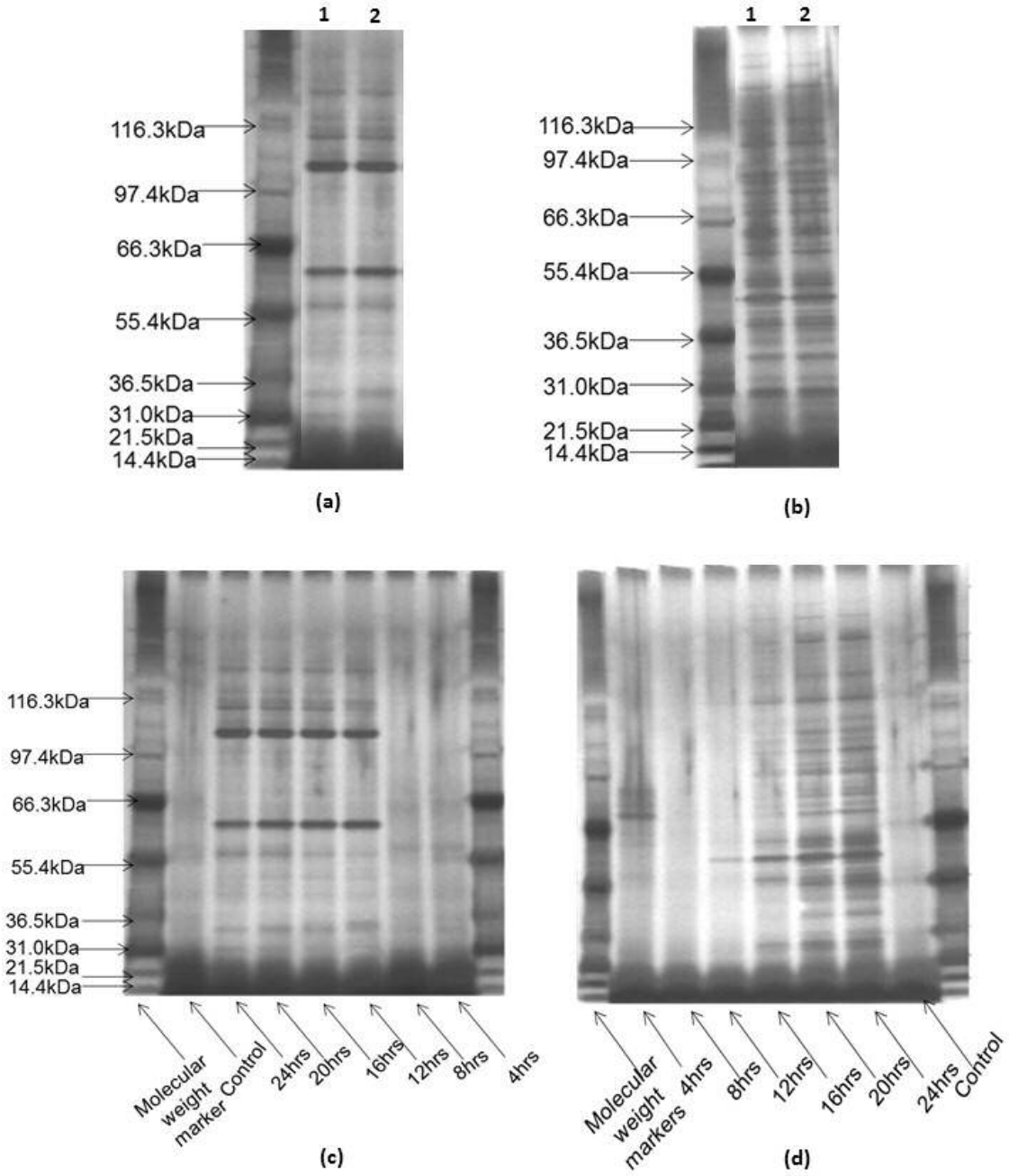


Figure 3.12: Effect of time of harvesting and method of separation on the composition of SAG supernatant harvested at 4 h intervals over 24 h.

a) comparison of *S. anginosus* 39/2/14a supernatant prepared by gravity separation and filtration b) comparison of *S. constellatus* 45386 supernatant prepared by gravity separation and filtration c) protein composition of *S. anginosus* 39/2/14a supernatant over 24 h compared to BHI d) protein composition of *S. constellatus* 45386 supernatant over 24 h compared to BHI. Key: lane 1, supernatant prepared by gravity separation; lane 2 supernatant prepared by filtration,

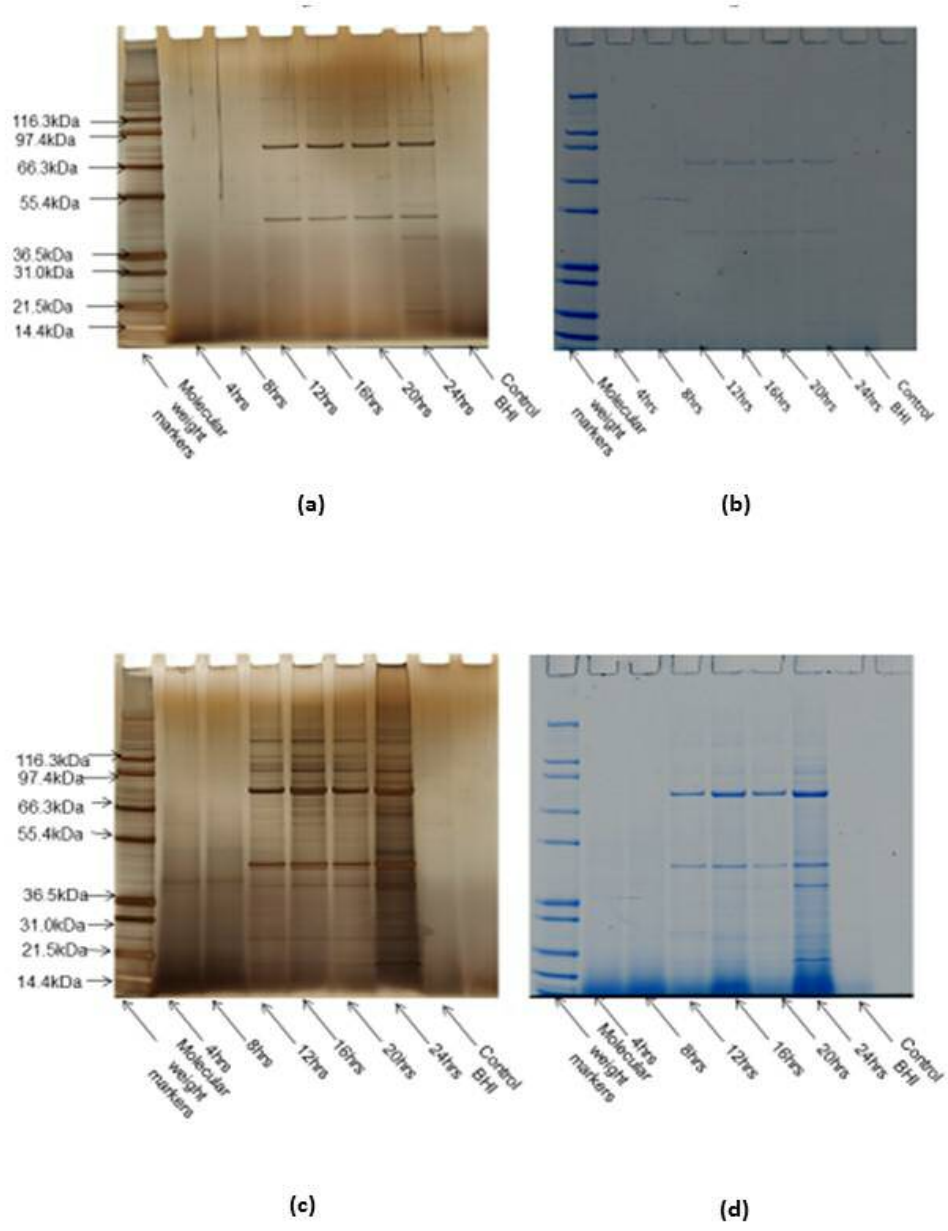


Figure 3.13: Change in protein concentration *S. anginosus* 39/2/14a of supernatant over 24 h analysis by SDS-PAGE.

a) neat supernatant gel-silver stained b) neat supernatant gel-Coomassie blue stained c) x12 supernatant gel - silver stained d) x12 supernatant gel-Coomassie blue stained.

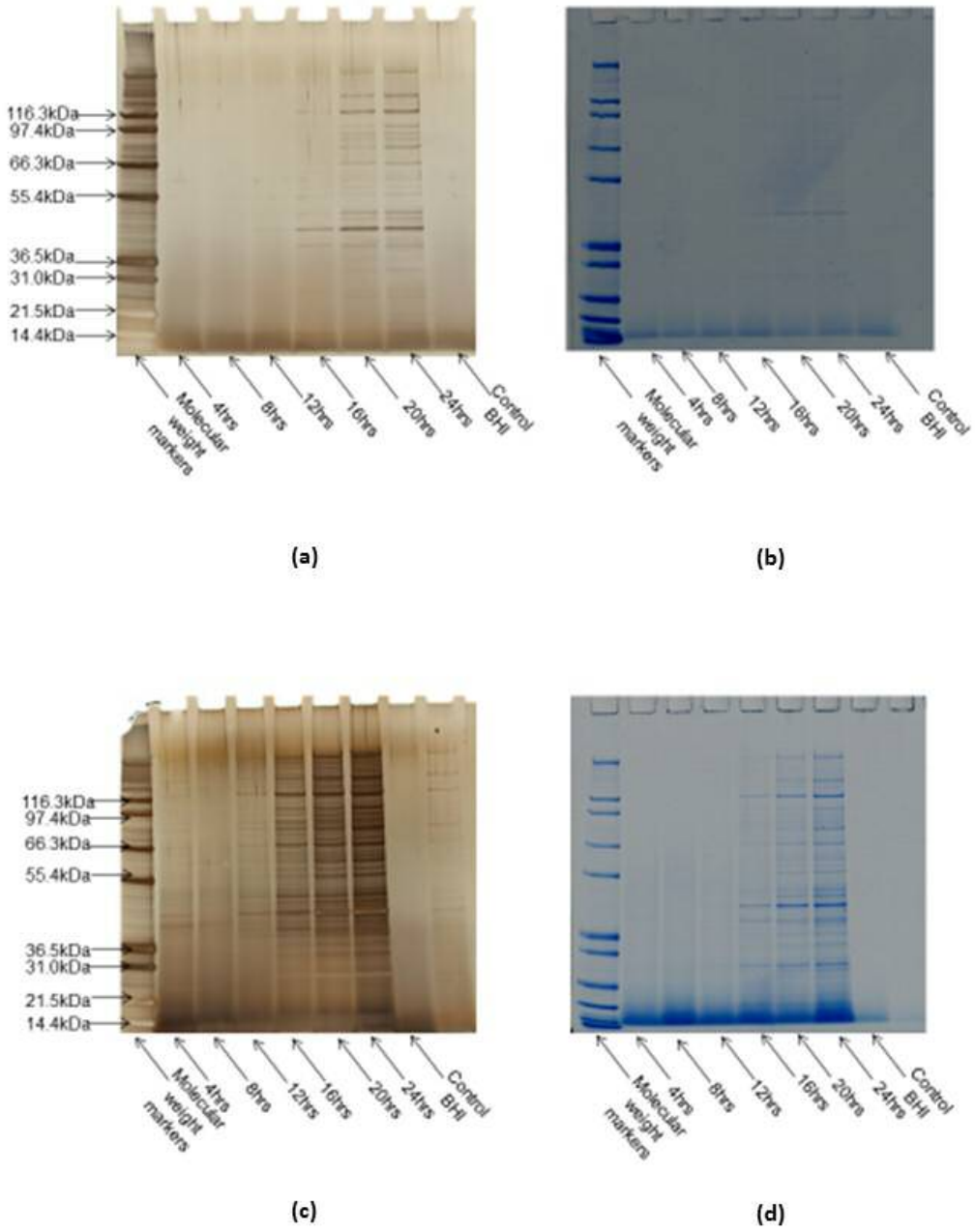


Figure 3.14: Change in protein concentration of *S. constellatus* 45386 supernatant over 24 h analysis by SDS-PAGE.

Band intensity also increases with time a) neat supernatant gel-silver stained b) neat supernatant gel-Coomassie blue stained c) x12 supernatant gel-silver stained d) x12 supernatant gel-Coomassie blue stained.

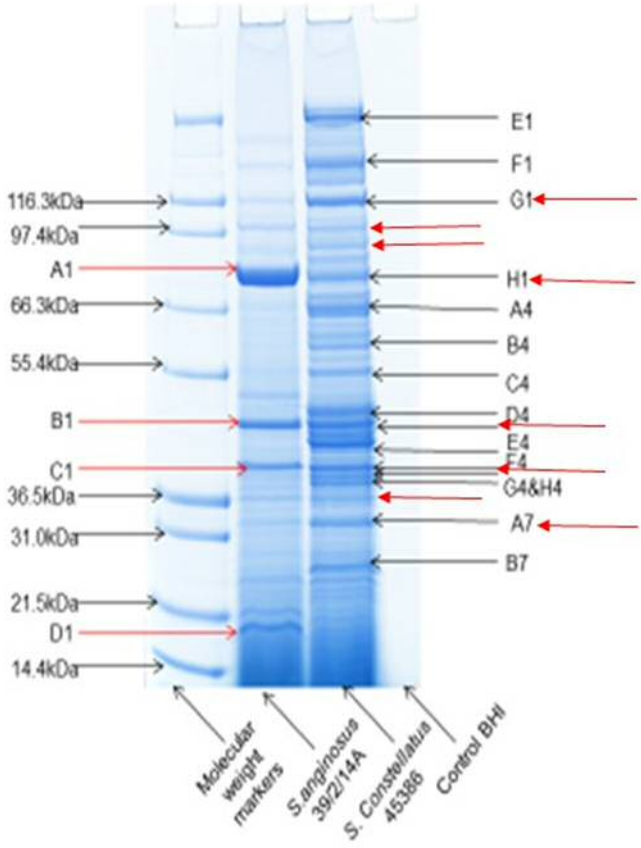


Figure 3.15: Proteins band in 24 h SAG supernatant concentrated x45 and separated by SDS-PAGE for mass spectrometry analysis.

SDS-PAGE analysis of 24h supernatant concentrated x45 from *S. anginosus* 39/2/14, *S. constellatus* 45386 and control BHI stained with Coomassie blue. Gel plugs of prominent protein bands were subject to mass spectrometry to identify the proteins in the supernatant **Table 3.2.**

3.3.9 Constituents of SAG supernatants identified by mass spectrometry

When the supernatants were concentrated to 45 times their original concentration and subjected to SDS-PAGE, there was an increase in the number of bands that became visible. Furthermore, the bands were darker compared to the 12 times concentrated supernatant. The IDs (A1 -H4) of the prominent protein bands identified in **Figure 3.15** that were isolated by cutting gel plugs and subjecting to mass spectrometry identified various metabolic enzymes of SAG, which included CHAP domain protein, general stress protein, peptidoglycan binding LysM, elongation factor, serine protease, bacterial extracellular binding protein, pyruvate kinase, glucose-6-phosphate isomerase, enolase, arginine deimerase, ornithine carbamoyltransferase tagatose1,6-diphosphate, fructose 1,6-biphosphate alsoase and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase **Table 3.2**. Some of the bands could not be identified and are shown as no ID **Table 3.2**.

3.3.10 Hyaluronidase-producing ability of SAG

The supernatants of SAG cultured in BHI with substrates hyaluronic acid sodium salt from bovine vitreous humour and bovine albumin fraction V were evaluated for hyaluronidase activity on agar plates containing hyaluronic acid. The positive control produced a clear zone indicating hyaluronidase activity **Figure 3.16a**. Supernatant from *S. anginosus* 39/2/14a strain did not show a clear zone **Figure 3.16c**; in contrast, *S. constellatus* 45386 showed a clear zone **Figure 3.16d**. The denatured supernatant from *S. constellatus* 45386 did not produce a clear zone compared to the *S. constellatus* 45386 bacterial suspension **Figure 3.16b**.

Table 3.2: IDs of prominent protein bands from the 45x concentrated SAG supernatants identified by mass spectrometry. Key: Categories C1 and C2 indicate the level of match of the identified sequence to the protein. The match is categorised C1-C6, C1 indicating a highly probable match and C6 being low.

Band	ID	Cat	Band	ID	Cat
A1	Putative uncharacterized protein OS=Streptococcus anginosus	C1	D4	Glucose-6-phosphate isomerase OS=Streptococcus anginosus F0211 Glucose-6-phosphate isomerase OS=Streptococcus anginosus 1_2_62CV Elongation factor Tu OS=Streptococcus anginosus F0211 Elongation factor Tu OS=Streptococcus anginosus 1_2_62CV	C2 C2 C2 C2
B1	CHAP domain protein OS=Streptococcus anginosus General stress protein GSP-781 OS=Streptococcus anginosus	C2 C2	E4	Enolase OS=Streptococcus anginosus F0211 Enolase OS=Streptococcus anginosus 1_2_62CV	C1 C1
C1	Trypsin OS=Streptococcus anginosus F0211 Serine protease OS=Streptococcus anginosus 1_2_62CV	C1 C1	F4	Arginine deiminase OS=Streptococcus anginosus F0211 CHAP domain protein OS=Streptococcus anginosus F0211 General stress protein GSP-781 OS=Streptococcus anginosus 1_2_62CV	C1 C1 C1
D1	Peptidoglycan-binding LysM OS=Streptococcus anginosus 1_2_62CV LysM domain protein OS=Streptococcus anginosus F0211	C1 C2	G4	Ornithine carbamoyltransferase OS=Streptococcus anginosus F0211 Ornithine carbamoyltransferase OS=Streptococcus anginosus 1_2_62CV	C1 C1
E1	No ID		H4	Tagatose 1,6-diphosphate aldolase 2 OS=Streptococcus anginosus F0211 Tagatose 1,6-diphosphate aldolase 1 OS=Streptococcus anginosus 1_2_62CV	C1 C1
F1	No ID		A7	Fructose-1,6-bisphosphate aldolase, class II OS=Streptococcus anginosus F0211 Fructose-bisphosphate aldolase OS=Streptococcus anginosus 1_2_62CV	C2 C2
G1	No ID		B7	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Streptococcus anginosus 1_2_62CV 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Streptococcus anginosus F0211	C1 C1
H1	Elongation factor G OS=Streptococcus anginosus F0211 Elongation factor G OS=Streptococcus anginosus 1_2_62CV	C1 C1			
A4	Bacterial extracellular solute-binding protein, family 5 OS=Streptococcus anginosus F0211 Oligopeptide-binding protein amiA OS=Streptococcus anginosus 1_2_62CV	C1 C1			
B4	No ID				
C4	Pyruvate kinase OS=Streptococcus anginosus F0211 Pyruvate kinase OS=Streptococcus anginosus 1_2_62CV	C1 C1			

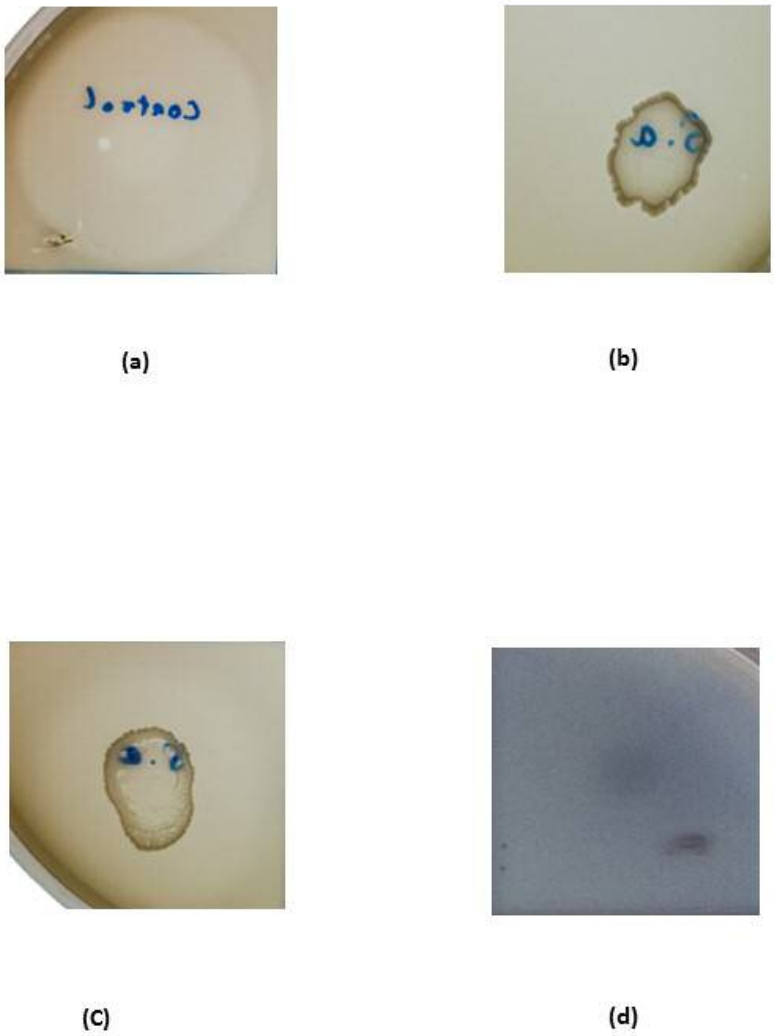


Figure 3.16: Evaluation of hyaluronidase activity of SAG supernatant.
Hyaluronidase activity of *S. anginosus* 39/2/14a and *S. constellatus* 45386 evaluated by formation of clear zones through degradation of hyaluronic acid. a) positive control; b) *S. anginosus* 39/2/14a; c) *S. constellatus* 45386; d) heat-inactivated *S. constellatus* 45386 supernatant.

3.4 Discussion

It was observed in Chapter 2 that SAG infection led to cell death and an increased inflammatory response in the dental pulp tissue compared to the uninfected control dental pulp tissue. It was also observed that when the tooth slices were stimulated with 100 µg/mL of 24 h SAG supernatant proteins, they produced histomorphometric changes similar to those produced by SAG infection. Current observations have demonstrated that *S. constellatus* 45386 supernatant proteins caused a significant reduction in odontoblast and dental pulp cells compared to that of *S. anginosus* 39/2/14a. This finding concurred with that seen when the tooth slices were infected with SAG bacteria, thereby demonstrating that the supernatant proteins have the same effect on the pulp as that of the infection and that the toxins produced by these bacteria may be the key factors for the tissue response in the pulp.

The *S. constellatus* 45386 supernatant proteins, even at a lower concentration of 25 µg/mL, caused death of odontoblasts compared to that of *S. anginosus* 39/2/14a. This could be due to the difference in the toxins produced by the two strains. In order to confirm that the effect seen in the pulp tissue is a result of the response to the SAG supernatant proteins, the SAG supernatants were subjected to heat inactivation.

As it was demonstrated that 100 µg/mL of SAG supernatant caused histomorphometric changes in the dental pulp tissue for both strains of SAG, this concentration of SAG bacterial supernatant was chosen as a standard concentration for stimulation of the tooth slices. When the tooth slices were stimulated with 100 µg/mL of SAG heat-inactivated supernatants, there was a reduction in odontoblast count compared to unstimulated pulp, but this was not significant, and the reduction was similar for both strains. The dental pulp cells showed no significant reduction ($p=0.85$ and $p=0.75$) for heat inactivated supernatant of *S. anginosus* 39/2/14a and *S. constellatus* 45386, respectively, compared to unstimulated control pulp. This result could be due to inactivation of the enzymes in the SAG bacterial supernatant by the heat treatment (Margosch *et al*. 2005).

When the tooth slices were stimulated with the heat-inactivated SAG bacterial supernatant to evaluate the inflammatory response of the pulp tissue, there was a significant increase in the expression of IL-1β ($p=0.01$ and $p=0.001$) and TNF-α ($p=0.03$ and $p=0.04$) for *S. anginosus* 39/2/14a and *S. constellatus* 45386 heat-inactivated supernatant, respectively, compared to the unstimulated tooth slices. IL-1α ($p= 0.18$ and $p=0.13$), IL-18 ($p=0.09$ and $p=0.21$) and IL-10 ($p=0.08$ and $p=0.06$) for *S. anginosus* 39/2/14a and *S. constellatus* 45386 heat-inactivated

supernatant, respectively, showed no significant difference compared to the unstimulated control. The expression of IL-1β and TNF-α may be due to the reduced activity of the SAG bacterial proteins caused by heat treatment (Margosch *et al*. 2005). The lack of cell death and expression of only IL-1β and TNF-α by the stimulation of pulp with heat-inactivated SAG supernatant in this work substantiates that SAG bacterial supernatant proteins have a role in causing cell death and an inflammatory response in the pulp. The inflammatory response of the pulp to SAG bacterial supernatant proteins was assessed by stimulating the tooth slices with 100 µg/mL of SAG supernatants over 24 h. The response was evaluated at 6, 12 and 24 h and compared to that of the unstimulated control tooth slices.

IL-1α produced by the SAG supernatant-stimulated pulp was not significantly different (p=0.53, p=0.21 and p=0.29) for *S. anginosus* 39/2/14a supernatant and (p= 0.48, p=0.66 and p=0.95) for *S. constellatus* 45386 at 6 h, 12 h and 24 h, respectively, compared to unstimulated pulp. IL-1β expression was significantly higher (p=0.03 and p=0.04) for *S. anginosus* 39/2/14a supernatant and (p=0.03 and p=0.04) for *S. constellatus* 45386 at 12 h and 24 h, respectively, compared to unstimulated pulp. There was up regulation of TNF-α expression at 12 h and 24 h in *S. constellatus* 45386 supernatant-stimulated pulp but the upregulation was noted in *S. anginosus* 39/2/14a supernatant only at 24 h. IL-18 expression was upregulated at 12 h and 24 h in pulp stimulated with supernatants from both strains of SAG. The expression of anti-inflammatory marker IL-10 was significantly reduced (p=0.05 and p=0.01) for *S. anginosus* 39/2/14a supernatant and (p=0.04 and p=0.04) for *S. constellatus* 45386 at 12 h and 24 h, respectively, compared to unstimulated pulp. These findings are similar to those observed in *in vivo* experiments (Renard *et al*. 2016) where rat pulp was stimulated with LPS. The only difference is that in our experiment, the upregulation of inflammatory markers persisted for 24 h in contrast to the *in vivo* study where the levels returned to baseline at 9 h. This early return of the level of inflammatory markers to baseline in the *in vivo* experiment could be due to a systemic clearing effect. Expression of all pro-inflammatory markers was slightly higher in *S. constellatus* 45386 supernatant stimulated pulp compared to *S. anginosus* 39/2/14a supernatant-stimulation. This could once again be due to the differences in proteins produced by the two strains of SAG (Grinwis *et al*. 2010; Asam and Spellerberg 2014).

Identifying and targeting specific SAG proteins could be useful in preventing cell death, matrix destruction and modulating the inflammatory response. In order to identify toxins present in the SAG bacterial supernatant, the proteins of the SAG supernatant were separated. Supernatants prepared by centrifugation still had some bacteria in them and when used to stimulate the tooth slices caused infection. This made it difficult to identify the true effect of

supernatants, so the supernatants were prepared by filtration of the bacterial suspension. In order to separate the protein constituents in the SAG supernatants and make sure that the filtration did not remove any of the proteins from the supernatant, supernatants were subjected to SDS-PAGE. Supernatants from both strains of SAG prepared by centrifugation and filtration were run on SDS-PAGE gels with BHI as control. There was no difference in the protein constituents of the supernatants prepared by either method. Therefore, supernatants prepared by filtration were used in all further experiments.

Supernatants, as mentioned before, were collected at 4 h intervals over 24 h to assess if the time of harvesting the supernatant had an effect on its protein composition. The bands become more intense over time and some bands only appeared at 24 h. This showed that time has an effect on the composition or concentration (or both) of the supernatant. Thus, it was the 24 h supernatant that was used in this work rather than any other time point. There were some protein bands that were common for both strains, and some of them were more strongly expressed in one SAG strain compared to the other. It was noted that the *S. constellatus* 45386 supernatant had more proteins compared to that of *S. anginosus* 39/2/14a. The greater number of bands in *S. constellatus* 45386 suggests that are a greater number of toxins and this could contribute to the increased cell death and inflammatory response observed in pulp infected with S*. constellatus* 45386, or its supernatant, compared to *S. anginosus* 39/2/14a, even though the total protein concentration was almost the same in both strains of SAG supernatants.

When SDS-PAGE gels were silver stained, these bands were prominent, but when stained with Coomassie blue for subjecting the protein bands to mass spectrometry they were very faint, and some bands were not even visible. In order to further improve the band quality and number in the Coomassie blue-stained gels, supernatants were concentrated 45-fold and subjected to SDS-PAGE. The gel plugs of the prominent band were excised and subjected to mass spectrometry.

The protein Identifications from mass spectrometry identified the proteins tested to be those of SAG bacteria. Degradative enzymes, like neuraminidase, α-galactosidase and N-acetyl-β-D-glucosaminidase have been identified in SAG supernatants (Willcox *et al*. 1995). These enzymes have the ability to cause tissue breakdown and spread of infection. Furthermore, some of the proteins produced by SAG bacteria, like sialidase, have been shown to be virulence factors and help in the colonisation of other bacteria by modifying the bacterial surface and host environment (Beighton and Whiley 1990; Takao *et al*. 2010)**.**

Streptococci have been known to produce proteases which degrade collagen and they have a molecular weight of around 120 kDa (Juarez and Stinson 1999). These proteases could be responsible for the matrix breakdown and degradation observed in SAG-infected tooth slices and those stimulated with SAG supernatant proteins as the pulp tissue is a loose connective tissue (Linde 1985). Mass spectrometry identified a protein of molecular weight around 40 kDa as a protease. It has been shown that proteases could also have different molecular weights (e.g. 98 kDa) as demonstrated by Juarez and Stinson (1999). This suggests that there may be differences in the proteases produced by different strains of Streptococci which might make it difficult to identify.

The protein band with a molecular weight of 48 kDa in *S. anginosus* 39/2/14a was identified as CHAPS domain protein but the corresponding band for *S. constellatus* 45386 was identified as enolase. It has been suggested that enolase produced by oral streptococci may have a plasminogen-binding effect which may contribute degradation of tissue and extracellular matrix by binding to plasmin (Itzek *et al*. 2010). This is because different proteins could have the same molecular weight but different structure. It has been shown that *S. constellatus* has a higher hyaluronidase activity than other SAG strains (Homer *et al*. 1993; Takao 2003). SAG bacteria are known to produce hyaluronidase and chondroitinase which have been shown to be responsible for matrix breakdown and abscess formation (Homer *et al*. 1993). Streptococcal hyaluronidase has been shown to have a molecular weight of 107 kDa (Berry *et al*. 1994). The same work also shows that a lower molecular weight, (around 89 kDa) variant of the same protein can also be expressed. Furthermore, the lack of substrate in the media like hyaluronic acid and chondroitin sulphate could have down regulated the expression of these enzymes. This could be the reason for the failure of these to appear in the supernatant. Therefore, the identification of proteins just by molecular weight of the band alone must be interpreted with caution. The mass spectrometry analysis of the band in *S. constellatus* 45386 corresponding 91 kDa did not give any identification.

L-cysteine desulphydrase (molecular weight 44 kDa) has been found to be produced by SAG bacteria (Yoshida *et al*. 2002). None of the bands corresponding to this molecular weight were identified as L-cysteine. Chondroitin sulphatase activity has been found to be associated with *S. constellatus* and *S. intermedius* and to a lesser extent with *S. anginosus* (Jacobs and Stobberingh 1995). This could have also contributed to the greater matrix destruction in *S. constellatus* 45386 infection, as chondroitin sulphate has been found in dental pulp (Shibutani *et al*. 1990). None of the bands subjected to mass spectrometry were identified as chondroitin sulphatase.

The proteins identified by mass spectrometry were mainly metabolic enzymes of SAG; and none of the identified proteins related to know virulence factors of SAG. It was speculated that it could be due to culturing SAG in the absence of specific substrates that would facilitate the production of these proteins. This could be the reason for greater matrix breakdown observed in SAG infections compared to supernatant stimulation as observed in the histomorphometric observation. Therefore, both strains of SAG were cultured on BHI agar containing hyaluronic acid. This enabled *S. constellatus* 45386 to produce hyaluronidase, which was apparent as a clear zone due to degradation of hyaluronic acid, as was observed in the positive control. It was noted that the clear zone was not seen in *S. anginosus* 39/2/14a, indicating a lack of hyaluronidase activity. This was further confirmed by culturing *S. constellatus* 45386 in BHI containing hyaluronic acid and then extracting the supernatant by filtration and heat inactivating it to affect its hyaluronidase activity. This showed that heat-inactivated supernatant had no hyaluronidase activity, as it did not produce a clear zone. The treatment of SAG pulpal infection should focus on both killing the bacteria and neutralising its supernatant toxins, thus, limiting tissue damage and spread of infection.

Chapter 4

4 Evaluation of triclosan as an anti-microbial and anti-inflammatory agent in the treatment of pulpal infection using the *ex vivo* tooth slice infection model

4.1 Introduction

Bacterial infection of the dental pulp is the most common injurious challenge to the pulp tissue due to caries, clinical operative procedures and trauma. As a consequence, mixed bacterial infection including gram-negative and anaerobic bacteria establish themselves in the pulp (Siqueira 2012). The infection process provokes an inflammatory response in the pulp tissue. This inflammatory process is important in the host's immune response to the injurious challenge, and it is represented by a broad array of cellular (Bruno *et al*. 2010) and molecular events (Hahn and Liewehr 2007a; Renard *et al*. 2016).

The innate immune response will trigger production of cytokines locally, which promote influx of other immune cells. Although the pulp is similar to other connective tissues the response to microbial infection is different due to its non-compliant environment of hard rigid covering shell and the non-self-cleansing environment due to lack of collateral circulation. The immune defences eventually lead to a chronic inflammatory state of the pulp. Chronic inflammation is central to the pathophysiology of a number of disorders frequently contributing to host damage irrespective of aetiology (Nathan 2002). Acute and chronic inflammation of the pulp leads to tissue damage and eventually compromises the vitality of the pulp, which may require time consuming and expensive root canal treatment, and restoration of the tooth. Furthermore, irrespective of the general immune response occurring in diseased or infected pulp, each individual case is unique in terms of disease activity and timing of the various defence responses. Thus, clinical management of inflammation is a challenge.

Traditionally tissue defence and repair/regeneration are considered as distinct processes, but clearly these processes should be considered as working in tandem (Cooper *et al*. 2014). Evidence suggests that if inflammation is resolved at its early stage relatively low levels of

pro-inflammatory mediators will promote repair (Cooper *et al*. 2010). Therefore, understanding of cellular and molecular events of inflammation and modulation of these inflammatory responses to enable the pulp to repair and regenerate is crucial to maintaining vitality of the pulp tissue.

Treatment of microbial diseases includes a range of therapeutic strategies in clinical practice. One therapeutic strategy that has found wide application is the use of antimicrobial agents with a primary effect on the organism but which also possess anti-inflammatory properties (Labro 2005). Pulpal disease, as mentioned above, has both infection and inflammation components. The management of pulpal disease would be more effective if agents used in its management have both antimicrobial and anti-inflammatory properties.

The use of triclosan in the past has been mainly targeted towards caries prevention and periodontal disease. Triclosan has been shown to possess antimicrobial activity against *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens* and *Fusobacterium nucleatum* (Haraszthy *et al*. 2010). *Enterococcus faecalis,* which is commonly implicated in endodontic infections, has been shown to be effectively inhibited by triclosan (Sun and Song 2011). Triclosan-containing toothpaste has been shown to reduce coronal caries and there have not been any safety concerns during three years of use (Kraglund 2014). There is evidence that triclosan/copolymer reduces dental biofilm, gingivitis, periodontitis, calculus and halitosis (Cury and Tenuta 2014). Triclosan was shown to be clinically effective in the local treatment of chronic periodontitis (Pleskanovskaia *et al*. 2013). *In vitro* studies on epithelial cells have demonstrated antimicrobial and anti-inflammatory activity of triclosan through regulation of the Toll-like receptor (TLR) signalling pathway (Wallet *et al*. 2013). Barros *et al*. (2010) demonstrated that triclosan is an inhibitor of acute and chronic inflammatory gene pathway. The use of triclosan toothpaste was reported not to cause any antimicrobial resistance in *S. anginosus* (Cullinan *et al*. 2014). Triclosan has demonstrated its antimicrobial and anti-inflammatory effect in various *in vitro, ex vivo* and *in vivo* studies (Waaler *et al*. 1993; Gaffar *et al*. 1995; Modeer *et al*. 1996; Mustafa *et al*. 1998; Barros *et al*. 2010; Ayad *et al*. 2015). The use of triclosan in the treatment of pulpal infection and inflammation is yet to be explored. Therefore, it would be worthwhile to explore the use of triclosan in the treatment of pulpal diseases.

The *ex vivo* SAG pulp infection model has been characterised and validated in chapter 2 to study tissue damage and assess inflammatory response. This model can now be used for testing the antimicrobial and anti-inflammatory effect of triclosan in pulpal treatment of pulpal disease before its effectiveness is studied in expensive *in vivo* studies.

The objectives of this chapter are:

- To establish the MIC and MBC of triclosan for SAG
- To evaluate the effect of BHI and MTCM on MIC and MBC of triclosan against SAG
- To evaluate the effect of triclosan on the pulp tissue using the *ex vivo* pulp model.
- To assess the effect of two protocols of triclosan treatment and evaluate its ability to limit odontoblast and dental pulp cell death caused by SAG infection using the *ex vivo* pulp infection model.
- To assess the ability of triclosan to eliminate or reduce pulp tissue colonisation when the SAG infection is treated with triclosan. This will be explored using FDA stained SAG bacteria to infect the pulp tissue.
- To evaluate the ability of triclosan to modulate the inflammatory response of pulp tissue to SAG infection. This will be assessed by evaluating the relative expression of pro-inflammatory markers IL-α, IL-β, IL-18 and TNF-α along with the level of expression of anti-inflammatory marker IL-10 using RT-qPCR.
- To establish if triclosan has the potential to neutralise hyaluronidase.

4.2 Materials and Methods

4.2.1 Preparation of stock and working concentrations of triclosan

Triclosan is a lipophilic (hydrophobic) compound and therefore a suitable solvent is needed to dissolve it and to hold it in solution without precipitation, particularly when added to the modified tissue culture media (Vandhana *et al*. 2010). Triclosan has been shown to be soluble without precipitation in dimethyl sulphoxide (DMDO) and has been previously used in solubilising triclosan for evaluating its anticarcinogenic effect (Vandhana *et al*. 2010). Triclosan was dissolved in DMSO to get a final concentration of 3.2 mg/mL.The following dilution method used for solubilising and diluting water-insoluble therapeutic agents was used to solubilise and dilute triclosan in media. Triclosan was diluted in DMSO and then this was diluted 1:100 in media using the dilution method shown in **Table 4.1** without precipitation. Thus, a concentration series of triclosan in media was generated to evaluate the MIC and MBC of triclosan for SAG.

Table 4.1: Intermediate concentration series of triclosan in DMSO and final concentration of triclosan when diluted in MTCM.

Step	Concentration in µg/mL	Source	Volume mL	Solvent volume mL (DMSO)	Intermediate concentration µg/mL (DMSO)	Final concentration 1:100 µg/mL in media
1	3200 (Stock)				3200	32
2	3200	Stock	0.5	0.5	1600	16
3	3200	Stock	0.5	1.5	800	8
4	3200	Stock	0.5	3.5	400	4
5	400	Step 4	0.5	0.5	200	2
6	400	Step 4	0.5	1.5	100	1
7	400	Step 4	0.5	3.5	50	0.5
8	46.6	Step 7	0.5	0.5	25	0.25
9	46.6	Step 7	0.5	1.5	12.5	0.12
10	46.6	Step 7	0.5	3.5	6.25	0.06
11	5.12	Step 10	0.5	0.5	3.12	0.03

4.2.2 Establishing the MIC and MBC of triclosan against SAG

MIC and MBC are the most commonly used antimicrobial outcome indicators. In order to establish the MIC of triclosan against S*. anginosus* 39/2/14A and *S. constellatus* 45386, a standard suspension of *S. anginosus* 39/2/14A and *S. constellatus* 45386 10^5cfu/mL was prepared in BHI as described in **section 2.2.3.1**. The same suspension was prepared in MTCM in order to evaluate the effect of the culture media on MIC and MBC. The bacterial suspension was substituted for media to develop a triclosan concentration series of 32 µg/mL–0.03 µg/mL in 10^5 cfu/mL SAG suspension in BHI or MTCM (**Table 4.2**) to produce a final concentration series of 32 µg/mL–0.03 µg/mL of triclosan in 10^5 cfu/mL SAG bacterial suspension. Two hundred µL of each of the concentrations of triclosan SAG bacterial suspension in the series were placed in triplicate in a 96-well plate.

Table 4.2*:* Concentration series of triclosan in bacterial suspension prepared in BHI and MTCM for evaluation of MIC and MBC

Step	Concentration in µg/mL	Source	Volume mL	Solvent volume mL (DMSO)	Intermediate concentration µg/mL (DMSO)	Final concentration 1:100 µg/mL SAG bacterial suspension
1	3200			1	3200	32
2	3200	Stock	0.5	0.5	1600	16
3	3200	Stock	0.5	1.5	800	8
4	3200	Stock	0.5	3.5	400	4
5	400	Step 4	0.5	0.5	200	2
6	400	Step 4	0.5	1.5	100	1
7	400	Step 4	0.5	3.5	50	0.5
8	46.6	Step 7	0.5	0.5	25	0.25
9	46.6	Step 7	0.5	1.5	12.5	0.12
10	46.6	Step 7	0.5	3.5	6.25	0.06
11	5.12	Step 10	0.5	0.5	3.12	0.03

The positive control was the SAG suspension treated with 2% chlorhexidine as 2% chlorhexidine has been proven to possess a bactericidal percentage as high as 99% against streptococcus species (Borges *et al*. 2012; Ruiz-Linares *et al*. 2014). The negative control was sterile water added to the SAG suspension. To evaluate if DMSO had any antimicrobial effect on SAG suspension a similar concentration series as described in **Table 4.2** was created in

DMSO with no triclosan. The plate was incubated at 37^{o}C in 5% CO_2 overnight. The MIC was recorded as the lowest concentration of triclosan at which growth of SAG cannot be visibly seen in the well following the overnight incubation. The same triclosan series was created for *Staphylococcus aureus* 9518 (*S. aureus* 9518). This was done in order to validate the dilution method used to produce different concentrations of triclosan **Table 4.1**. It was tested on *S. aureus* 9518 for which triclosan MIC and MBC is established in the literature (Al-Doori *et al*. 2003).

In order to establish the MBC, trypticase soy agar (TSA) containing lecithin and Tween 80 was prepared by mixing 40 g TSA powered agar, 0.7 g Lecithin, 5 g of Tween 80 in 1 L of deionised water, which was autoclaved at 121^{o}C for 15 min and then poured on to sterile petri dishes, in order to neutralise any residual active triclosan. 50 µL of SAG triclosan dilutions that did not show visible SAG growth in the well were plated on the TSA and incubated at 37^{o}C in 5% CO_2 for 24 h to detect inhibited but viable SAG. The MBC was the lowest concentration of triclosan that killed 99.9% of bacteria in the initial inoculum. The experiments were conducted in triplicate. A control to evaluate effectiveness of the neutralising agar was also performed. A fresh mix of SAG suspension in triclosan-containing medium was prepared and immediately plated out on media containing the neutralisers and incubated at 37^{o}C in 5% CO_2 for 24 h.

4.2.3 Evaluating the effect of triclosan and DMSO on the *ex vivo* tooth slice model

Tooth slices were prepared as described in **section 2.2.4**. Triclosan at a concentration of 8 µg/mL in MTCM was prepared as described in **section 4.2.1**. Tooth slices were incubated in MTCM containing triclosan at 37^{o}C in 5% CO_2 for 24 h. Tooth slices were then harvested and processed for histology as described in **section 2.2.6**. Viable odontoblast and dental pulp cells were then quantified as described in **section 2.2.7**. Five control tooth slices were incubated in MTCM without triclosan. Experiments were conducted using five tooth slices.

4.2.4 Triclosan treatment protocol for treatment of SAG pulpal infection

Two different triclosan treatment protocols were used to evaluate the benefit of triclosan in the treatment of SAG pulpal infection.

4.2.4.1 Triclosan pre-treatment protocol

Tooth slices were prepared and treated with 8 µg/mL triclosan for 24 h. Following this they were infected with 10^2 cfu/mL of SAG suspension over 24 h at 37^{o}C in 5% CO_2 on a rocking platform. Tooth slices were harvested at 0, 6, 12 and 24 h time points. The control was tooth slices infected with SAG but not treated with triclosan.

4.2.4.2 Triclosan post-treatment protocol

Tooth slices were prepared as described in **section 2.2.4** and were infected with 10^2 cfu/mL of SAG suspension then incubated for 6 h at 37^{o}C in 5% CO_2 on a rocking platform. They were transferred into MTCM containing 8 µg/mL of triclosan and incubated for 18 h. Tooth slices were harvested at 12 h and 24 h. The control was tooth slices infected with SAG but not treated with triclosan.

4.2.5 Evaluation of the effect of pre- and post-triclosan treatment of SAG infected pulp by viable cell count.

Two mm tooth slices were subjected to triclosan pre-treatment followed by SAG infection and post-treatment following initial infection with SAG as described in **section 4.2.4** were harvested and processed for histology as described in **section 2.2.6**) Viable odontoblast and dental pulp cells were quantified as described in **section 2.2.7**. They were compared against tooth slices infected with SAG over the same time period to evaluate the efficacy of triclosan treatment in preventing odontoblast and dental pulp cell death. Experiments were conducted in triplicate.

4.2.6 Evaluation of the effect of pre- and post-triclosan treatment on tooth slice colonisation of SAG

Two mm tooth slices were subjected to triclosan pre-treatment followed by SAG infection and post-treatment following initial infection with SAG as described in **section 4.2.4**. The only difference was that the SAG used to infect the tooth slices were stained with FDA as described in **section 2.2.8**. Tooth slices were harvested at 0, 6, 12 and 24 h for the triclosan pre-treatment group and 0, 12 and 24 h for the triclosan post-treatment group. Tooth sections were fixed and processed as described in **section 2.2.6** except that they were not subjected to H&E staining and viewed under fluorescent microscope with FITC filter. The area of SAG bacterial colonisation was quantified as described in **section 2.2.9**. Experiments were conducted on five tooth slices.

4.2.7 Evaluation of the effect of triclosan on expression of inflammatory markers in pulp tissue using the *ex vivo* SAG tooth slice infection model

Tooth slices of 4 mm length were prepared and cultured as described in **section 2.2.4**, **infected** with 10^2 cfu/mL of SAG suspension *S. anginosus* 39/2/14A or *S. constellatus* 45386 and incubated at 37^{o}C in 5% CO_2 over 6 h. Following this, they were subjected to the triclosan post-treatment protocol for 24 h at 37^{o}C in 5% CO_2. The effect of triclosan on inflammatory marker expression within the pulp tissue was assessed by treating uninfected tooth slices with 8 µg/mL triclosan. Tooth slices cultured in MTCM were used as negative controls.

Pulp tissue was extirpated from the tooth slice after 24 h and RNA extracted and expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 and IL-10 assessed as described in **section 2.2.10.2**. Experiments were conducted on five-tooth slices in triplicate.

4.2.8 Evaluation of anti-hyaluronidase activity of triclosan and DMSO

4.2.8.1 Culture of SAG in media containing substrate for promoting synthesis of hyaluronidase

Modified tissue culture media were prepared to enable SAG to produce hyaluronidase by adding 4 mg of hyaluronic acid sodium salt from bovine vitrious humor (Sigma-Aldrich) to 10 mL of media to give a final concentration of 400 µg/mL. A single colony of SAG from an overnight culture of SAG was inoculated into the media and incubated at 37°C, in 5% CO_2 overnight. This was then centrifuged at 16,000 x g for 30 s and the supernatant was filter sterilised with a 0.2 µm filter and used for evaluation of hyaluronidase activity.

4.2.8.2 Evaluation of anti-hyaluronidase activity of Triclosan

Ten µL of 800 µg/mL of triclosan in DMSO was added to 990 µL of bacterial supernatant as prepared in **section 4.2.8.1** and mixed well to give a final concentration of 8 µg/mL. The anti-hyaluronidase activity of DMSO was assessed by adding DMSO at a concentration of 1% to the supernatant and mixing it well. These samples were incubated at 37°C in 5% CO_2 for 24 h. Ten µL of the supernatants treated with triclosan or DMSO for 24 h were plated on BHI and hyaluronic acid agar plates, prepared to identify hyaluronidase activity as described in **section 3.2.8**. Ten µL of denatured supernatant was used as negative control and 10 µL of each S*. constellatus* 45386 supernatant without triclosan treatment and hyaluronidase from *S. pyogenes* (Sigma) was used as a positive control.

4.2.9 Statistical analysis

Data was statistically analysed to detect significances in difference between infected tooth slices and the effect of triclosan treatment on SAG infection by the Mann-Whitney U test using Graph Pad statistical software. Analysis was undertaken to determine any significant difference in cell counts, area of colonisation and inflammatory marker expression between the experimental group and the controls.

4.3 Results

4.3.1 Effect of media on MIC and MBC of triclosan

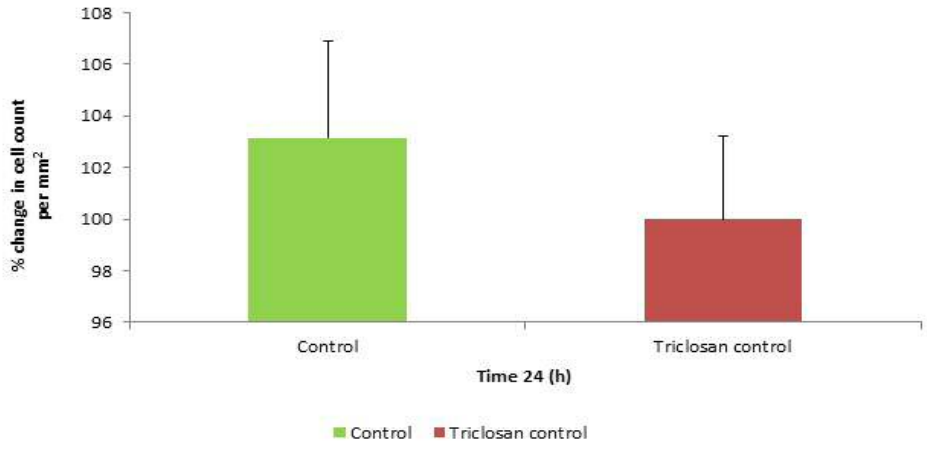
SAG bacteria approximately 10^5 cfu/mL in BHI and MTCM were incubated at 37°C, in 5% CO_2 overnight in the presence of a range of triclosan concentrations from 32 – 0.03 µg/mL prepared in DMSO. MIC was established as the lowest concentration of triclosan in which no visible growth of bacteria was observed. MBC was assessed by the presence of viable SAG when plated on TSA plates containing neutraliser and incubated at 37°C in 5% CO_2 for 24 h, despite the absence of turbidity in the wells. MBC was defined as the concentration of triclosan, which produced 99.9% reduction in cfu/mL compared to the initial inoculum of SAG when plated on TSA plate and incubated at 37°C in 5% CO_2 for 24 h. MIC and MBC for both strains of SAG in BHI was lower than in MTCM **Table 4.3**. Two percent chlorhexidine (the positive control) was bactericidal to SAG, whereas the negative control water, did not affect the growth of SAG. The validation with *S. aureus* 9518 in BHI gave an MIC of 0.5 µg/mL and MBC of 2 µg/mL.

Table 4.3: MIC and MBC of triclosan for SAG in BHI and MTCM

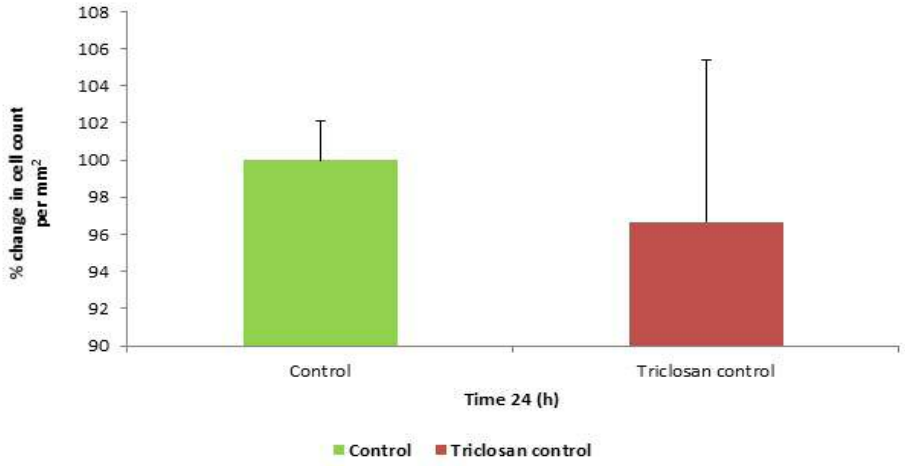
Triclosan	*S. anginosus* 39/2/14a		*S. constellatus* 45386	
	BHI	MTCM	BHI	MTCM
MIC	0.5 µg/mL	4 µg/mL	0.5 µg/mL	4 µg/mL
MBC	0.5 µg/mL	4 µg/mL	0.5 µg/mL	8 µg/mL

4.3.2 Effect of triclosan on pulp cell viability

Tooth slices were incubated at 37°C in 5% CO_2 with triclosan for 24 h and then processed. Viable odontoblast and dental pulp cells were counted and compared with the odontoblast and dental pulp cell count of the control tooth slices. There was a decrease in odontoblast and dental pulp cell count in tooth slices treated with triclosan (**Figure 4.1**). The difference was not statistically significant for either odontoblasts (p=0.556) or dental pulp cells (p=0.730) compared to the control tooth slice cell counts.



(a)



(b)

Figure 4.1 Evaluation of the effect of triclosan on odontoblast and dental pulp cell viability after 24h.

Odontoblast and dental pulp cell counts performed on transverse sections of tooth slices. Odontoblast & dental pulp cell counts were estimated as % change in cell count per mm² of the pulp relative to control. Error bars indicate SE for experiments conducted in triplicate. Tooth slices were treated with triclosan in MTCM (triclosan controls) and controls in just MTCM a) odontoblasts; b) dental pulp cells (*=$p<0.05$ was considered significant from Mann–Whitney U test).

4.3.3 Effect of triclosan on pulp cell viability: of triclosan treated SAG infection

4.3.3.1 Effect of triclosan pre-treatment

When tooth slices were treated with triclosan overnight, infected with SAG for 24 h, and processed for counting of viable odontoblast and dental pulp cells, it was observed that triclosan pre-treatment caused more cells to survive compared to the untreated infection **Figures 4.2 & 4.3**. This difference for odontoblasts was not statistically significant in *S. anginosus* 39/2/14a infection, but for *S. constellatus* 45386 infection showed statistically significant higher cell viability when treated with triclosan only for the 24 h time point **Table 4.4, Figure 4.2b**. In the case of dental pulp cells, the pre-treatment significantly reduced the cell death at 12 h and 24 h for infections with both strains of SAG **Table 4.4, Figure 4.3**.

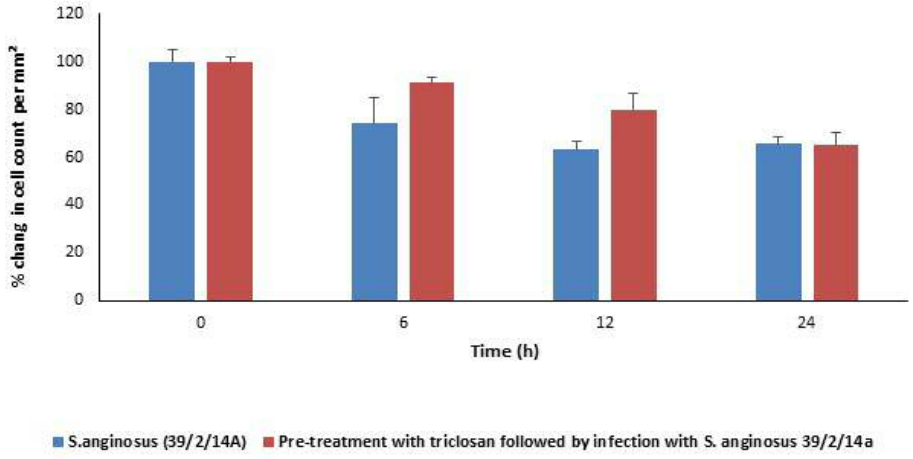
Table 4.4: Statistical comparison of cell viability from triclosan pre-treatment protocol for SAG pulpal infection to uninfected pulp.
Mann–Whitney *U* test comparing cell viability in tooth slices treated with 8 µg/mL triclosan followed by infection with SAG compared to untreated SAG infection.

Type of cell	***S. anginosus* 39/2/14a**			***S. constellatus* 45386**		
	6 h	**12 h**	**24 h**	**6 h**	**12 h**	**24 h**
Odontoblast	p=0.189	p=0.092	p=0.352	p=0.826	p=0.947	p=0.028*
Dental pulp cells	p=0.374	p=0.016*	p=0.005**	p=0.769	p=0.033*	p=0.028*

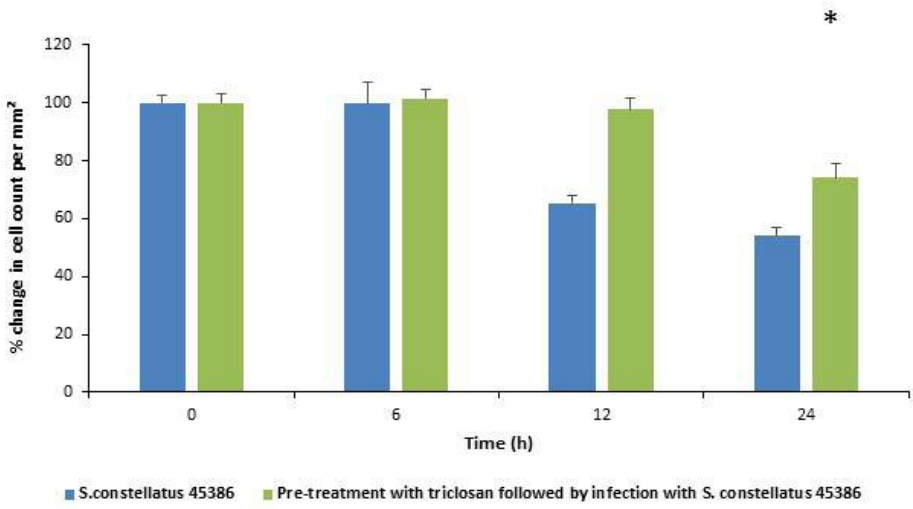
*Statistically significant

4.3.3.2 Effect of triclosan post-treatment

Treatment of SAG with triclosan caused a reduction in odontoblast cell death **Figure 4.4** and this reduction was significant compared to untreated infection for *S. anginosus* 39/2/14a at 24 h and for *S. constellatus* 45386 at 12 h and 24 h **Table 4.5**. The reduction in dental pulp cell death was observed at 12 h and 24 h for both strains of SAG **Figure 4.5**. The reduction in odontoblast and dental pulp cell death was significant compared to untreated infection for these time points as well **Table 4.5**.

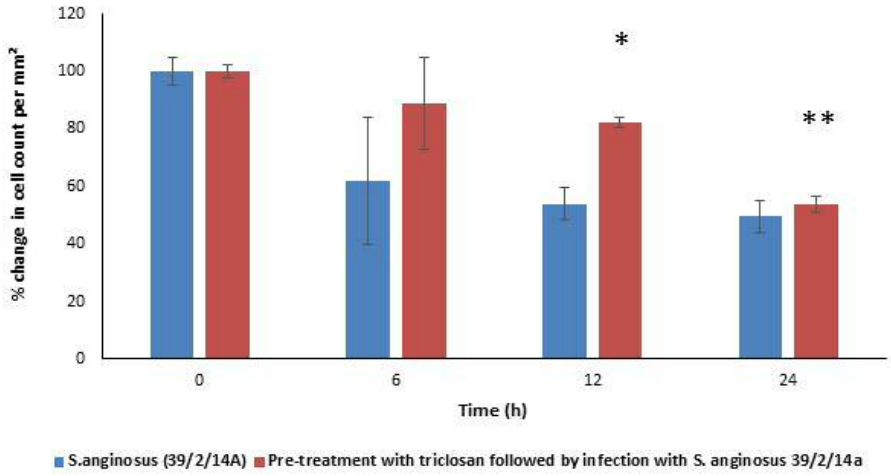


(a)

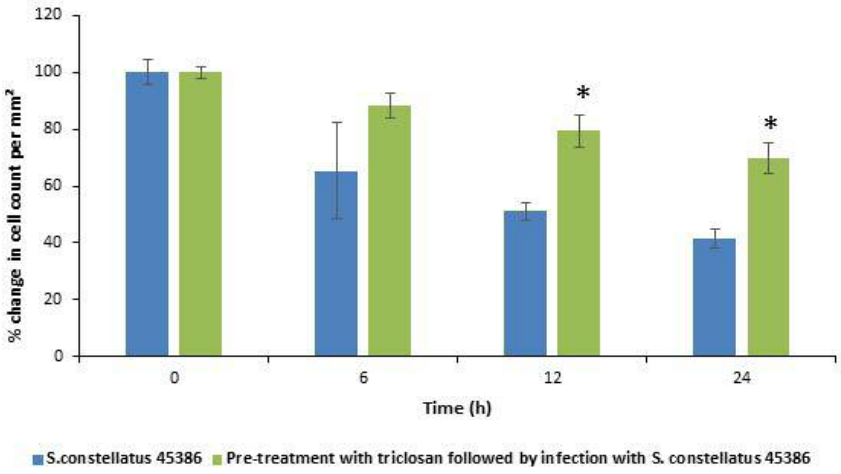


(b)

Figure 4.2: Effect of triclosan pre-treatment protocol on odontoblast count over 24 h.
Odontoblast count performed on transverse section of tooth slices and odontoblast count estimated as % change in cell count per mm² of the pulp relative to untreated pulpal infection. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05).

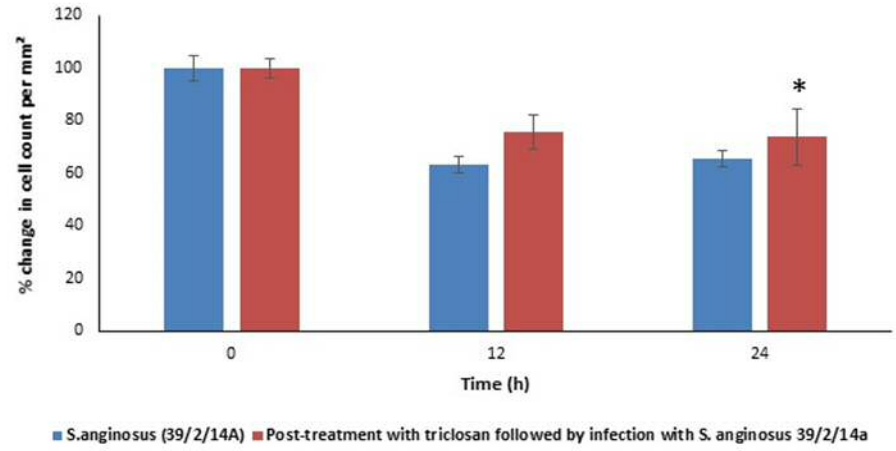


(a)

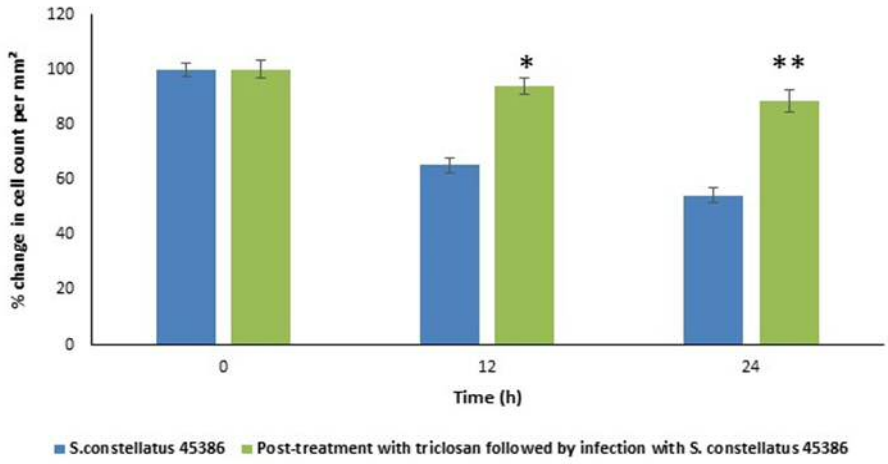


(b)

Figure 4.3: Effect of triclosan pre-treatment protocol on the dental pulp cell count over 24 h. Dental pulp cell count performed on transverse sections of tooth slices and dental pulp cell count estimated at % change in count per mm² of the pulp relative to untreated pulpal infection. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.005).

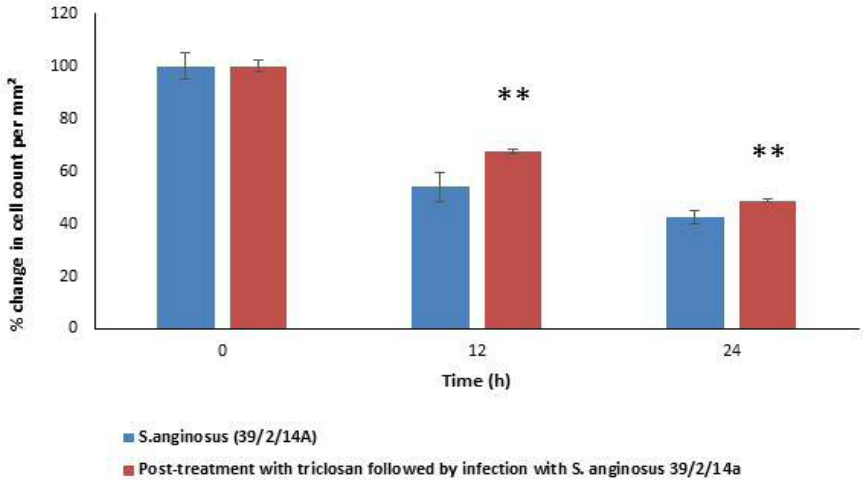


(a)



(b)

Figure 4.4: Effect of triclosan post-treatment protocol on the odontoblast count over 24 h. Odontoblast cell count performed on transverse sections of tooth slices and odontoblast count estimated per mm² of the pulp relative to untreated pulpal infection. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.005)



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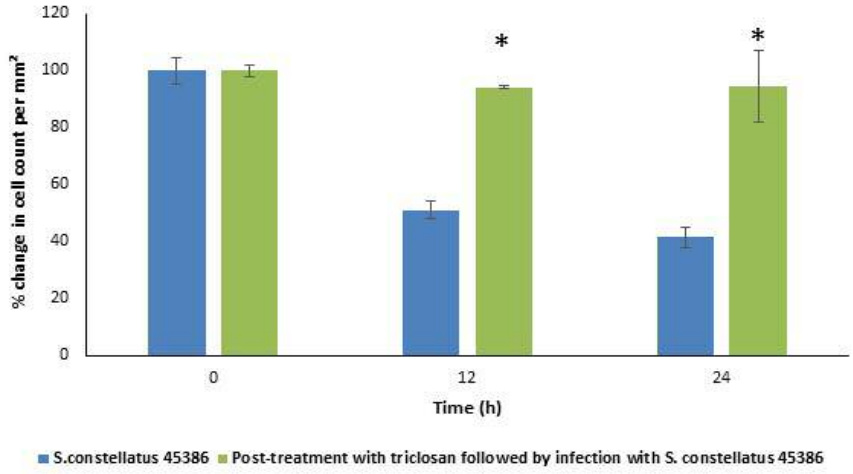


(b)

Figure 4.5: Effect of triclosan post-treatment on the dental pulp cell count over 24 h.
Dental pulp cell count performed on transverse sections of tooth slices and dental pulp cell count estimated as % change per mm² of the pulp relative to untreated pulpal infection. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.01).

Table 4.5: Statistical comparison of cell viability from triclosan post-treatment protocol for SAG pulpal infection to uninfected pulp.
Mann–Whitney *U* test comparing cell viability in tooth slices infection with SAG followed by treatment with 8 µg/mL triclosan to untreated infection showed.

Type of cell	***S. anginosus* 39/2/14a**		***S. constellatus* 45386**	
	12 h	**24 h**	**12 h**	**24 h**
Odontoblasts	p=0.157	p=0.023*	p=0.041*	p=0.002*
Dental pulp cells	p=0.008**	p=0.006**	p=0.048*	p=0.024*

*Statistically significant

4.3.4 Effect of triclosan on SAG bacterial colonisation of dental pulp

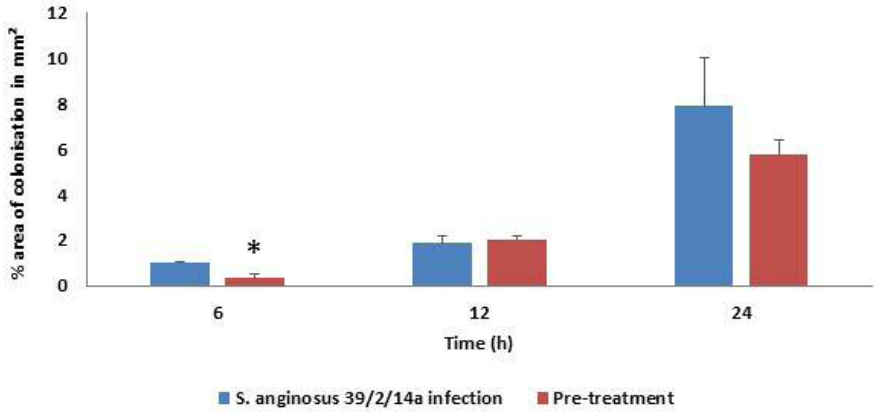
4.3.4.1 Effect of triclosan pre-treatment on SAG bacterial colonisation

When the tooth slices were pre-treated with triclosan and infected with SAG bacteria for 24 h and the pulp tissue assessed for the area of colonisation by *S. anginosus* 39/2/14a, there was a reduction in area of colonisation compared to SAG infection in untreated pulp **Figure 4.6** but this was statistically significant only for the 6 h time point (p=0.019) **Table 4.6**. In *S. constellatus* 45386 infection there was an increase in area of SAG colonisation in triclosan pre-treated and untreated pulp at 12 h and 24 h **Figure 4.6** but an initial significant reduction in area of colonisation was noted only at 6 h (p=0.22) **Table 4.6**.

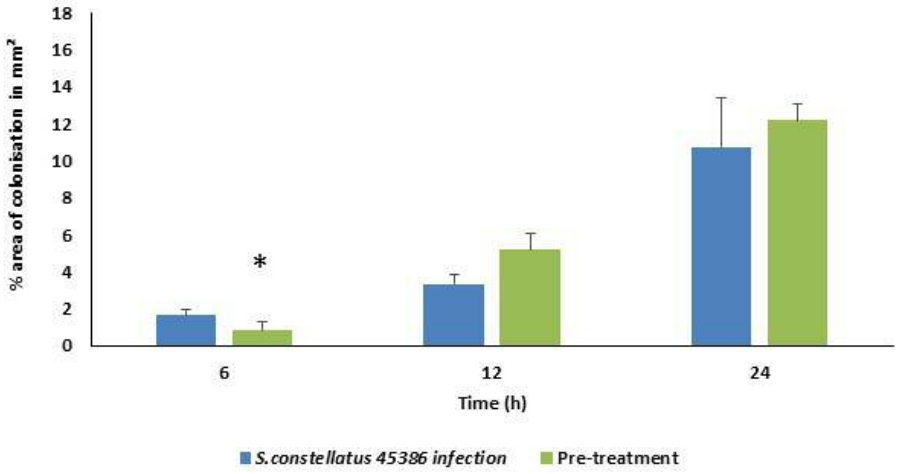
Table 4.6: Statistical comparison of the effect of triclosan pre-treatment on area of colonisation of SAG.
Mann–Whitney *U* test comparing area of colonisation of the pulp tissue by SAG when pre-treated with triclosan before SAG infection compared to untreated infection.

Triclosan treatment	***S. anginosus* 39/2/14a**			***S. constellatus* 45386**		
	6 h	12 h	24 h	6 h	12 h	24 h
Pre-treatment	p=0.019* ▲	p=0.66	p=0.079	p=0.22* ▲	p=0.151	p=0.642

*Statistically significant ▲ decreased expression



(a)



(b)

Figure 4.6: Effect of triclosan pre-treatment on SAG colonisation of pulp tissue over 24 h.
The area of fluorescence from FDA stained SAG measured relative to the total area of the pulp. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=$p<0.05$).

4.3.4.2 Effect of triclosan post-treatment on SAG bacterial colonisation

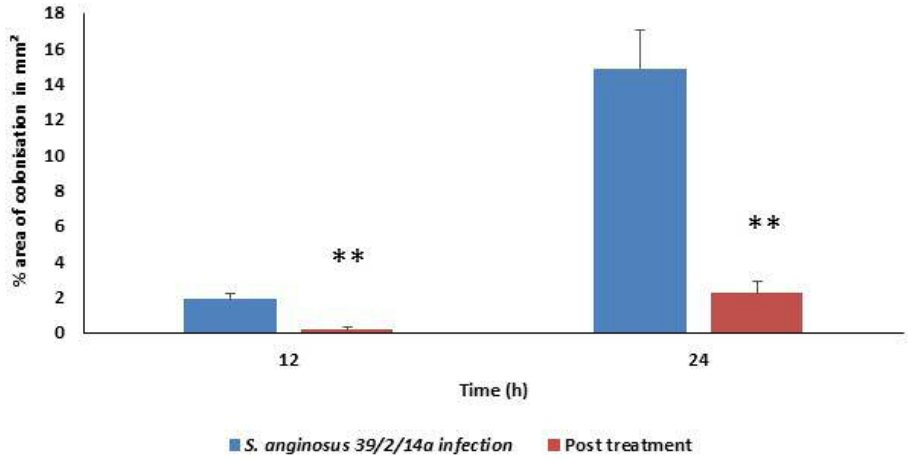
The treatment of SAG infection with triclosan over 24 h caused a significant reduction in the area of colonisation of the pulp by both strains of SAG compared to untreated SAG infection **Figure 4.7**. This was statistically significant for 12 h and 24 h time points **Table 4.7**.

Table 4.7: Statistical comparison of the effect of triclosan post-treatment on area of colonisation of SAG.

Mann–Whitney *U* test comparing area of colonisation of the pulp tissue by SAG when established SAG infection was treated with 8 μg/mL triclosan and compared to untreated infection.

Triclosan treatment	***S. anginosus* 39/2/14a**		***S. constellatus* 45386**	
	12 h	24 h	12 h	24 h
Post-treatment	p=0.004* ▲	p=0.005* ▲	p=0.003* ▲	p=0.041* ▲

*Statistically significant ▲ decreased expression



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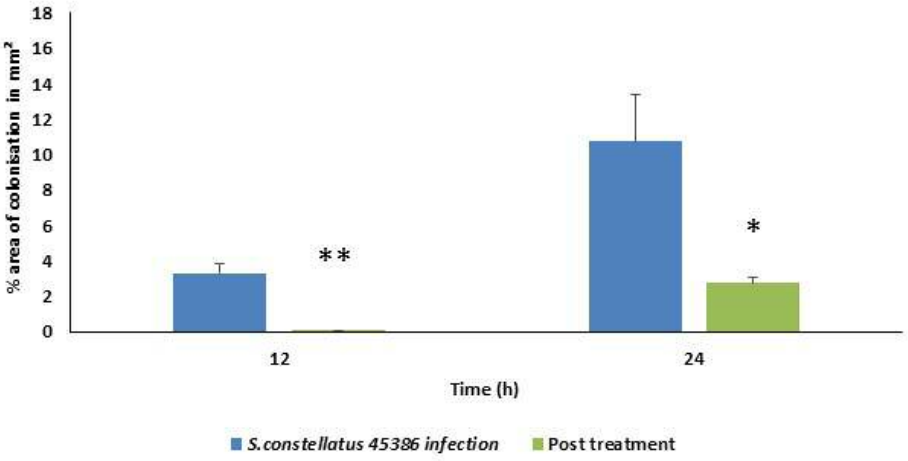


(b)

Figure 4.7: Effect of triclosan post-treatment on SAG colonisation of pulp tissue over 24 h.
The area of fluorescence from FDA-stained SAG measured relative to the total area of the pulp. Error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.005).

4.3.5 Modulation of inflammatory response by triclosan in *ex vivo* pulpal infection

Pulp tissue treated with triclosan was assessed for expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 and IL-10. These expressions were compared to expression of the same markers by control tooth slices not treated with triclosan **Figures 4.8 & 4.9**, the difference in expression was not statistically significant **Table 4.8**.

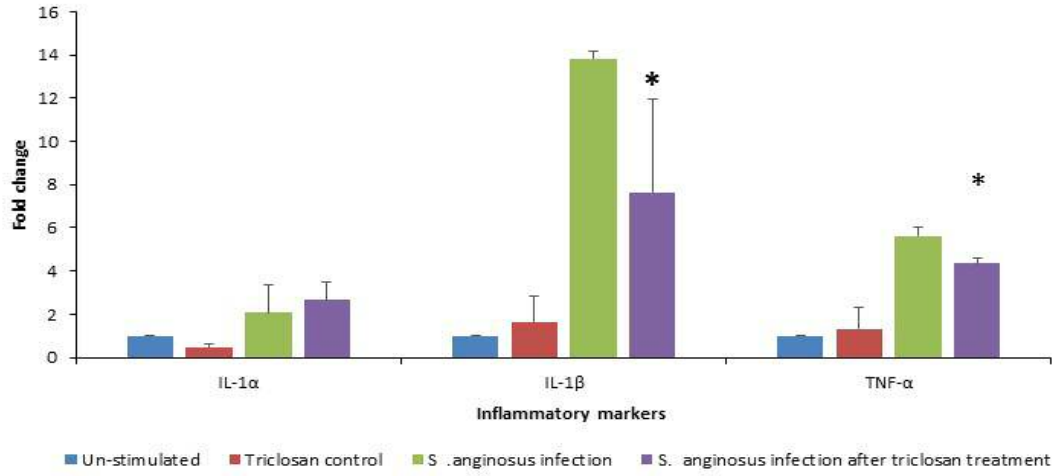
Table 4.8: Statistical comparison of inflammatory marker expression in triclosan treated pulp and pulp not treated with triclosan.

Mann–Whitney *U* test comparing expression of inflammatory markers expressed by triclosan treated uninfected pulp to uninfected pulp not treated with triclosan.

Inflammatory markers	Triclosan control
IL-α	p= 0.062
IL-1β	p= 0.401
TNF-α	p= 0.603
IL-10	p= 0.115
IL-18	p= 0.056

*Statistically significant

When the expression of inflammatory markers IL-1α, IL-1β, TNF-α, IL-18 by the triclosan control pulp tissue was lower than in tissue without triclosan treatment **Figures 4.8 & 4.9** In the case of anti-inflammatory marker IL-10, the reduction was significant only for *S. constellatus* 45386 (p=0.033) and not for *S. anginosus* 39/2/14a (p= 0.096). This increase was statistically significant for all pro-inflammatory markers except IL-1α **Table 4.9**.



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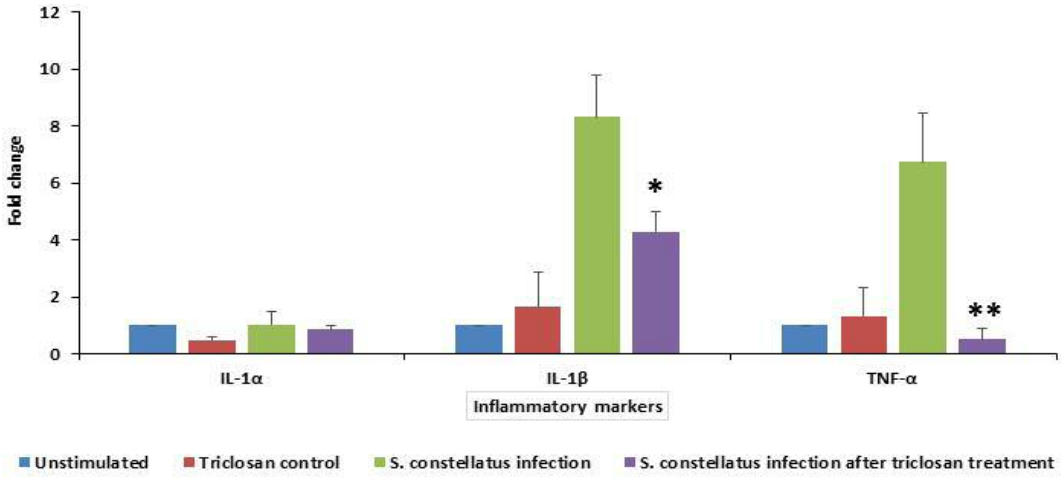


(b)

Figure 4.8: Effect of triclosan post-treatment on IL-1α, IL-1β and TNF-α expression in *ex vivo* SAG infection.

Expression of IL-1α, IL-1β and TNF-α in dental pulp tissue infected with SAG bacteria compared with SAG infected pulp treated with triclosan relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.005).

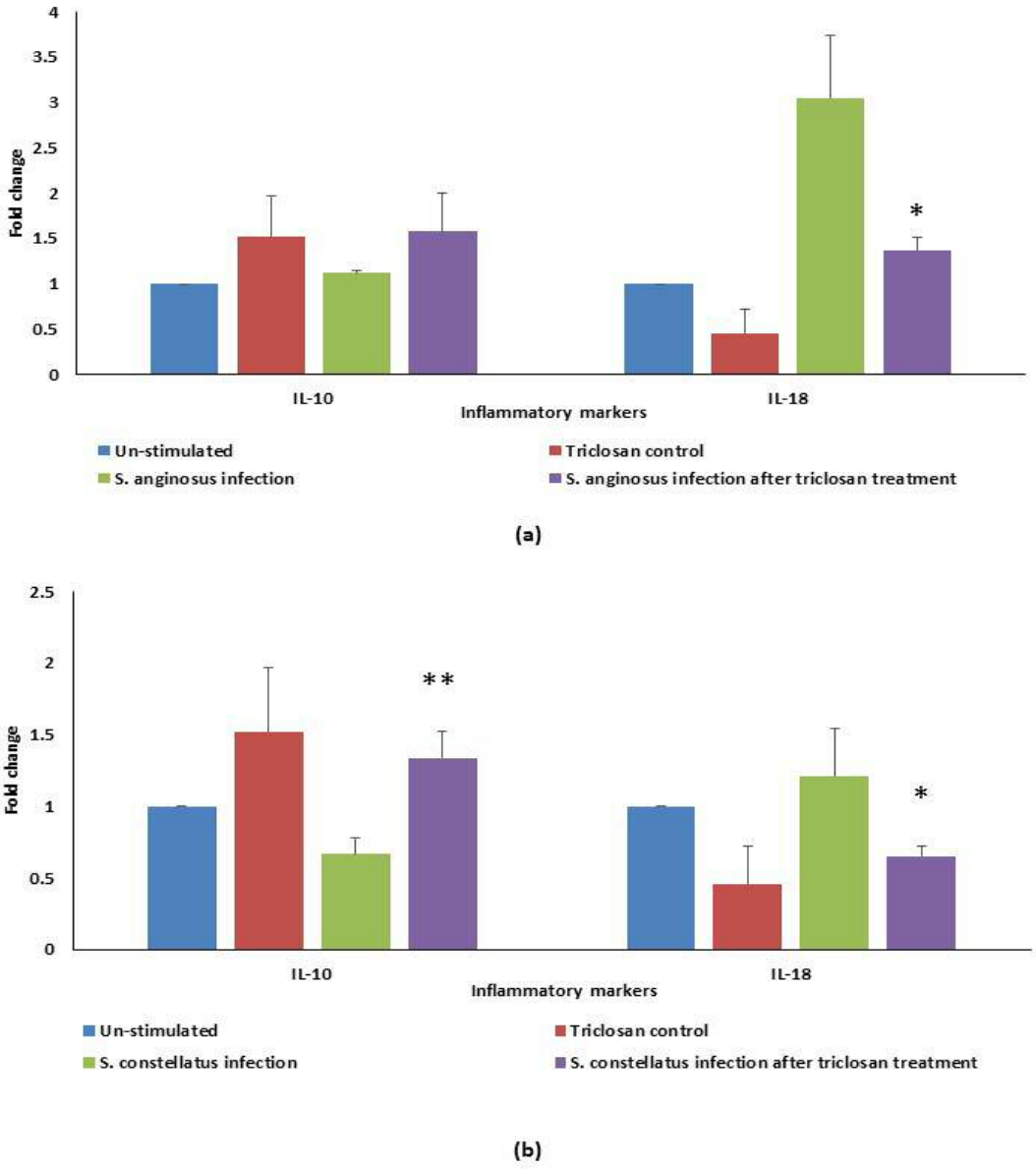


Figure 4.9: Effect of triclosan post–treatment on IL-18 and IL-10 expression in *ex vivo* SAG infection. Expression of IL-18 and IL-10 in dental pulp tissue infected with SAG bacteria compared with SAG infected pulp treated with triclosan relative to β-actin, assessed by RT-qPCR over a period over 24 h. Each bar represents fold change compared to the housekeeping gene β-actin. The error bars indicate SE for experiments conducted in triplicate. a) *S. anginosus* 39/2/14a; b) *S. constellatus* 45386 (*=p<0.05, **=p<0.005).

Table 4.9: Statistical comparison of inflammatory marker expression in triclosan treated SAG infected pulp to triclosan treated uninfected pulp.

Mann–Whitney *U* test comparing expression of inflammatory markers expressed by triclosan treated control pulp to SAG infected pulp.

Inflammatory markers	***S. anginosus* 39/2/14a**	***S. constellatus* 45386**
IL-α	p=0.092	p=0.133
IL-1β	p= 0.002*	p=0.003*
TNF-α	p= 0.002*	p=0.009*
IL-10	p= 0.096	p=0.033* ▲
IL-18	p= 0.003*	p=0.035*

*Statistically significant ▲ decreased expression

When the effect of triclosan treatment on the expression of pro-inflammatory markers in SAG infection of the pulp was evaluated and compared to untreated SAG infection **Figures 4.8 & 4.9**, the triclosan treatment of SAG pulpal infection significantly reduced the expression of IL-1β, TNF-α and IL-18, but change in expression of IL-1α was not statistically significant **Table 4.10**. The triclosan treatment increased expression of the anti-inflammatory marker IL-10 but this was significant only for *S. constellatus* 45386 infection (p=0.006).

Table 4.10: Statistical comparison of inflammatory marker expression in triclosan treated SAG infected pulp to untreated infected pulp.

Mann–Whitney *U* test comparing effect of triclosan treatment of SAG infection on the expression of inflammatory markers to that of untreated infection.

Inflammatory markers	***S. anginosus* 39/2/14a treated with triclosan**	***S. constellatus* 45386 treated with triclosan**
IL-α	p=0.552	p=0.587
IL-1β	p=0.011* ▲	p=0.013* ▲
TNF-α	p=0.010* ▲	p=0.003* ▲
IL-10	p=0.133	p=0.006**
IL-18	p=0.014* ▲	p=0.043* ▲

*Statistically significant ▲ decreased expression

4.3.6 Triclosan and DMSO interaction with SAG bacterial supernatant proteins

Triclosan treatment of the *S. constellatus* 45386 supernatant did not affect the activity of hyaluronidase, which was manifested by the appearance of the clear zone **Figure 4.10d** due to degradation of the hyaluronic acid as seen with the positive control **Figure 4.10b**. DMSO also did not have any anti-hyaluronidase activity as it also produced a clear zone **Figure 4.10c**. Heat-inactivated *S. constellatus* 45386 supernatant showed very little hyaluronidase activity as it produced a very small clear zone as demonstrated in **Figure 4.10a**.

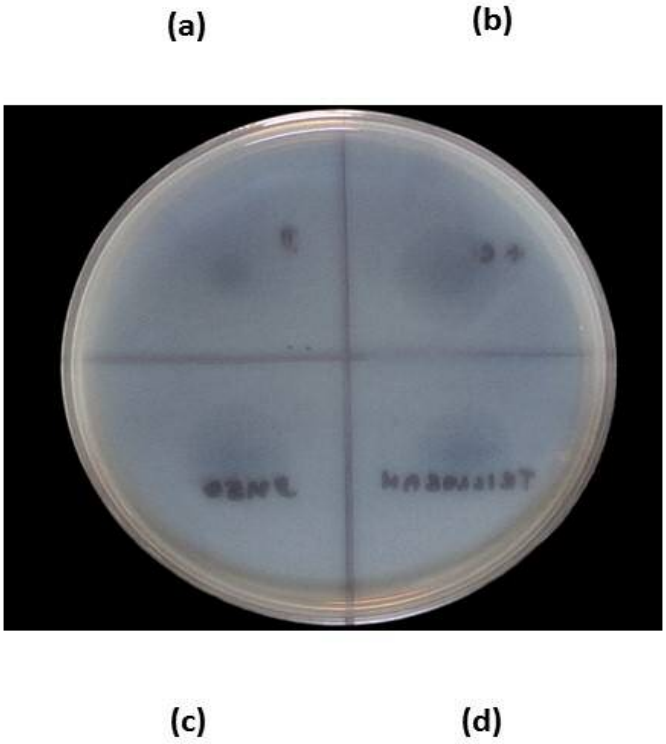


Figure 4.10: Evaluation of the anti-hyaluronidase activity of triclosan on *S. constellatus* 45386 supernatant.
Anti-hyaluronidase activity of triclosan through the prevention of clear zone formation by degradation of hyaluronic acid**.** a) heat -nactivated *S. constellatus* 45386 supernatant; b) positive control; c) *S. constellatus* 45386 supernatant treated with DMSO; d) *S. constellatus* 45386 supernatant treated with triclosan.

4.4 Discussion

When triclosan was solubilised in media using the technique to dissolve hydrophobic antimycotic agents, it was not clear that this protocol would be sufficient to determine MIC and MBC. Therefore, the protocol was validated against *S. aureus* 9518, whose triclosan MIC and MBC was known from the literature (Schmid and Kaplan 2004). The MIC and MBC for triclosan against *S. aureus* 9518 assessed by the method used in this work concurred with previous published values (Al-Doori *et al*. 2003). Through the evaluation of MIC and MBC of triclosan by culture of SAG in BHI and MTCM, it was observed that the MIC and MBC of triclosan for SAG were affected by the type of medium in which the bacteria were cultured. MIC and MBC were higher for SAG cultured in MTCM. It has been shown that FBS contains a wide range of proteins and mineral ions (Lindl 2002), which may be bind to the active sites of triclosan and reduce the free active triclosan, leading to a higher triclosan concentration.

It was previously estimated that the triclosan MIC for SAG was 125 µg/mL (Roberts 2010). This concentration of triclosan was very high compared to the reported MIC for streptococci (Schmid and Kaplan 2004). An MIC of 0.5 µg/mL and 4 µg/mL for BHI and MTCM, respectively, for both strains of SAG was established in this work. Furthermore, *S. constellatus* 45386 and *S. anginosus* 39/2/14a had an MBC of 8 µg/mL and 4 µg/mL, respectively, in MTCM. These concentrations, established by this work, were more consistent with the literature. Therefore, the concentration of triclosan used in this work was set at 8 µg/mL to make it effective against both strains of SAG. Furthermore, previous work (Roberts 2010) only assessed the antimicrobial activity of triclosan against SAG, but did not evaluate its effectiveness as an antimicrobial and anti-inflammatory agent in the management of pulpal infection(Roberts 2010) .

It was necessary to evaluate the toxicity of triclosan dissolved in DMSO on pulp tissue before deciding to use it in the treatment of pulpal infection. It has been shown that triclosan is toxic to epithelial cells but not to fibroblasts (Babich and Babich 1997). In order to evaluate toxic effects against a more complex tissue model, tooth slices were incubated in 8 µg/mL (MBC) of triclosan overnight and the viable odontoblast and dental pulp cells were counted. There was no significant difference between controls and tooth slices treated with triclosan ($p=0.556$ and $p=0.730$) for odontoblasts and dental pulp cells, respectively. It was accepted that triclosan was not toxic to pulp tissue. Furthermore, the fact that triclosan was not toxic

to human beings has been substantiated by the use of triclosan in the treatment of periodontal and oral mucosal diseases by its presence in various toothpastes (Trombelli and Farina 2013).

Pulpal inflammation is caused by bacterial challenge to the pulp. Deep carious lesions have been shown to cause pulpal inflammation (Reeves and Stanley 1966). In situations where bleeding from the pulp occurs during excavation of deep carious lesions in an asymptomatic tooth where pulp capping is performed, bacteria may be sealed in the pulp horn and eventually the pulp becomes infected. In these cases, the successful resolution of pulpal infection was markedly reduced (Bjørndal *et al*. 2010). Therefore, bacteria may be present in pulp tissue even though the pulp may be asymptomatic. Furthermore, in situations where there may be risk of pulp exposure on complete caries excavation in an asymptomatic tooth, a procedure called step-wise excavation or selective excavation may be carried out (Banava 2011). This involves incomplete excavation of caries and sealing part of the caries and bacteria in the deeper part of the lesion with a restoration. Therefore, treatment of these deep lesions and any microscopic exposure of the pulp with antimicrobials as a preventive measure to protect the pulp from bacterial colonisation may improve the prognosis of the pulp tissue. On the other hand, in frank infection of pulp by caries, the pulp is already infected with bacteria, and this infection should be controlled to prevent necrosis of the pulp. This work evaluated the effectiveness of two modalities of treatment. The first modality was to evaluate if pre-treatment of the pulp with triclosan could be used prophylactically to prevent infection in an environment where bacteria cannot be eliminated as in deep carious lesions without risk of pulp exposure. The second modality was the treatment of established infection where triclosan is applied on the infected pulp to kill the bacteria and modulate the inflammation of the pulp, thereby helping it to heal and repair.

When the effect on cell viability was assessed to evaluate the effect of pre-treatment with 8µg/mL triclosan, it was noted that cell viability was higher for odontoblasts of the treated pulp compared to the untreated infection group, but the difference was not statistically significant. This was not the case with dental pulp cells as there was a significant reduction ($p=0.005$ and $p=0.028$) in cell death for and *S. anginosus* 39/2/14a and *S. constellatus* 45386, respectively, at 24 h. There was also significant reduction in dental pulp cell death at 12 h for *S. anginosus* 39/2/14a ($p=0.016$). This could be due to differences in susceptibility of different cells of the pulp to infection. With respect to the area of colonisation, pre-treatment with

triclosan only prevented or reduced colonisation for the first 6 hours. Pre-treatment was not effective in this model as the bacterial load did not remain the same and increased. In addition, the amount of triclosan in the pulp tissue was the only triclosan present and it may have leached out of the tissue or was effective only to prevent early colonisation. There may also be growth of the bacteria on the pulp from the bacteria that attach after the effect of triclosan has been lost from it leaching out from the pulp tissue. Once the bacterial load increased, there may not have been enough triclosan to be effective. Furthermore, the load of bacteria in a clinical infection would be reduced by mechanical debridement, which might make this treatment protocol work clinically. This would be the case in procedures such as vital pulp therapy and step-wise excavation as most bacteria would be removed and only some would be left due to risk of pulp exposure, therefore requiring triclosan to be effective only for the period immediately after restoration, whereby it can combat any residual bacteria.

In the treatment of established SAG pulp infection with triclosan, there was a significant increase in the viability of the odontoblasts ($p=0.023$ and $p=002$) for *S. anginosus* 39/2/14a and *S. constellatus* 45386 infections, respectively, at 24 h. Dental pulp cells showed significant retention of viability at 12 h and 24 h for both SAG strains. As for the effect on colonisation, there was a very significant reduction in the area of colonisation by both *S. anginosus* 39/2/14a ($p=0.004$ and $p=0.005$) and *S. constellatus* 45386 ($p=0.003$ and $p=0.041$) at 12 and 24 h, respectively. In contrast to pre-treatment, the post-treatment modality was effective in the current model. This may be because the bacterial load was immediately reduced by the addition of triclosan to the system and further there was more triclosan in the media to penetrate the pulp tissue and kill the bacteria that had colonised the pulp. Post-treatment, to some extent, also has the benefits of pre-treatment, as while controlling the infection the presence of triclosan in the immediate environment of the pulp will exert the pre-treatment effect of triclosan on the pulp tissue. Therefore, with respect to studying the effectiveness of triclosan on the infection model, the post-treatment modality was considered effective and this treatment was used to assess the anti-inflammatory effect of triclosan.

As mentioned in chapter 1 modulation of inflammation is necessary to limit tissue damage and promote healing. IL-1 markers are produced as an innate immune response and they have been shown to cause inflammatory tissue damage by direct activation of T helper cells

even without the adaptive immune component (Zeng 2014). Furthermore, TNF-α has been shown to possess the ability to stimulate dental pulp cells to differentiate into odontoblasts (Paula-Silva *et al*. 2009). When the anti-inflammatory effect of triclosan was assessed against the control pulp tissue, it was found that the level of inflammatory marker expression was higher in pulp treated with triclosan, but this was only marginal and not statistically significant. Pulp infected with SAG showed a significant increase in expression of pro-inflammatory markers IL-1β, TNF-α and IL-18 compared to the expression in the control pulp and pulp treated with triclosan. This was the same for both strains of SAG. To evaluate the anti-inflammatory effect of triclosan on the SAG infection of the pulp, the infection was treated with triclosan and then assessed for expression of anti-inflammatory marker IL-10. IL-10 was down-regulated for both strains of SAG infection compared to control pulp tissue, but it was significantly downregulated only for *S. constellatus* 45386. It was also noted that treatment of uninfected pulp tissue with triclosan increased expression of IL-10, but this was not statistically significant (p=0.115) compared to the control pulp tissue. The anti-inflammatory marker IL-10 expression was up-regulated by triclosan treatment of SAG infection. This up-regulation was statistically significant only for S. *constellatus* 45386 (p=0.006) but not for S. *anginosus* 39/2/14a (p=0.133). This is still a promising finding, as even though there was no significant upregulation of IL-10 there was no drop in the level of the anti-inflammatory marker in triclosan treated *S. anginosus* 39/2/14a infection compared to the uninfected control.

The fact that *S. constellatus* 45386 supernatant treatment with triclosan produced a clear zone on the plate confirmed that triclosan was not effective in neutralising the effect of hyaluronidase. This was substantiated by the fact that heat-inactivated *S. constellatus* 45386 supernatant produced a very small clear zone for the same volume of supernatant as the heat-inactivated hyaluronidase. Therefore, it can be concluded that the anti-inflammatory activity of triclosan is due to its antimicrobial activity where the killing of the bacteria prevents the production of the toxins rather than by triclosan neutralisation of the toxins. Further, it has been shown that triclosan has the ability to inhibit pathways for the production of collagen-degrading enzymes in response to TNF-α stimulation (Arancibia *et al*. 2009). These mechanisms of triclosan need to be investigated further to evaluate its usefulness in the treatment of pulpal infection.

Chapter 5

5 Developing an *ex vivo* polymicrobial infection model with *Streptococcus anginosus* and *Enterococcus faecalis*

5.1 Introduction

Infections advancing to the pulp tissue are a common finding in teeth with irreversible pulpitis but are rarely observed in normal/reversibly inflamed pulps (Ricucci *et al*. 2014). The oral cavity has about 500-700 bacterial species (Jenkinson and Lamont 2005; Paster *et al*. 2006) and the healthy mount has about 100-300 different species in different areas of the oral cavity (Bik *et al*. 2010). Endodontic infections are polymicrobial infections (Rocas *et al*. 2016), with various bacterial profiles identified in both primary and secondary infections.

The previous chapters demonstrated the influence of SAG bacteria on the pulp in terms of cell viability, colonisation of the pulp tissue and its ability to produce an immune-inflammatory response using the *ex vivo* pulp infection model. In a clinical situation, pulpal infections are usually polymicrobial (Hahn *et al*. 1991; Rocas *et al*. 2016). Furthermore, there is a change in the type of microorganisms seen in carious lesions as they progress due to pH change, availability of nutrients and oxygen levels (Hahn and Liewehr 2007b). Different bacteria have different virulence factors which contribute to their pathogenicity (Peterson 1996). In multi-bacterial infections, the virulence factor of one bacterium may have the ability to facilitate or inhibit the virulence factor of another bacterium. Symptomatic and asymptomatic endodontic infections have been shown to have a clear association with distinct bacterial communities (Anderson *et al*. 2013). The difference in symptoms may be due the different virulence factors and the type of response they produce in the pulp and periapical tissue.

Therefore, studying pulpal infection with just SAG may not give a full picture of the pulpal response likely to be encountered clinically. As the *ex vivo,* pulpal infection model has been validated and characterised, it can now be improved to support a polymicrobial infection. This will allow a better understanding of the likely pulpal response to a polymicrobial infection encountered clinically. *Streptococci* are primary colonisers in the oral cavity that attach to salivary pellicle and co-aggregate with other bacteria (Jenkinson and Demuth 1997). It has been reported that bacteria in endodontic infections may also exist as aggregates (Nair 1987), which was observed with SAG colonisation of the pulp.

Furthermore, both primary and secondary endodontic infections show the presence of SAG and *E. faecalis* (Komiyama *et al*. 2016), which are both components of the normal human commensal flora, with the former predominating in primary infections and the latter in secondary infections (Jung *et al*. 2000). Though *E. faecalis* is implicated in secondary endodontic infections it has also been identified in necrotic pulps (Salah *et al*. 2008). This suggests that *E. faecalis* is present in established pulpal infections. The virulence factors of *E. faecalis* include aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase and cytolysin (hemolysin) (Kayaoglu and Ørstavik 2004). Some of the bacteria isolated from endodontic infections can survive only in mixed infection and not in isolation; in contrast *E. faecalis* survives in isolation and in mixed root canal infections (Fabricius *et al*. 1982). SAG possesses its own set of virulence factors through the production of chondroitinase and hyaluronidase (Olson *et al*. 2013; Asam and Spellerberg 2014). Therefore, co-culturing these two bacteria with the tooth slice may alter the tissue response and simulate a more clinically accurate polymicrobial infection.

Deep carious lesions and carious lesions with pulp exposure are associated with bacterial biofilms (Mohammadi *et al*. 2013). Even though the bacteria exist as biofilms, they may also exist as a planktonic suspension within the fluid phase of the infection (Ørstavik and Ford 2008). The model would allow both scenarios, whereby polymicrobial biofilms are allowed to form on the pulpal matrix with a planktonic suspension in the culture media.

The objectives of this chapter are:

- To study the growth kinetics of *E. faecalis* in BHI and MTCM.
- To study the growth kinetics of co-culture of SAG and *E. faecalis* to evaluate how one strain influences the growth of the other.
- To evaluate the effect of the polymicrobial infection on odontoblast and dental pulp cell viability.
- To use FISH staining to identify SAG and *E. faecalis* within a co-culture polymicrobial infection.

5.2 Material and Methods

5.2.1 Bacterial identification

E. faecalis used in this study were clinical isolate from the Oral Microbiology Unit, School of Dentistry, Cardiff. Confirmation of the identity of *E. faecalis* was done through gram staining and assessing the colony appearance on blood agar, haemolysis and catalase test. 16S r RNA sequencing was also performed for *E. faecalis* using the protocol described in **section 2.2.1.1.2** for SAG.

5.2.2 Growth kinetics of *Enterococcus faecalis* in BHI and MTCM

A suspension of *E. faecalis* with an absorbance of 0.1 at 600 nm was plated on FAA and incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h. Colony counts were performed to establish the number of CFU/mL. This was performed in triplicate and the average value was accepted as the CFU/mL. It was established that a suspension of *E. faecalis* with an absorbance value of 0.1 at 600 nm corresponded to 10^8 CFU/mL.

An overnight *E. faecalis* culture in BHI was used to prepare a 10^8 CFU/mL suspension in BHI and this was diluted to produce a final suspension of 10^2 CFU/mL. Two hundred µL of this suspension was added to 9 wells of a 96 well plate and the plate was incubated in a CO_2 incubator (5% CO2) at 37^{o}C for 24 h on a mechanical rocker at 75 rpm/min. Fifty µL of the sample from one of the 9 wells was harvested at 3 h intervals and plated on FAA plates using a Wasp-spiral plater (Dan Whitley Scientific Limited, Shipley, UK) and incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h. Colonies were counted using the counting method in the Wasp user manual and the CFU/mL for each time point was estimated. Log_{10} CFU/mL change was calculated and plotted against time to generate a growth curve. The experiment was repeated in triplicate and the standard error calculated for each time point. The growth curve for *E. faecalis* in MTCM was generated in exactly the same way as described above except that MTCM substituted for BHI.

5.2.3 Growth kinetics of *Streptococcus anginosus* 39/2/14a in MTCM

The growth kinetics of *S. anginosus* 39/2/14a was performed using the same procedure as above except that *S. anginosus* 39/2/14a was employed instead of *E. faecalis* and the media used was MTCM.

5.2.4 Growth kinetics of co-culture of SAG with *E. faecalis* in MTCM

A standard 10^2 CFU/mL culture of *S. anginosus* 39/2/14A in MTCM was prepared as described in **section 2.2.5**. A standard 10^2 CFU/mL culture of *E. faecalis* in MTCM was also prepared as described in **section 5.2.2**. An equal volume of 10^2 CFU/mL *S. anginosus* 39/2/14A in MTCM and 10^2 CFU/mL *E. faecalis* in MTCM was mixed by vortexing to produce a 50%:50% suspension. Two hundred µL of this suspension was added to 9 wells of a 96-well plate and the plate was incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h on a mechanical rocker at 75 rpm/min. Fifty µL of the sample from one of the 9 wells was harvested at 3 h intervals and plated on FAA plates using a Wasp-spiral plater (Dan Whitley Scientific Limited) and incubated in a CO_2 incubator (5% CO_2) at 37^{o}C for 24 h. Another 50 µL of the sample from the same well was spiral plated on another FAA plate and the second plate was incubated at 10 ^{o}C for 7 days. Colonies were counted at the end of 24 h incubation at 37^{o}C and at the end of the 7 days incubation at 10^{o}C using the counting method in the Wasp user manual. The CFU/mL for each time points and bacterium was calculated. Both *S. anginosus* 39/2/14A and *E. faecalis* grow at 37^{o}C, but 10^{o}C incubation allows growth of only *E. faecalis*. Therefore, the plate incubated at 37^{o}C gives the total count of *S. anginosus* 39/2/14A and *E. faecalis* for the particulate time point and the corresponding plate incubated at 10^{o}C gives the CFU/mL of *E. faecalis* for the same time point in the mixed culture. By subtracting the CFU/mL of *E. faecalis* from the total count of the mixed culture, the CFU/mL of *S. anginosus* 39/2/14A for the particular time point can be established. The log change over time was calculated and plotted. The experiment was repeated in triplicate and the standard error was calculated for each time point.

Another suspension was prepared by mixing 9 mL of 10^2 CFU/mL *S. anginosus* 39/2/14A in MTCM to 1 mL of 10^2 CFU/mL *E. faecalis* in MTCM producing a 90%:10% suspension. In order to assess the effect that the change in proportion was the growth kinetics, the same procedure performed above for the 50%:50% suspension was repeated for the 90%:10% suspension and the CFU/mL calculated as above. The log change over time was calculated and plotted. This was performed in triplicate and the standard error calculated for each time point.

5.2.5 Quantification of cell viability of dental pulp infected with *E. faecalis*

Maxillary and mandibular incisors from 28-day old male Wistar rats were extracted, cut into 2 mm thick sections and cultured as described **section 2.2.4**. Tooth slices were washed overnight with antibiotic-free DMEM with no BHI to remove any residual antibiotics.

An overnight culture of *E. faecalis* was prepared in BHI. This was inoculated into MTCM and incubated at 37^{o}C in 5% CO_2 on a mechanical rocker to reach its exponential phase at approximately 5-6 h. This *E. faecalis* planktonic suspension was diluted in MTCM to produce a suspension of 10^2 CFU/mL. Each well of a 12-well plate was filled with 2 mL of 10^2 CFU/mL *E. faecalis* in MTCM. To each of these wells, one washed tooth slice, as prepared in **section 2.2.4**, was added and incubated at 37^{o}C in 5% CO_2 for 24 h on a rocker at 75 rpm/min. Control tooth slices were incubated in MTCM only at 37^{o}C in 5% CO_2 for 24 h.

After 24 h incubation the specimens were harvested, fixed in formalin and processed as described in **section 2.2.6**. Viable odontoblasts and fibroblasts were counted as described in **section 2.2.7** and compared to uninfected control tooth slices to evaluate the effect of *E. faecalis* infection on the pulp. The experiment was repeated with *S. anginosus* 39/2/14A and the cells counted.

5.2.6 Ex vivo pulpal infection model with stained *E. faecalis*

FDA (Sigma Aldrich) stain for *E. faecalis* was prepared using the same protocol described in **section 2.2.8** for staining SAG. Stained *E. faecalis* was incubated at 37^{o}C in 5% CO_2 for 24 h as described in **section 5.2.5** on tooth slices and processed as described in **section 2.2.6**, but the omitting staining with H&E. This tooth slice was then viewed under fluorescence microscope with a FITC filter and the images captured using an Olympus AX70 upright compound microscope (Olympus Corporation) and image analysis software PAX-it (PAX-it^{TM}*)* at 100x magnification to observe the pattern of attachment of *E. faecalis* to pulp tissue.

5.2.7 Quantification of cell viability of dental pulp infected with SAG and *Enterococcus faecalis*

A 90%:10% mixed suspension of *S. anginosus* 39/2/14A and *E. faecalis* in MTCM was prepared as described in **section 5.2.4**. Two mL of this suspension was added to each well of a 12-well plate. Tooth slices prepared as described in **section 2.2.5** were washed overnight with antibiotic-free DMEM with no BHI to remove any residual antibiotics. One tooth slice was added to each

well and incubated at 37^{o}C for 5% CO_2 for 24 h on a mechanical rocker at 75 rpm/min. Control tooth slices were incubated in MTCM only at 37^{o}C in 5% CO_2 for 24 h. After 24 h incubation, specimens were harvested, fixed in formalin and processed as described in **section 2.2.6**. Viable odontoblasts and fibroblasts were counted as described in **section 2.2.7** and compared to uninfected control tooth slices to evaluate the effect of mixed infection of the pulp compared to mono-infection with *S. anginosus* 39/2/14A and *E. faecalis*.

5.2.8 Fluorescence *in-situ* hybridisation (FISH) labelling of SAG *and Enterococcus faecalis*

Ten µl of 10^2 CFU/mL mixture of 50%:50% mixture of *S. anginosus* 39/2/14A and *E. faecalis* was spread on a glass slide and left to air dry. The slide was incubated for 10 min at 30°C with hybridisation solution (20mM Tris-HCl, 0,9 M NaCl, 0,1% SDS, 20% formamide pH 7.2 1mg/mL lysozyme). This was followed by incubation for 60 min at 55°C with respective probes (Eurofins Genomics, Anzinger Straße, Munich, Germany). **Table 5.1** in hybridization solution (100 ng of probe in 20µL). Slides were then washed by incubating for 10 min at 50°C in wash buffer (20 mM Tris-HCl, 0.18 NaCl pH=7.2). The slides were air dried and analysed with a fluorescence microscope and FITC filter for visualisation of *E. faecalis* and *S. anginosus* 39/2/14A as visualised through Tetramethylrhodamine (TRITC) for the rhodamine conjugate.

Table 5.1: Details of the FISH probes used for staining *S. anginosus* 39/2/14A and *E. faecalis.*

Oligonucleotide Name	**Sequence (5'→3')**	**Tm ^{o}C**	**MW (g/mol)**	**GC Content**	**Modification**
S. anginosus Rho	AGT TAA ACA GTT TCC AAA GCC TAC	57.6	8138	37.5%	5'-RRE
E. faecalis FITC	TCT AGC GAC TCG TTG TAC TTC	57.9	6931	47.6%	5'-FITC

5.2.9 Statistical analysis

The significance in difference between infected tooth slice and control were analysed by one-way Mann-Whitney U test using Graph Pad statistical software.

5.3 Results

5.3.1 Bacterial identification

The *E. faecalis* displayed alpha haemolysis with colonies of 1-2 mm, confirmed to be Gram-positive and catalase-negative. The 16S rRNA sequence revealed a 97% homology with *E. faecalis*. These findings confirmed the species to be *E. faecalis*.

5.3.2 Comparison of the growth kinetics of SAG with *Enterococcus faecalis* in different growth media

When the effect of BHI and MTCM on the growth of *E. faecalis* was evaluated, normal bacterial growth characteristics were demonstrated by *E. faecalis* in both media at 37^{o}C and 5% CO_2. The different phases of growth occurred at the same time points in both media **Figure 5.1**. *E. faecalis* replicated faster in BHI compared to MTCM. This was higher during the log phase of growth or exponential phase between 6 h and 9 h compared to the stationary phase **Figure 5.1a**.

5.3.3 Comparison of the growth of SAG and *Enterococcus faecalis* in MTCM

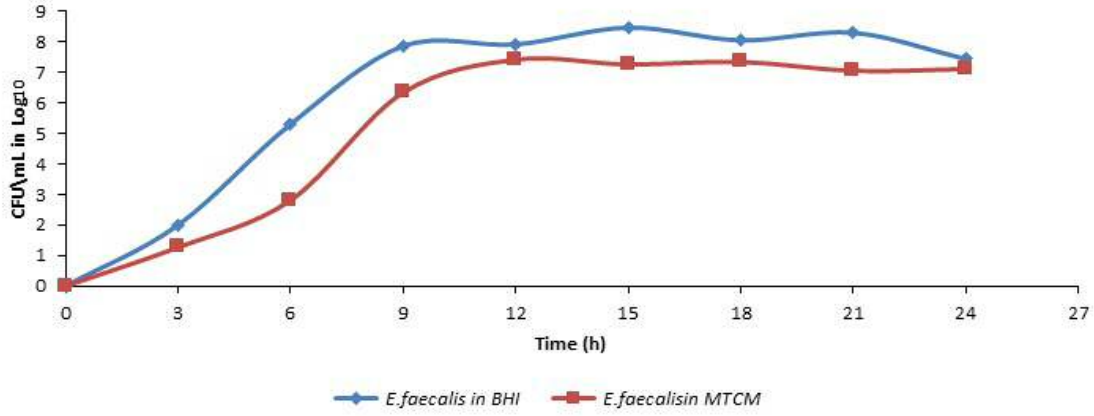
When the growth of *S. anginosus* 39/2/14A in MTCM was compared against the rate of growth of *E. faecalis*, it was observed that *E. faecalis* reached the mid-log phase at 8 h whereas *S. anginosus* 39/2/14A reached the mid-log phase at 10 h **Figure 5.1b**.

5.3.4 Effect of SAG on *Enterococcus faecalis* growth in co-culture

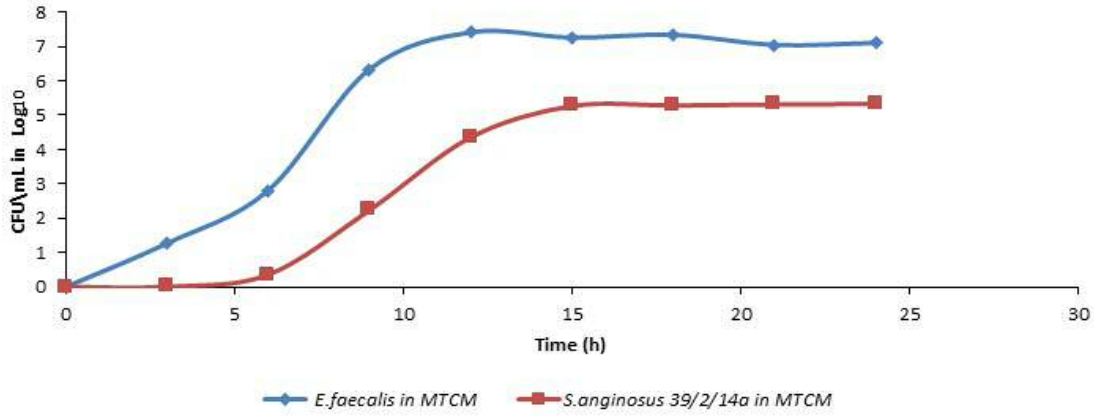
When *S. anginosus* 39/2/14A and *E. faecalis* where mixed at the ratio of 50%:50% and cultured for 24 h at 37^{o}C and 5% CO_2, this did not affect the growth of *E. faecalis* in the mixed culture in comparison to the growth of *E. faecalis* in monoculture under the same conditions. There was no difference in the change of $\log_{10}$ CFU/mL at any time point between the monoculture and mixed culture **Figure 5.2a**. When the ratio of bacteria in the mixed culture was changed to 90%:10% for *S. anginosus* 39/2/14A and *E. faecalis* respectively, there was no difference in the $\log_{10}$ CFU/mL for any time point and these findings were similar to that observed in the 50% :50% mixed culture **Figure 5.2b**.

5.3.5 Effect of *Enterococcus faecalis* on SAG in co-culture

When the effect of *E. faecalis* on the growth of *S. anginosus* 39/2/14A was assessed in a 50%:50% mixed culture, *S. anginosus* 39/2/14A reached the mid-log phase earlier at 8 h than *S. anginosus* 39/2/14A in monoculture and reached the mid-log phase of growth at around 10 h **Figure 5.3a**. It was noted in the stationary phase that on average the growth was 4.5 log CFU/mL higher in the mixed culture compared to the monoculture. In the 90% :10% ratio for *S. anginosus* 39/2/14A and *E. faecalis,* the trend in growth was exactly the same as that in the 50%:50% mixed culture **Figure 5.3b**.



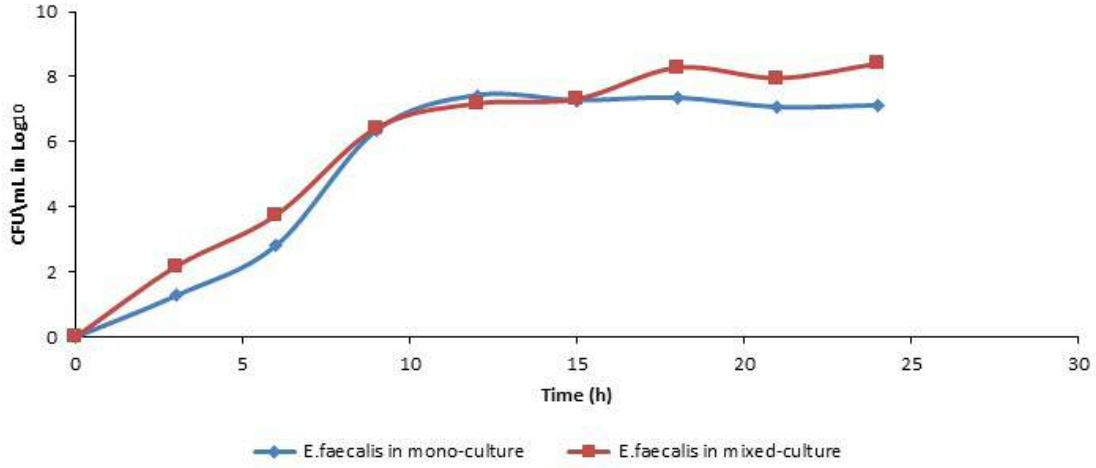
(a)



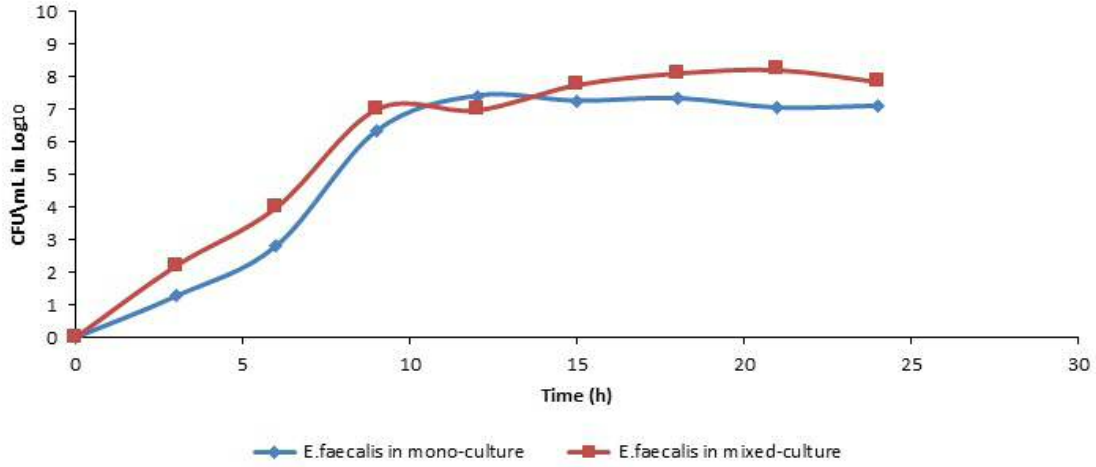
(b)

Figure 5.1: Effect of media on the growth of *E. faecalis* and its comparison to *S. anginosus* 39/2/14a growth in MTCM over 24 h.

a) Comparison of the effect of BHI and MTCM on the growth of *E. faecalis* over 24 h b) Comparison of the growth of *E. faecalis* and *S. anginosus* 39/2/14a in MTCM over 24 h. The experiments was conducted in triplicate.

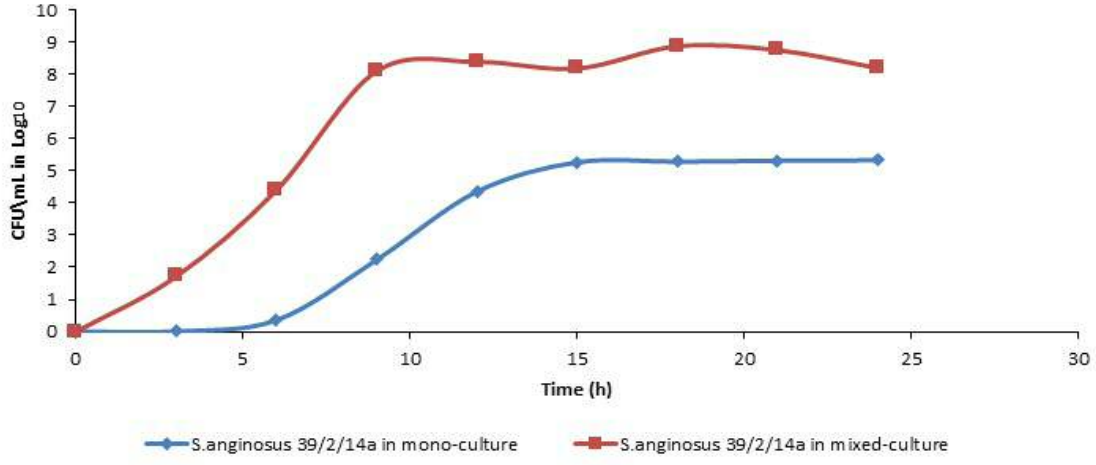


(a)

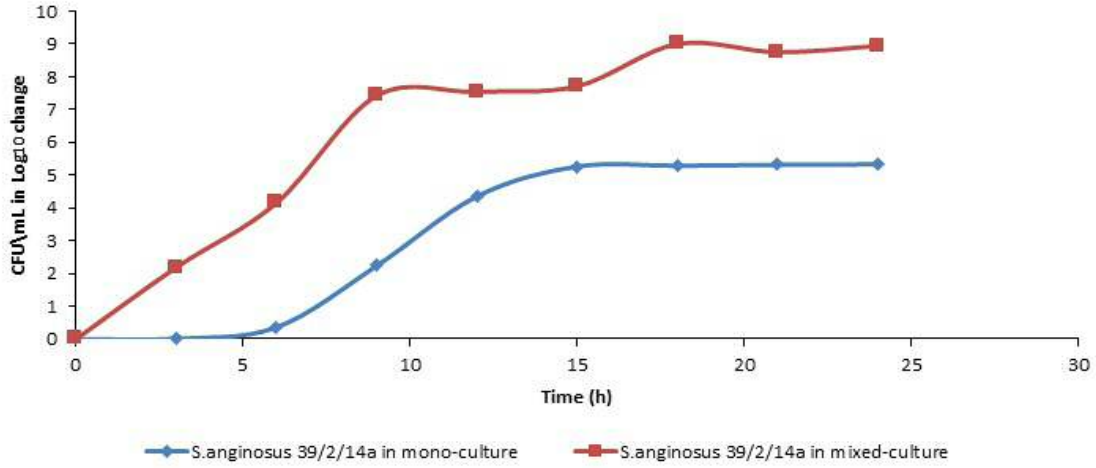


(b)

Figure 5.2: Effect of different proportions of *S. anginosus* 39/2/14a on the growth of *E. faecalis.*
a) Comparison of the effect of *S. anginosus* 39/2/14a on the growth of *E. faecalis* in 50%:50% mixed culture over 24 h, b) Comparison of the effect *of S. anginosus* 39/2/14a on the growth of *E. faecalis* in 90%:10 % mixed culture over 24 h. The experiments were conducted in triplicate.



(a)



(b)

Figure 5.3: Effect of different proportions of *E. faecalis* on the growth of *S. anginosus* 39/2/14a.
a) Comparison of the effect *of E. faecalis* on the growth of *S. anginosus* 39/2/14a in a 50% :50% mixed culture over 24 h; b) Comparison of the effect *of E. faecalis* on the growth of *S. anginosus* 39/2/14a in a 90% :10 % mixed culture over 24 h. The experiment was conducted in triplicate.

5.3.6 Histomorphometrics of the *ex vivo* pulp infection model.

When the H&E stained *E. faecalis*-infected tooth section was compared under the light microscope with un-infected control tooth slices **Figure 5.4a,b** it showed that the density of dental pulp cells was reduced in the cell-rich layer of Höhl: just below the odontoblast layer was from death of odontoblasts caused by *E. faecalis* infection. The pulpal fibroblasts also appeared to be sparsely distributed, indicating cell death in a degraded pulp matrix following infection compared to that of the control tooth slices. An interesting observation was a well-defined area of bacterial colonisation in the pulp associated with pulpal vasculature **Figure 5.4b, c**.

5.3.7 Effect of poly-microbial infection on dental pulp cell viability

Cell counts revealed a statistically significant reduction in odontoblast count compared to the uninfected tooth slices (p=0.003). When the odontoblast count of the *E. faecalis* mono-infection was compared to that of the odontoblast count of the *S. anginosus* 39/2/14A mono-infection, the former produced a significant reduction in odontoblast count (p=0.01) **Figure 5.5a**. When the tooth slices were infected with a 50%:50% mixed culture of *S. anginosus* 39/2/14A and *E. faecalis* over 24 h, there was a statistically significant reduction in odontoblast count compared to the uninfected controls (p=0.001). The mixed infection had a significant effect on the odontoblast count compared to that of *S. anginosus* 39/2/14A mono-infection (p=0.009) **Figure 5.5a**. When comparing the effect of mixed infection to that of the *E. faecalis* mono-infection there was no significant reduction in the difference in odontoblast count (p=0.7424) **Figure 5.5a**.

When the tooth slices were infected with a 90%:10% mixed culture of *S. anginosus* 39/2/14A and *E. faecalis* over 24 h, there was a significant reduction in odontoblast count after mixed infection compared to that of the *S. anginosus* 39/2/14A mono-infection (p=0.04) **Figure 5.5b**, but when the effect of *E. faecalis* mono-infection was evaluated against the mixed infection there was no significant reduction in odontoblast count (p=0.463) **Figure 5.5b**. When the effect of a 50%:50% infection was compared to the effect of a 90%:10% infection, the former produced a significant reduction compared to the latter (p=0.004).

When the effects of the infections were evaluated on dental pulp cells, both *S. anginosus* 39/2/14A and *E. faecalis* mono-infections caused significant loss of dental pulp cells (p=0.04 and p=0.03). *E. faecalis* mono-infection caused greater pulp cell death than *S. anginosus* 39/2/14A mono-infection (p=0.023) **Figure 5.6a**. When the tooth slices were infected with a 50%:50% mixed culture of *S. anginosus* 39/2/14A and *E. faecalis* over 24 h, there was a significant

reduction in cell counts compared to the uninfected controls. The mixed infection caused significantly higher cell death than isolated mono-infections (p= 0.003 and p=0.04) for *S. anginosus* 39/2/14A and *E. faecalis,* respectively, **Figure 5.6a**.

In the case of infection with 90%:10% mixed culture of *S. anginosus* 39/2/14A and *E. faecalis* over 24 h, a similar tread to that of the 50% :50% mixed infection was noted. The 90%:10% mixed infection caused significantly higher cell death compared to mono-infection p=0.004 and p=0.007, for *S. anginosus* 39/2/14A and *E. faecalis,* respectively, **Figure 5.6b**. When the effect of a 50%:50% infection was compared to the effect of a 90%:10% infection, there was no significant difference (p=0.119).

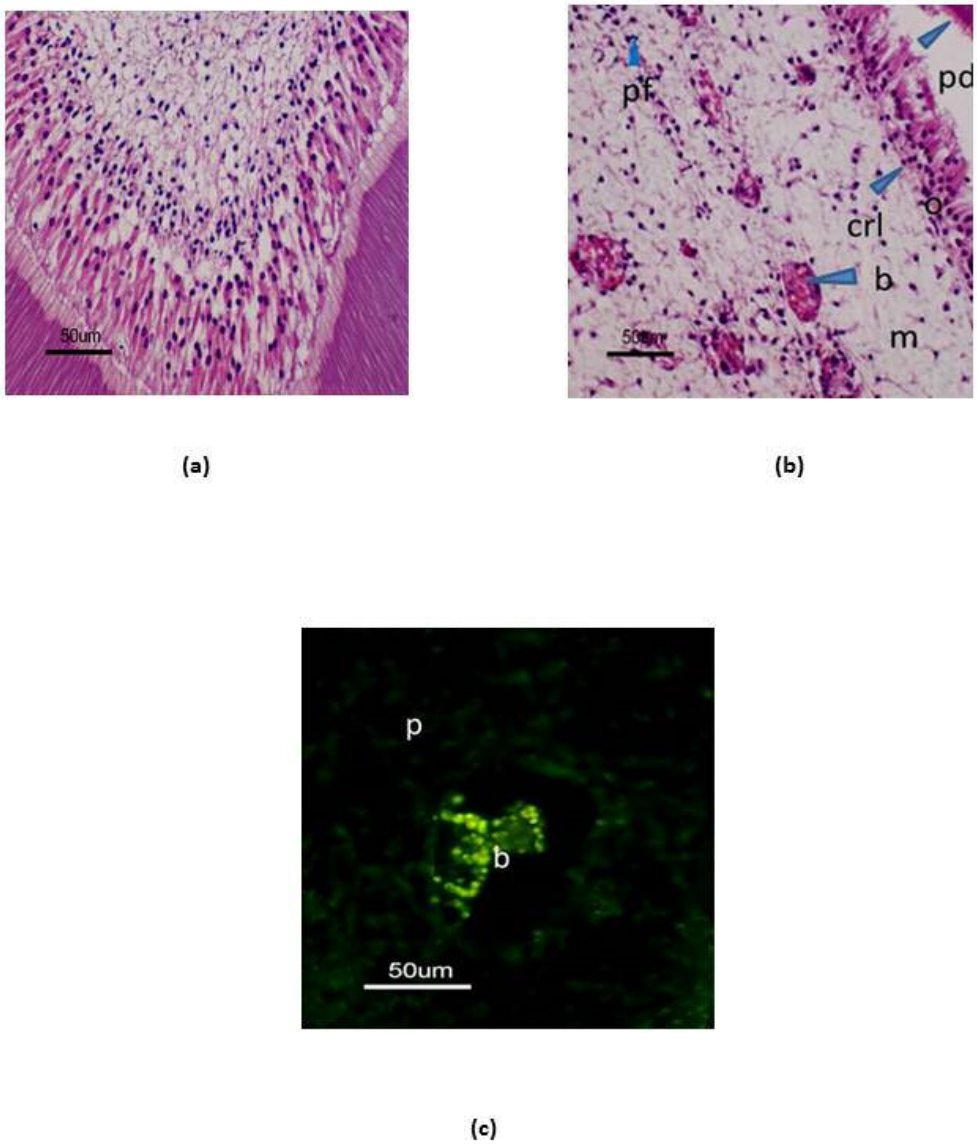
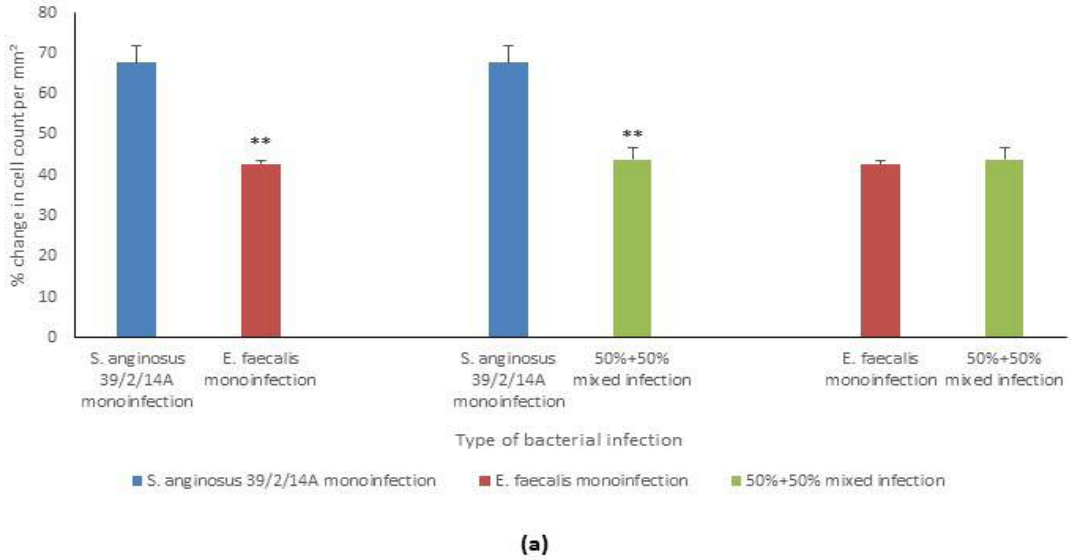
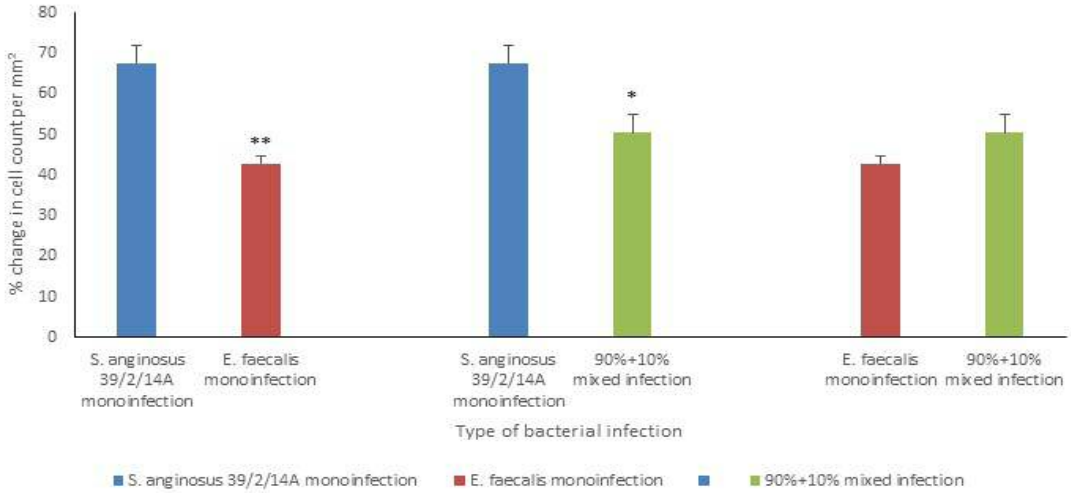


Figure 5.4: Histology of *E. faecalis* co-cultured on tooth slices for 24 h.
a) control x40 magnification; b) *E. faecalis* co-culture on tooth slice x40 magnification; c) FDA-stained *E. faecalis* in the blood vessel x100 magnification. Key: o, odontoblast; pd, predentine; crl, cell rich layer of Höhl; b, bacterial colonies in blood vessels; m, pulp matrix; pf, pulp fibroblast; p, pulp tissue.



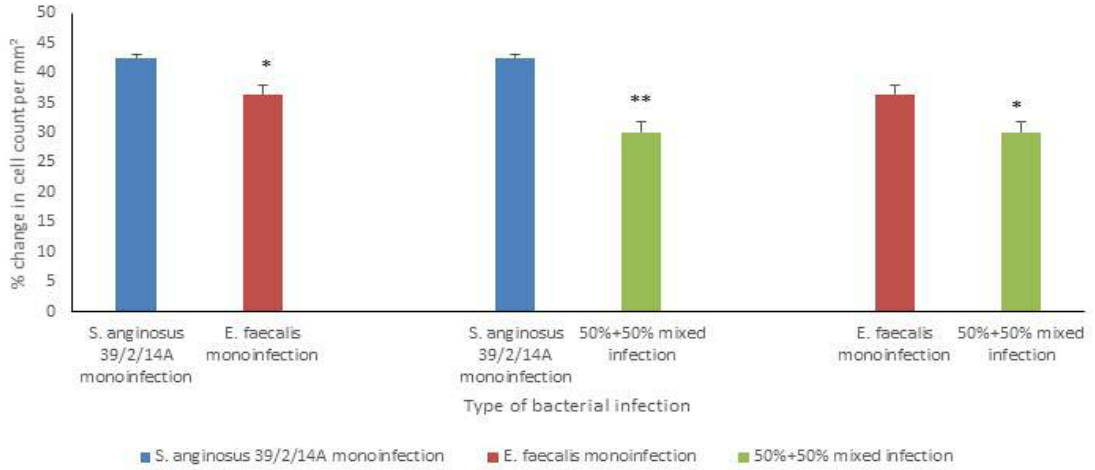
(a)



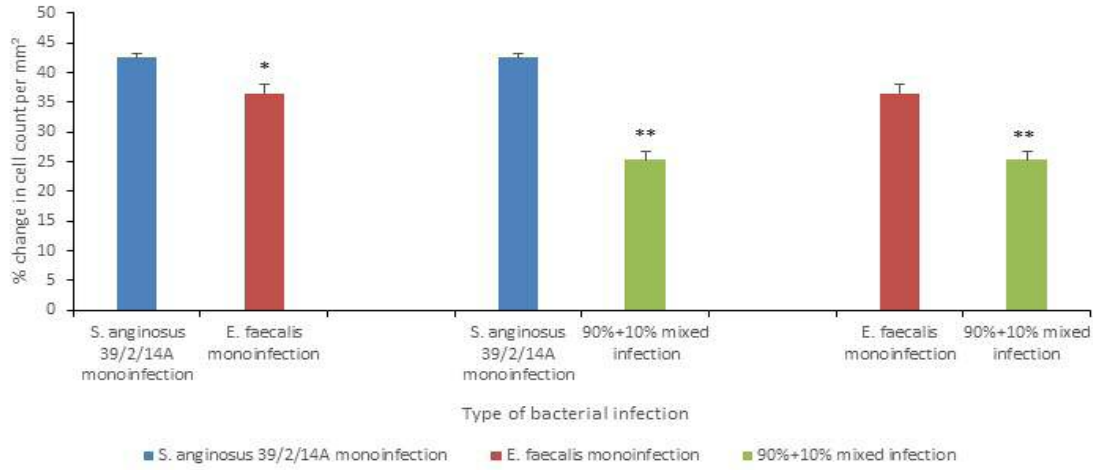
(b)

Figure 5.5: Effect of mixed infection of *S. anginosus* 39/2/14a and *E. faecali*s on the odontoblast count over 24 h.

Odontoblast count performed on transverse sections of tooth slices and odontoblast count estimated as % change per mm² of the pulp compared to uninfected pulp. Error bars indicate SE for experiments conducted in triplicate. a) 50%:50%; b) 90%:10% (Mann–Whitney U test; **=p<0.01, *=p<0.05).



(a)



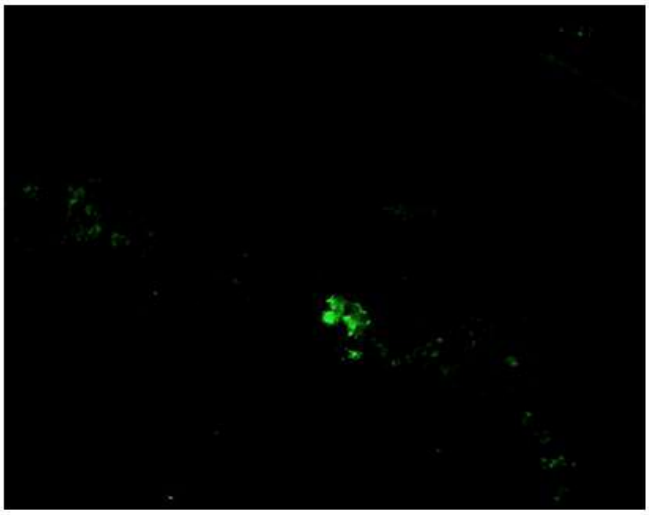
(b)

Figure 5.6: Effect of mixed infection of *S. anginosus* 39/2/14a and *E. faecalis* on dental pulp cell count over 24 h.

Odontoblast count performed on transverse sections of tooth slices and odontoblast count estimated as % change per mm² of the pulp compared to uninfected pulp. It can be seen that the presence of *E. faecalis* in the mixed infection caused greater pulp cell death and greater proportion of *S. anginosus* 39/2/14a and a synergistic effect of dental pulp cell death. Error bars indicate SE for experiments conducted in triplicate. a) 50% :50%; b) 90% :10% (Mann–Whitney U test; *=$p<0.05$, **=$p<0.01$).

5.3.8 Identification of SAG and *Enterococcus faecalis* in mixed culture using FISH labelling

During FISH labelling, *E. faecalis* appeared fluorescent green **Figure 5.7**. *S. anginosus* 39/2/14A did not take up the FISH labelling and therefore was not visualised.



(a)

(b)

Figure 5.7: Selective identification of *E. faecalis* and *S. anginosus* 39/2/14a in 50%:50 % mixed culture FISH staining.
a) Fluorescent green areas represent *E. faecalis* labelled by the FISH staining at X40 magnification; b) *S. anginosus* 39/2/14a did not take up the FISH stain could not be not identified.
.

5.4 Discussion

E. faecalis grown in MTCM did not replicate as rapidly as when grown in BHI. This may be due to the presence of FBS and DMEM. This observation was in contrast to that of *S. anginosus* 39/2/14A which did not show any significant difference between BHI and MTCM. In contrast, when *E. faecalis* growth kinetics in MTCM were compared to that of *S. anginosus* 39/2/14A, the former exhibited a significantly higher log change compared to the latter. This may be due to the very short initial lag phase of growth in *E. faecalis* in contrast to *S. anginosus* 39/2/14A, which was shown to have a lag phase of up to 3 h.

The growth of *E. faecalis* was more rapid than *S. anginosus* 39/2/14A as observed from the individual growth kinetics of *E. faecalis* and *S. anginosus* 39/2/14A. Therefore, it was decided to evaluate the effect that each bacterium would have on the growth of the other, as would be encountered in a clinical situation of polymicrobial infections. In order to account for the difference in growth kinetics of *S. anginosus* 39/2/14A and *E. faecalis,* two different proportions of the bacteria were mixed together. One was a 50%:50% mixture of *S. anginosus* 39/2/14A and *E. faecalis* and the other was a 90%:10% mixture of S. anginosus 39/2/14A and *E. faecalis*. Neither of the proportions of *S. anginosus* 39/2/14A affected the growth kinetics of *E. faecalis*. The growth of *E. faecalis* was similar to that of the mono-culture. This is consistent with the observation that *E. faecalis* can survive in isolation and in mixed infection without being affected (Fabricius *et al*. 1982). In contrast, the presence of *E. faecalis* in the culture significantly increased the log change of *S. anginosus* 39/2/14A when compared to the mono-culture. This may be due to some interaction between the two bacteria through synergistic relationships to facilitate co-habitation and efficient utilisation of metabolic by-products (Peters *et al*. 2012). It has been demonstrated in primates that the addition of *E. faecalis* to a mixture of 4 strains of bacteria increased the survival of the four strains compared to culture of the four strains without *E. faecalis* (Ramsey *et al*. 2011). Contact-dependent attachment, cell-cell communication *via* quorum-sensing cross talk, an enhancement of colonisation, augmented virulence phenotypes in trans, immunomodulation or a combination of these events may contribute to this synergistic effect (Peters *et al*. 2012; Cook *et al*. 2013). One of these mechanisms or a combination of these mechanisms may facilitate *E. faecalis* to promote the growth of *S. anginosus* 39/2/14A. Furthermore, SAG and *E. faecalis* have been shown to exist in high caries risk subjects (Becker *et al*. 2002; Preza *et al*. 2008). Irrespective of the proportion of *S. anginosus* 39/2/14A in the mixed culture, *E. faecalis* exhibited the same growth kinetic pattern. Therefore, it may be the mere presence of *E. faecalis* and not its proportion that matters.

An interesting finding during the histomorphometric analysis of tooth slices infected with FDA-stained *E. faecalis* was its association with pulpal vasculature, and this has also been demonstrated in H&E stained sections. This observation may be due to the affinity of *E. faecalis* to collagen. This affinity may be caused by Adhesin to collagen of *E. faecalis* (ACE) which is a collagen binding protein belonging to the Microbial surface component recognising adhesive matrix molecule (MSCRAMM) family. *E. faecalis* mutated to prevent production of ACE showed a significant reduction in attachment to collagen type I and IV (Nallapareddy *et al*. 2000; Singh *et al*. 2010). Attachment to the pulpal vasculature may be due to the expression of fibrinogen and fibronectin from injury to blood vessels (Clark *et al*. 1982; Gijo *et al*. 2015). Attachment may also be due to the carbohydrate antigen in the cell wall of *E. faecalis* which may modulate its attachment to endothelial cells (Guzman *et al*. 1991).

The effect of the 50%:50% and 90%:10% mixtures of *S. anginosus* 39/2/14A and *E. faecalis* on the pulp tissue was assessed through viability counts of the odontoblasts and dental pulp cells. *E. faecalis* mono-infection caused greater loss of odontoblasts than *S. anginosu*s 39/2/14A mono-infection. This may be due to the difference in virulence factors between the two strains of bacteria or their growth rates. Furthermore, *Enterococci* are known to produce virulence factors, such as the extracellular protein Esp and aggregation substances, which aid in colonisation of the host tissue, which may be the mechanism for attachment of *E. faecalis* to the pulp tissue (Fisher and Phillips 2009). Loss of odontoblasts was significantly higher in the 50%:50% mixed infection compared to *S. anginosus* 39/2/14A mono-infection and in the 90%:10% group there was a loss of odontoblasts, but it was less than that observed for the 50%:50% infection, but the difference was not significant. It can be inferred from this that the presence of *E. faecalis* in the culture promotes replication of *S. anginosus* 39/2/14A, enabling it to cause cell destruction. This agrees with the finding of the growth kinetics of *S. anginosus* 39/2/14A in mixed culture being perpetuated compared to mono-culture. Furthermore, the presence of a higher number of *E. faecalis* early in the 50%:50% infection may have also contributed to the greater loss of odontoblasts in this group compared to the 90%:10% infection, even though the loss in both groups were statistically significant compared to uninfected controls. When the *E. faecalis* mono-infection was compared to the 50%:50% and 90%:10% mixed infections, there was no significant difference. This means that *E. faecalis* mono-infection did not influence the loss of odontoblasts in the mixed culture or by itself in isolation. Therefore, the addition of *S. anginosus* 39/2/14A to an *E. faecalis* culture does not increase tissue destruction. This could also be due to the differential interaction between the odontoblasts and the two strains of bacteria.

When the influence of infection on dental pulp cells was assessed, the *E. faecalis* mono-infection caused a significant increase in cell death compared to the *S. anginosus* 39/2/14A mono-infection. This may be due to the virulence factors of *E. faecalis* AS, ESP and surface adhesins that facilitate colonisation (Gijo *et al*. 2015). In contrast to the odontoblasts, the 50%:50% and the 90%:10% mixed infections caused significant dental pulp cell death when compared to either mono-infection of *E. faecalis* or *S. anginosus* 39/2/14A. Furthermore, there was no significant difference in the proportion of dental pulp cell death when the 50%:50% and 90%:10% infections were compared. This may be due to the fact that different cells of the pulp respond to the bacterial challenge differently or could influence one another as demonstrated by an *in vitro* model (Bedran *et al*. 2014).

In order to evaluate the interaction between the two strains of bacteria when co-cultured, and to study the co-aggregation of the bacteria and evaluate the pattern of differential colonisation by the two bacteria within the pulp in a poly-microbial infection, FISH labelling protocols were tested on planktonic cultures. Co-aggregation is an important mechanism and has previously been shown to cause radical changes in gene and protein expression (Costerton *et al*. 1999). Only *E. faecalis* was successfully labelled whereas the procedure for *S. anginosus* 39/2/14A labelling was not successful. Future work should focus on modification of the protocol and fine tune it to stain *the S. anginosus* 39/2/14A. Once the FISH stains are made to work on the bacteria the infected pulp tissue can be labelled to study the interaction between the different strains of the poly-microbial infection and between the bacteria and the pulp tissue. This will form part of future work in further developing the poly-microbial pulp infection model.

Chapter 6

6 General Discussion

The dental pulp is a complex tissue located in a confined environment that makes it unique in the way it responds to infection and inflammation. The most common causes of dental pain are pulpitis and apical periodontitis, caused by endodontic infections (Mansour and Cox 2006). The pulp responds to infection through inflammation (pulpitis); in its early stages it is treated conservatively by VPT. The use of a suitable model system will help understand the disease process and investigate treatment modalities.

Therefore, the present study characterised and validated the SAG *ex vivo* pulp infection model through the assessment of cell viability, SAG colonisation of the pulp and immunoinflammatory response. This was followed by the use of the *ex vivo* model to evaluate the effect of bacterial proteins on the dental pulp and assessed its response through evaluation of cell viability and immunoinflammatory response and then identified possible virulence factors that may be responsible for the pathogenicity of SAG. The study also investigated the effectiveness of triclosan as an antimicrobial and anti-inflammatory agent in the treatment of SAG-induced pulpitis using the *ex vivo* pulp infection model. The effect of triclosan was assessed through two modalities of treatment: pre-treatment of tooth slices with triclosan and then infection with SAG; and post-treatment where established SAG infection was treated. The effect of triclosan treatment was evaluated through the assessment of its ability in preventing pulp cell death, colonisation of the pulp by SAG and modulation of pro- and anti-inflammatory responses. The effect of triclosan to neutralise the SAG toxin was also investigated. Finally, the study attempted to develop a poly-microbial *ex vivo* pulp infection model through co-culture of *S. anginosus* and *E. faecalis* on the tooth slices.

Previously, Roberts *et al.* (2013) developed an *ex vivo* pulpal infection model as a development of the *in vitro* tooth slice model by Sloan *et al* (1998). The study by Roberts and co-workers established and validated the culture media and conditions suitable for sustaining both SAG and the tooth slice. This study evaluated the cell viability by selecting five 50µm^2 areas randomly on the tooth slice as a representation of the whole tooth slice. This may not be the true representation of the viability of cells in the whole tooth slice and has the possibility of influence from investigators unconscious bias in choosing the area for counting cell viability. In our current study, we developed a method to count cells from the whole tooth slice and also considered the area occupied by the odontoblasts and the pulp cells to arrive at the count of odontoblasts per

mm^2 and pulp cells per mm^2 which are true representations of the cell count. This step is believed to minimise error or bias on the part of the investigator. This method makes the quantification of viable cells more reproducible for other researchers that would wish to use this model. Furthermore, Robert and co-workers stained the SAG bacteria with FDA to identify them in pulp tissue, but in this study, we have we have semi quantitatively calculated the area of colonisation of the pulp by the SAG. The previous study only identified the expression of inflammatory markers IL-1β and TNF-α, but did not quantify their expression. In our study we used RT-qPCR to quantitatively assess the expression of pro-inflammatory markers IL-1α, IL-1β, IL-18 and the anti-inflammatory marker IL-10.

SAG are a commensal bacteria in the oral cavity (Whiley *et al*. 1992), which when favourable conditions arise can become pathogenic and cause infections. They have been commonly identified in dental abscesses (Shweta and Prakash 2013). Having been identified in early pulpal infection, their role in pulpal infection is worth investigating (Roberts *et al*. 2013). The SAG *ex vivo* model facilitated this in a reproducible and economical way. The model responded by loss of cell viability of odontoblast and dental pulp cells caused by SAG infection over 24 h, with a greater loss caused by *S. constellatus* 45386. These findings were in contrast to those observed by Roberts *et al*. (2013). This difference could be explained by the difference in the way the cell count was evaluated in our study. Histomorphometric analysis of both the studies showed that S*. constellatus* 45386 caused more disintegration of the matrix. This is substantiated by the fact that *S. constellatus* and *S. intermedius* are commonly associated with abscess formation through tissue destruction (Claridge *et al*. 2001). The capsule of *S. constellatus* has been demonstrated to be a virulence factor for causing abscesses in *in vivo* mice experiments compared to unencapsulated strains (Kanamori *et al*. 2004). Matrix degradation may be due to the adhesins of streptococci causing them to adhere to extracellular matrix components such as fibronectin, fibrinogen and laminin (Willcox and Knox 1990; Allen *et al*. 2002a). Another mechanism has been suggested, namely through attachment of serine protease plasmin to bacteria causing the plasmin to retain its activity, whereby it causes extracellular matrix degradation. This activity is constituted by the enolase enzyme produced by SAG (Itzek *et al*. 2010). Furthermore, Robert *et al.* (2013) concluded that *S. anginosus* (Ref 39/2/14A) had a greater ability to colonise the pulp but we have now proved this quantitatively. The present study also showed an increase in expression IL-18 and a decrease in expression in IL-10 over 24 h in addition to IL-1β and TNF-α investigated in the previous study. These differences in response of the pulp tissue to the two strains of SAG suggested a difference in the virulence factors between the two strains. Furthermore, the responses observed in the model of infection are consistent with those observed in clinical pulpal infections.

In an effort to investigate the difference in response and to simulate the effect of SAG in deep carious lesions and identify virulence factors, the effect of SAG supernatants was evaluated using the *ex vivo* tooth slice model. The present study also identified the proteins in SAG supernatants in order to target them and neutralise them to prevent severe inflammatory response which could cause tissue damage. The histomorphometric changes in response to SAG supernatants were similar to those following SAG infections and the extent of matrix destruction and cell death in a dose-dependent manner was observed. A more pronounced response to *S. constellatus* 45386 supernatant was demonstrated. These findings substantiated the difference observed in the response to *S. constellatus* 45386 bacterial infection in the *ex vivo* model. These effects were confirmed to be those of SAG as heat-inactivated supernatant had very little effect on the pulp tissue and showed only a slightly elevated level of pro-inflammatory and anti-inflammatory markers, but significance was only noted in IL-1β and TNF-α. Even though there was increase in IL-10 expression, which is consistent with clinical observations in the early stages of inflammation, it was not significant. When neat SAG supernatant was used to stimulate pulp tissue, the response was similar to that of SAG infection. This experiment simulates the *in vivo* scenario of bacterial toxins penetrating the dentinal tubules in deep carious lesions where there is no bacterial invasion of the pulp. It was also identified that the number of protein constituents increased with time, especially in *S. constellatus* 45386. The virulence may be because of the hyaluronidase activity of S*. constellatus* 45386, causing tissue destruction. This is consistent with the identification of hyaluronidase as a virulence factor of SAG (Asam and Spellerberg 2014). This makes this model useful to study the effect of deep carious lesions on the pulp.

The characterised and validated *ex vivo* pulp infection model was used to evaluate the usefulness of the triclosan as an anti-inflammatory and anti-microbial agent in the treatment of pulpal infection and inflammation. Treatment of SAG infection with 8 μg/mL of triclosan reduced odontoblast and pulpal cell death, reduced the colonisation of the pulp by SAG and down-regulated expression of IL-1α, IL-1β, and IL-18 and prevented a drop in IL-10 levels but failed to show any effect against hyaluronidase. These findings suggest a potential use of triclosan as a less expensive alternative treatment agent in vital pulp therapy.

In the study developing the polymicrobial *ex vivo* pulp infection model, *E. faecalis* was found to potentiate the growth of *S. anginosus*. Furthermore, there was no difference in odontoblast cell death caused by *E. faecalis* mono-infection or poly-microbial infection of *S. anginosus* and *E. faecalis*. It was demonstrated that just the presence of *E. faecalis* and not its proportion was important for this effect. In the case of dental pulp cells, the mixed infection caused greater cell death than *S. anginosus* 39/2/14A. In our recent publication we have shown that there is no

significant difference in cell viability between mono-infection and poly-microbial infection using this *ex vivo* pulp infection model (Nishio Ayre *et al*. 2018). This difference may be the results of overall cell counts rather than attempting to quantify different cell types. Our published study also shows that the inflammatory response caused by *E. faecalis* in isolation to be greater than that caused by infection with *S. anginosus* + *E. faecalis* or *S. anginosus* in isolation. This effect was not due to supernatant of water-soluble cell wall virulence factors of *E. faecalis* as demonstrated in our publication. In our publication, we have confirmed the expression of inflammatory markers through immunohistochemistry. We have also shown the affinity of poly-microbial infection to pulp vasculature, which could be a mechanism for pathogenesis in pulpal infection and thus needs further investigation.

The current study has some limitations. The effect of acquired immunity on pulpal infection was not evaluated as the model is a static model and not dynamic with the supply of immune cells through circulation, which could influence the immuno-inflammatory response. The study only evaluated the expression of three IL-1 family markers, TNF-α and IL-10. Expression of other inflammatory markers such as IL-6 and IL-8 was not evaluated. Even though the antimicrobial and anti-inflammatory effect of triclosan on SAG infected tissue was evaluated, the effect of this treatment on repair and regeneration was not evaluated. The mechanism of attachment of the bacteria to the pulp was also not investigated. Future work should add immune cells into the model and evaluate the acquired inflammatory response of the *ex vivo* pulp infection model. It would also be helpful to evaluate through immunohistochemistry the localisation of different inflammatory markers in the pulp tissue. Further research should focus on identification of other virulence proteins expressed by SAG and other bacteria. In order to confirm the toxic effect of the SAG bacterial enzymes, SAG strains that have been knocked-out for genes specific to suspected virulence factors could be used in the model, then their role confirmed in tissue damage and inflammatory response.

It would be worthwhile to evaluate if the antimicrobial and anti-inflammatory effect of triclosan has a potentially reparative or regenerative effect on the pulp by thorough investigation for secretion of non-collagenous matrix proteins or their expression at gene level. The used of FISH probes should be refined to allow for differential identification of different bacteria in the polymicrobial infection. The mechanism of attachment of bacteria to pulpal vasculature should investigated through the use of bacteria which have mutated so as not to express the suspected virulence factor, e.g. ACE. The poly-microbial infection needs to be further developed by the addition of different types of bacteria as would be found in a clinical pulpal infection to better mirror the *in vivo* response. The effect of antimicrobial and anti-inflammatory agents like

triclosan should be evaluated on poly-microbial models to assess their usefulness in the management of clinical pulpal infection.

In conclusion, this study has characterised and validated the previously developed *ex vivo* SAG pulp infection model using more reliable and reproducible methods to quantify cell viability, bacterial colonisation and expression of inflammatory markers. The responses expressed by the model were consistent with *in vivo* clinical findings of pulpal infection to microbes for the responses assessed, making the model useful for assessment of these responses in dental pulp studies. The study has also identified SAG bacterial enzymes that could be the cause of tissue damage and inflammatory response using the model, thus making it suitable for the investigation of pulpal responses to direct pulp exposure. The model has been successfully used to evaluate the antimicrobial and anti-inflammatory effects of triclosan in the treatment of SAG pulpal infections. A foundation has been laid for developing the model into a poly-microbial *ex vivo* pulp infection model.

References

AAE. 2016. AAE Guide to Clinical Endodontics. 6th ed.

AAoPD. 2016. Guideline on Pulp Therapy for Primary and Immature Permanent Teeth. *Pediatric Dentistry* 38(6), pp. 280-288.

Abd-Elmeguid, A. et al. 2013. Osteocalcin expression in pulp inflammation. *J Endod* 39(7), pp. 865-872.

Aberg, T. et al. 1997. Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Dev Dyn* 210(4), pp. 383-396.

About, I. and Mitsiadis, T. A. 2001. Molecular aspects of tooth pathogenesis and repair: in vivo and in vitro models. *Adv Dent Res* 15, pp. 59-62.

Acosta-Perez, G. et al. 2008. Differential dependence of the ingestion of necrotic cells and TNF-alpha / IL-1beta production by murine macrophages on lipid rafts. *Scand J Immunol* 68(4), pp. 423-429.

Adams, G. B. et al. 2006. Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* 439(7076), pp. 599-603.

Aeinehchi, M. et al. 2007. Randomized controlled trial of mineral trioxide aggregate and formocresol for pulpotomy in primary molar teeth. *Int Endod J* 40(4), pp. 261-267.

Aguilar, P. and Linsuwanont, P. 2011. Vital pulp therapy in vital permanent teeth with cariously exposed pulp: a systematic review. *J Endod* 37(5), pp. 581-587.

Ahmed, N. A. et al. 2007. AI-2 quorum sensing affects antibiotic susceptibility in Streptococcus anginosus. *J Antimicrob Chemother* 60(1), pp. 49-53.

Akira, S. et al. 2006. Pathogen recognition and innate immunity. *Cell* 124(4), pp. 783-801.

Al-Doori, Z. et al. 2003. Susceptibility of MRSA to triclosan. *J Antimicrob Chemother* 51(1), pp. 185-186.

Al-Hiyasat, A. S. et al. 2006. The radiographic outcomes of direct pulp-capping procedures performed by dental students: a retrospective study. *J Am Dent Assoc* 137(12), pp. 1699-1705.

Al-Quran, F. A. et al. 2011. Single-tooth replacement: factors affecting different prosthetic treatment modalities. *BMC Oral Health* 11, p. 34.

Al Habashneh, R. et al. 2017. The effect of a triclosan/copolymer/fluoride toothpaste on plaque formation, gingivitis, and dentin hypersensitivity: A single-blinded randomized clinical study. *Quintessence Int* 48(2), pp. 123-130.

Alison, B. 2014. Martindale: The Complete Drug Reference Brayfield Alison (Ed) Martindale: The Complete Drug Reference pound459 4,688pp Pharmaceutical Press 9780857111395 0857111396 [Formula: see text]. *Emerg Nurse* 22(5), p. 12.

Allen, B. L. and Hook, M. 2002. Isolation of a putative laminin binding protein from Streptococcus anginosus. *Microb Pathog* 33(1), pp. 23-31.

Allen, B. L. et al. 2002a. Streptococcus anginosus adheres to vascular endothelium basement membrane and purified extracellular matrix proteins. *Microb Pathog* 32(4), pp. 191-204.

Allen, D. R. et al. 2002b. A clinical study to compare the anticalculus efficacy of three dentifrice formulations. *J Clin Dent* 13(2), pp. 69-72.

Alqaderi, H. et al. 2016. Coronal pulpotomy for cariously exposed permanent posterior teeth with closed apices: A systematic review and meta-analysis. *J Dent* 44, pp. 1-7.

Altenburg, A. et al. 2014. The treatment of chronic recurrent oral aphthous ulcers. *Dtsch Arztebl Int* 111(40), pp. 665-673.

Anderson, A. C. et al. 2013. Comparison of the bacterial composition and structure in symptomatic and asymptomatic endodontic infections associated with root-filled teeth using pyrosequencing. *PLoS One* 8(12), p. e84960.

Arana-Chavez, V.E and Massa, L.F. 2004. Odontoblasts: the cells forming and maintaining dentine. *Int J Biochem Cell Biol* 36(8), pp.1367-1373

Arancibia, R. et al. 2009. Triclosan inhibits tumor necrosis factor-alpha-stimulated urokinase production in human gingival fibroblasts. *J Periodontal Res* 44(6), pp. 726-735.

Arenholt-Bindslev, D. and Horsted-Bindslev, P. 1989. A simple model for evaluating relative toxicity of root filling materials in cultures of human oral fibroblasts. *Endod Dent Traumatol* 5(5), pp. 219-226.

Asam, D. and Spellerberg, B. 2014. Molecular pathogenicity of Streptococcus anginosus. *Mol Oral Microbiol* 29(4), pp. 145-155.

Asgary, S. and Eghbal, M. J. 2013. Treatment outcomes of pulpotomy in permanent molars with irreversible pulpitis using biomaterials: a multi-center randomized controlled trial. *Acta Odontol Scand* 71(1), pp. 130-136.

Asgary, S. et al. 2015. Five-year results of vital pulp therapy in permanent molars with irreversible pulpitis: a non-inferiority multicenter randomized clinical trial. *Clin Oral Investig* 19(2), pp. 335-341.

Asgary, S. et al. 2017. Treatment Outcomes of Full Pulpotomy as an Alternative to Tooth Extraction in Molars with Hyperplastic/Irreversible Pulpitis: A Case Report. *Iran Endod J* 12(2), pp. 261-265.

Ata-Ali, J. et al. 2015. Treatment of periimplant mucositis: a systematic review of randomized controlled trials. *Implant Dent* 24(1), pp. 13-18.

Avila, M. et al. 2009. The oral microbiota: living with a permanent guest. *DNA Cell Biol* 28(8), pp. 405-411.

Ayad, F. et al. 2015. Randomized clinical trial of two oral care regimens in reducing and controlling established dental plaque and gingivitis. *Am J Dent* 28 Spec No A, pp. 27A-32A.

Aylikci, B. U. and Colak, H. 2013. Halitosis: From diagnosis to management. *J Nat Sci Bio Med* 4(1), pp. 14-23

Babbar, A. et al. 2017. Members of a new subgroup of Streptococcus anginosus harbor virulence related genes previously observed in Streptococcus pyogenes. *Int J Med Microbiol* 307(3), pp. 174-181.

Babich, H. and Babich, J. P. 1997. Sodium lauryl sulfate and triclosan: in vitro cytotoxicity studies with gingival cells. *Toxicol Lett* 91(3), pp. 189-196.

Baik, J. E. et al. 2008. Lipoteichoic acid partially contributes to the inflammatory responses to Enterococcus faecalis. *J Endod* 34(8), pp. 975-982.

Banava, S. 2011. Stepwise excavation: a conservative community-based dental treatment of deep caries to inhibit pulpal exposure. *Iran J Public Health* 40(3), p. 140.

Barros, S. P. et al. 2010. Triclosan inhibition of acute and chronic inflammatory gene pathways. *J Clin Periodontol* 37(5), pp. 412-418.

Barthel, C. R. et al. 2000. Pulp capping of carious exposures: treatment outcome after 5 and 10 years: a retrospective study. *J Endod* 26(9), pp. 525-528.

Batouli, S. et al. 2003. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. *J Dent Res* 82(12), pp. 976-981.

Baume, L. J. 1980. The biology of pulp and dentine. A historic, terminologic-taxonomic, histologic-biochemical, embryonic and clinical survey. *Monogr Oral Sci* 8, pp. 1-220.

Becker, M. R. et al. 2002. Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol* 40(3), pp. 1001-1009.

Bedran, T. B. et al. 2014. Synergistic anti-inflammatory activity of the antimicrobial peptides human beta-defensin-3 (hBD-3) and cathelicidin (LL-37) in a three-dimensional co-culture model of gingival epithelial cells and fibroblasts. *PLoS One* 9(9), p. e106766.

Begue-Kirn, C. et al. 1992. Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. *Int J Dev Biol* 36(4), pp. 491-503.

Beighton, D. and Whiley, R. A. 1990. Sialidase activity of the "Streptococcus milleri group" and other viridans group streptococci. *J Clin Microbiol* 28(6), pp. 1431-1433.

Bergenholtz, G. 1977. Effect of bacterial products on inflammatory reactions in the dental pulp. *Scand J Dent Res* 85(2), pp. 122-129.

Bergenholtz, G. 1981. Inflammatory response of the dental pulp to bacterial irritation. *J Endod* 7(3), pp. 100-104.

Bergenholtz, G. and Lindhe, J. 1975. Effect of soluble plaque factors on inflammatory reactions in the dental pulp. *Scand J Dent Res* 83(3), pp. 153-158.

Berry, A. M. et al. 1994. Cloning and nucleotide sequence of the Streptococcus pneumoniae hyaluronidase gene and purification of the enzyme from recombinant Escherichia coli. *Infect Immun* 62(3), pp. 1101-1108.

Beyer, T. A. et al. 2013. The TGFbeta superfamily in stem cell biology and early mammalian embryonic development. *Biochim Biophys Acta* 1830(2), pp. 2268-2279.

Bienvenut, W. V. et al. 2002. Matrix-assisted laser desorption/ionization-tandem mass spectrometry with high resolution and sensitivity for identification and characterization of proteins. *Proteomics* 2(7), pp. 868-876.

Bik, E. M. et al. 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. *ISME J* 4(8), pp. 962-974.

Bjørndal, L. et al. 1998. A quantitative light microscopic study of the odontoblast and subodontoblastic reactions to active and arrested enamel caries without cavitation. *Caries Res* 32(1), pp. 59-69.

Bjørndal, L. and Mjor, I. A. 2002. *Dental caries--characteristics of lesions and pulpal reactions*. *Quintessence Int* 32(9),pp.717-736.

Bjørndal, L. et al. 2010. Treatment of deep caries lesions in adults: randomized clinical trials comparing stepwise vs. direct complete excavation, and direct pulp capping vs. partial pulpotomy. *Eur J Oral Sci* 118(3), pp. 290-297.

Blancou, P. and Anegon, I. 2010. Editorial: Heme oxygenase-1 and dendritic cells: what else? *J Leukoc Biol* 87(2), pp. 185-187.

Bletsa, A. et al. 2006. Cytokine signalling in rat pulp interstitial fluid and transcapillary fluid exchange during lipopolysaccharide-induced acute inflammation. *J Physiol* 573(Pt 1), pp. 225-236.

Blinkhorn, A. et al. 2009. Is there a role for triclosan/copolymer toothpaste in the management of periodontal disease? *Br Dent J* 207(3), pp. 117-125.

Bogen, G. et al. 2008. Direct pulp capping with mineral trioxide aggregate: an observational study. *J Am Dent Assoc* 139(3), pp. 305-315; quiz 305-315.

Bonucci, E. 2002. Crystal ghosts and biological mineralization: fancy spectres in an old castle, or neglected structures worthy of belief? *J Bone Miner Metab* 20(5), pp. 249-265.

Borges, F. M. et al. 2012. Antimicrobial effect of chlorhexidine digluconate in dentin: In vitro and in situ study. *J Conserv Dent* 15(1), pp. 22-26.

Botero, T. M. et al. 2010. MAPK signaling is required for LPS-induced VEGF in pulp stem cells. *J Dent Res* 89(3), pp. 264-269.

Bowden, G. H. et al. 2000. The microbial ecology of dental caries. *J Microbial Ecology in Heath and Disease* 12(3), pp.138-148

Brannstrom, M. and Lind, P. O. 1965. Pulpal response to early dental caries. *J Dent Res* 44(5), pp. 1045-1050.

Brennan, D. S. and Spencer, A. J. 2004. Disability weights for the burden of oral disease in South Australia. *Popul Health Metr* 2(1), p. 7.

Brennan, D. S. et al. 2008. Tooth loss, chewing ability and quality of life. *Qual Life Res* 17(2), pp. 227-235.

Brennan, K. and Jefferies, C. A. 2009. Proteomic analysis of protein complexes in Toll-like receptor biology. *Methods Mol Biol* 517, pp. 91-104.

Bright, R. et al. 2018. The effect of triclosan on posttranslational modification of proteins through citrullination and carbamylation. *Clin Oral Investig* 22(1), pp.487-493.

Brown, L. R. et al. 1986. Quantitative comparisons of potentially cariogenic microorganisms cultured from noncarious and carious root and coronal tooth surfaces. *Infect Immun* 51(3), pp. 765-770.

Bruno, K. F. et al. 2010. Characterization of inflammatory cell infiltrate in human dental pulpitis. *Int Endod J* 43(11), pp. 1013-1021.

Buckley, C. D. et al. 2013. The resolution of inflammation. *Nat Rev Immunol* 13(1), pp. 59-66.

Burry, J. C. et al. 2016. Outcomes of Primary Endodontic Therapy Provided by Endodontic Specialists Compared with Other Providers. *J Endod* 42(5), pp. 702-705.

Butler, W. T. and Ritchie, H. 1995. The nature and functional significance of dentin extracellular matrix proteins. *Int J Dev Biol* 39(1), pp. 169-179.

Bystrom, A. et al. 1985. The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals. *Endod Dent Traumatol* 1(5), pp. 170-175.

Byun, R. et al. 2004. Quantitative analysis of diverse Lactobacillus species present in advanced dental caries. *J Clin Microbiol* 42(7), pp. 3128-3136.

Cachovan, G. et al. 2014. Radiography-based score indicative for the pathogenicity of bacteria in odontogenic infections. *Acta Odontol Scand* 72(7), pp. 530-536.

Caliskan, M. K. and Guneri, P. 2016. Prognostic factors in direct pulp capping with mineral trioxide aggregate or calcium hydroxide: 2- to 6-year follow-up. *Clin Oral Investig* 21(1), pp. 357-367

Camilleri, J. 2008. Characterization of hydration products of mineral trioxide aggregate. *Int Endod J* 41(5), pp. 408-417.

Camilleri, J. et al. 2005. The constitution of mineral trioxide aggregate. *Dent Mater* 21(4), pp. 297-303.

Carneiro-Neto, J. N. et al. 2017. Protocols for management of oral complications of chemotherapy and/or radiotherapy for oral cancer: Systematic review and meta-analysis current. *Med Oral Patol Oral Cir Bucal* 22(1), pp. e15-e23.

Carrouel, F. et al. 2013. Lipopolysaccharide-binding protein inhibits toll-like receptor 2 activation by lipoteichoic acid in human odontoblast-like cells. *J Endod* 39(8), pp. 1008-1014.

Cassidy, N. et al. 1997. Comparative analysis of transforming growth factor-beta isoforms 1-3 in human and rabbit dentine matrices. *Arch Oral Biol* 42(3), pp. 219-223.

Chacko, V. and Kurikose, S. 2006. Human pulpal response to mineral trioxide aggregate (MTA): a histologic study. *J Clin Pediatr Dent* 30(3), pp. 203-209.

Chailertvanitkul, P. et al. 2014. Randomized control trial comparing calcium hydroxide and mineral trioxide aggregate for partial pulpotomies in cariously exposed pulps of permanent molars. *Int Endod J* 47(9), pp. 835-842.

Chang, S. W. et al. 2015. Odontoblastic Differentiation, Inflammatory Response, and Angiogenic Potential of 4 Calcium Silicate-based Cements: Micromega MTA, ProRoot MTA, RetroMTA, and Experimental Calcium Silicate Cement. *J Endod* 41(9), pp. 1524-1529.

Chang, S. W. et al. 2014. Effects of calcium silicate endodontic cements on biocompatibility and mineralization-inducing potentials in human dental pulp cells. *J Endod* 40(8), pp. 1194-1200.

Charadram, N. et al. 2013. Structural analysis of reactionary dentin formed in response to polymicrobial invasion. *J Struct Biol* 181(3), pp. 207-222.

Chaudhry, A. et al. 2011. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 34(4), pp. 566-578.

Chauhan, A. et al. 2018. In vivo Outcomes of Indirect Pulp Treatment in Primary Posterior Teeth: 6 Months' Follow-up. Contemp Clin Dent 9(Suppl10, pp.S69-S73.

Chen, J. et al. 2017. Treated dentin matrix paste as a novel pulp capping agent for dentin regeneration. *J Tissue Eng Regen Med* 11(12) pp. 3428-3436.

Chin, J. S. et al. 2016. A survey of dental practitioners in Wales to evaluate the management of deep carious lesions with vital pulp therapy in permanent teeth. *Br Dent J* 221(6), pp. 331-338.

Chino, T.et al. 2009. Effects of oral commensal and pathogenic bacteria on human dentritic cells. *Oral Microbiol Immunol* 24(2), pp. 96-103.

Chmilewsky, F. et al. 2015. LPS induces pulp progenitor cell recruitment via complement activation. *J Dent Res* 94(1), pp. 166-174.

Chmilewsky, F. et al. 2013. Pulp progenitor cell recruitment is selectively guided by a C5a gradient. *J Dent Res* 92(6), pp. 532-539.

Claridge, J. E., 3rd et al. 2001. Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus ("Streptococcus milleri group") are of different clinical importance and are not equally associated with abscess. *Clin Infect Dis* 32(10), pp. 1511-1515.

Clark, R. A. et al. 1982. Fibronectin is produced by blood vessels in response to injury. *J Exp Med* 156(2), pp. 646-651.

Clewell, D. B. and Weaver, K. E. 1989. Sex pheromones and plasmid transfer in Enterococcus faecalis. *Plasmid* 21(3), pp. 175-184.

Coia, J. E. et al. 2006. Guidelines for the control and prevention of meticillin-resistant Staphylococcus aureus (MRSA) in healthcare facilities. *J Hosp Infect* 63 Suppl 1, pp. S1-44.

Coll, J. A. et al. 2017. Primary Tooth Vital Pulp Therapy: A Systematic Review and Meta-analysis. *Pediatr Dent* 39(1), pp. 16-123.

Colombo, J. S. et al. 2014. Scaffolds to control inflammation and facilitate dental pulp regeneration. *J Endod* 40(4 Suppl), pp. S6-12.

Connelly, L. et al. 2001. Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol* 166(6), pp. 3873-3881.

Cook, L. C. et al. 2013. Interspecies communication among commensal and pathogenic streptococci. *MBio* 4(4), pp. 1-11

Cooper, P. R. et al. 2017. Inflammation and Regeneration in the Dentin-pulp Complex: Net Gain or Net Loss? *J Endod* 43(9S), pp. S87-S94.

Cooper, P. R. et al. 2014. Inflammation and regeneration in the dentin-pulp complex: a double-edged sword. *J Endod* 40(4 Suppl), pp. S46-51.

Cooper, P. R. et al. 2011. Mediators of inflammation and regeneration. *Adv Dent Res* 23(3), pp. 290-295.

Cooper, P. R., Smith, A.J. . 2013. Molecular mediators of pulp inflammation and regeneration. *Endod Top* (28), pp. 90–105.

Cooper, P. R. et al. 2010. Inflammation-regeneration interplay in the dentine-pulp complex. *J Dent* 38(9), pp. 687-697.

Cordeiro, M. M. et al. 2008. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. *J Endod* 34(8), pp. 962-969.

Cornish, J. et al. 1997. Adrenomedullin is a potent stimulator of osteoblastic activity in vitro and in vivo. *Am J Physiol* 273(6 Pt 1), pp. E1113-1120.

Costerton, J. W. et al. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418), pp. 1318-1322.

Couve, E. et al. 2014. Reactionary Dentinogenesis and Neuroimmune Response in Dental Caries. *J Dent Res* 93(8), pp. 788-793.

Cox, C. F. et al. 1985. Pulp capping of dental pulp mechanically exposed to oral microflora: a 1-2 year observation of wound healing in the monkey. *J Oral Pathol* 14(2), pp. 156-168.

Cox, C. F. et al. 1996. Tunnel defects in dentin bridges: their formation following direct pulp capping. *Oper Dent* 21(1), pp. 4-11.

Crozat, K. et al. 2009. Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. *Immunol Rev* 227(1), pp. 129-149.

Cullinan, M. P. et al. 2014. No evidence of triclosan-resistant bacteria following long-term use of triclosan-containing toothpaste. *J Periodontal Res* 49(2), pp. 220-225.

Cummins, D. 1991. Zinc citrate/Triclosan: a new anti-plaque system for the control of plaque and the prevention of gingivitis: short-term clinical and mode of action studies. *J Clin Periodontol* 18(6), pp. 455-461.

Cury, J. A. and Tenuta, L. M. 2014. Evidence-based recommendation on toothpaste use. *Braz Oral Res* 28 Spec No, pp. 1-7.

Cvek, M. 1992. Prognosis of luxated non-vital maxillary incisors treated with calcium hydroxide and filled with gutta-percha. A retrospective clinical study. *Endod Dent Traumatol* 8(2), pp. 45-55.

Cvek, M. et al. 1987. Hard tissue barrier formation in pulpotomized monkey teeth capped with cyanoacrylate or calcium hydroxide for 10 and 60 minutes. *J Dent Res* 66(6), pp. 1166-1174.

da Rosa, W. L. O. et al. 2018. Current trends and future perspectives of dental pulp capping materials: A systematic review. *J Biomed Mater Res B Appl Biomater* 106(3), pp. 1358-1368

da Silva, L. A. et al. 2008. Radiographic evaluation of pulpal and periapical response of dogs' teeth after pulpotomy and use of recombinant human bone morphogenetic protein-7 as a capping agent. *J Dent Child (Chic)* 75(1), pp. 14-19.

Dagley,S. et al. 1950. Factors including the early phases of growth of Aerobacter aerogenes. *J Gen Microbiol* 4(3), pp. 437-447.

Dammaschke, T. et al. 2010. Long-term evaluation of direct pulp capping--treatment outcomes over an average period of 6.1 years. *Clin Oral Investig* 14(5), pp. 559-567.

Decup, F. et al. 2000. Bone sialoprotein-induced reparative dentinogenesis in the pulp of rat's molar. *Clin Oral Investig* 4(2), pp. 110-119.

Denyer, S. P. and Maillard, J. Y. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. *J Appl Microbiol* 92 Suppl, pp. 35S-45S.

Dobie, K. et al. 2002. Effects of alginate hydrogels and TGF-beta 1 on human dental pulp repair in vitro. *Connect Tissue Res* 43(2-3), pp. 387-390.

Drucker, D. B. and Green, R. M. 1977. Dental caries induced by Streptococcus milleri. *J Dent Res* 56(9), p. 1062.

Dummer, P. M. et al. 1980. Clinical signs and symptoms in pulp disease. *Int Endod J* 13(1), pp. 27-35.

El Karim, I. A. et al. 2016. Biodentine Reduces Tumor Necrosis Factor Alpha-induced TRPA1 Expression in Odontoblastlike Cells. *J Endod* 42(4), pp. 589-595

Elsalhy, M. et al. 2013. Cytokines as diagnostic markers of pulpal inflammation. *Int Endod J* 46(6), pp. 573-580.

Embery, G. and Smalley, J. W. 1980. The influence of fluoride on the uptake of radiosulphate by rat incisor odontoblasts in vitro. *Arch Oral Biol* 25(10), pp. 659-662.

Eming, S. A. et al. 2007. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 127(3), pp. 514-525.

Estrela, C. et al. 2011. Diagnostic and clinical factors associated with pulpal and periapical pain. *Braz Dent J* 22(4), pp. 306-311.

Fabricius, L. et al. 1982. Influence of combinations of oral bacteria on periapical tissues of monkeys. *Scand J Dent Res* 90(3), pp. 200-206.

Facklam, R. 2002. What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin Microbiol Rev* 15(4), pp. 613-630.

Farges, J. C. et al. 2013. Odontoblast control of dental pulp inflammation triggered by cariogenic bacteria. *Front Physiol* 4, p. 326.

Farges, J. C. et al. 2015. Human odontoblast-like cells produce nitric oxide with antibacterial activity upon TLR2 activation. *Front Physiol* 6, p. 185.

Farges, J. C. et al. 2015. Dental pulp Defence and Repair Mechanisms in Dental Caries *Mediators Inflammation* 2015, pp. 230-251.

Farges, J. C. et al. 2011. Cytokine production by human odontoblast-like cells upon Toll-like receptor-2 engagement. *Immunobiology* 216(4), pp. 513-517.

Farges, J. C. et al. 2009. Odontoblasts in the dental pulp immune response. *J Exp Zool B Mol Dev Evol* 312B(5), pp. 425-436.

Featherstone, J. D. 2000. The science and practice of caries prevention. *J Am Dent Assoc* 131(7), pp. 887-899.

Featherstone, J. D. 2004. The continuum of dental caries--evidence for a dynamic disease process. *J Dent Res* 83 Spec No C, pp. C39-42.

Finkelman, R. D. et al. 1990. Quantitation of growth factors IGF-I, SGF/IGF-II, and TGF-beta in human dentin. *J Bone Miner Res* 5(7), pp. 717-723.

Finney, M. et al. 2003. Antimicrobial effects of a novel Triclosan/zinc citrate dentifrice against mixed culture oral biofilms. *Int Dent J* 53(6 Suppl 1), pp. 371-378.

Fisher, K. and Phillips, C. 2009. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* 155(Pt 6), pp. 1749-1757.

Fitzgerald, M. et al. 1990. Autoradiographic analysis of odontoblast replacement following pulp exposure in primate teeth. *Arch Oral Biol* 35(9), pp. 707-715.

Fletcher, A. M. et al. 2010. Incisor degeneration in rats induced by vascular endothelial growth factor/fibroblast growth factor receptor tyrosine kinase inhibition. *Toxicol Pathol* 38(2), pp. 267-279.

Fong, J. N. C. and Yildiz, F. H. 2015. Biofilm Matrix Proteins. *Microbiol Spectr* 3(2), pp. 1-16

Fransson, H. 2012. On the repair of the dentine barrier. *Swed Dent J Suppl* (226), pp. 9-84.

Franz, C. M. et al. 2003. Enterococci in foods--a conundrum for food safety. *Int J Food Microbiol* 88(2-3), pp. 105-122.

Franz, F. E. et al. 1984. Ultrastructure (SEM) of dentine bridging in the human dental pulp. *J Biol Buccale* 12(3), pp. 239-246.

Franz, F. E. et al. 1985. Microradiographic assessment of neodentinal bridging following direct pulp capping in human teeth. *J Endod* 11(1), pp. 6-10.

Fujiyoshi, T. et al. 2001. [Clinical and bacteriological significance of the Streptococcus milleri group in deep neck abscesses]. *Nihon Jibiinkoka Gakkai Kaiho* 104(2), pp. 147-156.

Furia, J., E. & Schenkel, A. G. 1968. Broad spectrum bacteriostatic. *Soap & Chemical Specialties* 44(1), pp. 47-50, 116, 118, 120, 122.

Gafa, V. et al. 2007. In vitro infection of human dendritic cells by Aspergillus fumigatus conidia triggers the secretion of chemokines for neutrophil and Th1 lymphocyte recruitment. *Microbes Infect* 9(8), pp. 971-980.

Gaffar, A. et al. 1995. The effect of triclosan on mediators of gingival inflammation. *J Clin Periodontol* 22(6), pp. 480-484.

Gale, Z. et al. 2011. Effects of glial cell line-derived neurotrophic factor on dental pulp cells. *J Dent Res* 90(10), pp. 1240-1245.

Garrocho-Rangel, A. et al. 2009. Efficacy of EMD versus calcium hydroxide in direct pulp capping of primary molars: a randomized controlled clinical trial. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 107(5), pp. 733-738.

Genge, B. R. et al. 2007a. In vitro modeling of matrix vesicle nucleation: synergistic stimulation of mineral formation by annexin A5 and phosphatidylserine. *J Biol Chem* 282(36), pp. 26035-26045.

Genge, B. R. et al. 2007b. Kinetic analysis of mineral formation during in vitro modeling of matrix vesicle mineralization: effect of annexin A5, phosphatidylserine, and type II collagen. *Anal Biochem* 367(2), pp. 159-166.

Ghoddusi, J. et al. 2014. New approaches in vital pulp therapy in permanent teeth. *Iran Endod J* 9(1), pp. 15-22.

Gijo, J. et al. 2015. Enterococcus faecalis, a nightmare to endodontist: A systematic review. *African Journal of Microbiology Research* 9, pp. 898-908.

Glickman, G. N. 2009. AAE Consensus Conference on Diagnostic Terminology: background and perspectives. *J Endod* 35(12), pp. 1619-1620.

Goldberg, M. et al. 2008. Inflammatory and immunological aspects of dental pulp repair. *Pharmacol Res* 58(2), pp. 137-147.

Goldberg, M. et al. 2011. Dentin: structure, composition and mineralization. *Front Biosci (Elite Ed)* 3, pp. 711-735.

Goldberg, M. et al. 2015. Is Pulp Inflammation a Prerequisite for Pulp Healing and Regeneration? *Mediators Inflamm* 2015, p. 347649.

Goldberg, M. et al. 2001. Application of bioactive molecules in pulp-capping situations. *Adv Dent Res* 15, pp. 91-95.

Goldberg, M. et al. 2003. Bioactive molecules and the future of pulp therapy. *Am J Dent* 16(1), pp. 66-76.

Goldberg, M. and Smith, A. J. 2004. cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. *Crit Rev Oral Biol Med* 15(1), pp. 13-27.

Golub, E. E. 2009. Role of matrix vesicles in biomineralization. *Biochim Biophys Acta* 1790(12), pp. 1592-1598.

Gomes-Filho, J. E. et al. 2016. Evaluation of photodynamic therapy on fibroblast viability and cytokine production. *Photodiagnosis Photodyn Ther* 13, pp. 97-100.

Gomes-Filho, J. E. et al. 2009. Evaluation of the effects of endodontic materials on fibroblast viability and cytokine production. *J Endod* 35(11), pp. 1577-1579.

Goncalves, L. F. et al. 2016. Effect of EDTA on TGF-beta1 released from the dentin matrix and its influence on dental pulp stem cell migration. *Braz Oral Res* 30(1), p. e131.

Goncalves, S. B. et al. 2007. Tooth slice-based models for the study of human dental pulp angiogenesis. *J Endod* 33(7), pp. 811-814.

Goto, Y. et al. 2009. Restorations of endodontically treated teeth: new concepts, materials, and aesthetics. *Pract Proced Aesthet Dent* 21(2), pp. 81-89.

Graham, L. et al. 2006. The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. *Biomaterials* 27(14), pp. 2865-2873.

Graham, L. W. et al. 2007. Cytokine release from human dentine. *J Dent Res*, p. 86.

Grando Mattuella, L. et al. 2007. Vascular endothelial growth factor and its relationship with the dental pulp. *J Endod* 33(5), pp. 524-530.

Grinwis, M. E. et al. 2010. Characterization of Streptococcus milleri group isolates from expectorated sputum of adult patients with cystic fibrosis. *J Clin Microbiol* 48(2), pp. 395-401.

Gronthos, S. et al. 2002. Stem cell properties of human dental pulp stem cells. *J Dent Res* 81(8), pp. 531-535.

Guillen, J. et al. 2004. Location and orientation of Triclosan in phospholipid model membranes. *Eur Biophys J* 33(5), pp. 448-453.

Guthrie, T. J. et al. 1965. dental pulp hemogram. *J Dent Res* 44, pp. 678-682.

Guzman, C. A. et al. 1991. Serum dependent expression of Enterococcus faecalis adhesins involved in the colonization of heart cells. *Microb Pathog* 11(6), pp. 399-409.

Hahn, C. L. et al. 2000. Cytokine induction by Streptococcus mutans and pulpal pathogenesis. *Infect Immun* 68(12), pp. 6785-6789.

Hahn, C. L. et al. 1991. Microbiological studies of carious dentine from human teeth with irreversible pulpitis. *Arch Oral Biol* 36(2), pp. 147-153.

Hahn, C. L. and Liewehr, F. R. 2007a. Innate immune responses of the dental pulp to caries. *J Endod* 33(6), pp. 643-651.

Hahn, C. L. and Liewehr, F. R. 2007b. Relationships between caries bacteria, host responses, and clinical signs and symptoms of pulpitis. *J Endod* 33(3), pp. 213-219.

Hahn, C. L. and Liewehr, F. R. 2007c. Update on the adaptive immune responses of the dental pulp. *J Endod* 33(7), pp. 773-781.

Hannahan, J. P. and Eleazer, P. D. 2008. Comparison of success of implants versus endodontically treated teeth. *J Endod* 34(11), pp. 1302-1305.

Haraszthy, V. I. et al. 2010. Evaluation of the antimicrobial activity of dentifrices on human oral bacteria. *J Clin Dent* 21(4), pp. 96-100.

Hargreaves. K.M, G. H. E., Seltzer. S, and Bender. I. B.,. 2002. *Seltzer and Bender's Dental Pulp*. Chicago, Ill,USA: Quintessence. chapter 10 by José F, pp.205-240

Hasegawa, N. 1989. Effects of various culture conditions on matrix formative functions of rat incisor odontoblasts in a pulp-dentin slice culture system. *Shika Kiso Igakkai Zasshi* 31(4), pp. 392-403.

He, P. et al. 2010. Ameloblast differentiation in the human developing tooth: effects of extracellular matrices. *Matrix Biol* 29(5), pp. 411-419.

He, W. et al. 2013. LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. *Int Endod J* 46(2), pp. 128-136.

Heikinheimo, K. 1994. Stage-specific expression of decapentaplegic-Vg-related genes 2, 4, and 6 (bone morphogenetic proteins 2, 4, and 6) during human tooth morphogenesis. *J Dent Res* 73(3), pp. 590-597.

Heikinheimo, K. et al. 1993. Transforming growth factor beta 2 in epithelial differentiation of developing teeth and odontogenic tumors. *J Clin Invest* 91(3), pp. 1019-1027.

Hessam, S. et al. 2016. Combination of oral zinc gluconate and topical triclosan: An anti-inflammatory treatment modality for initial hidradenitis suppurativa. *J Dermatol Sci* 84(2), pp. 197-202.

Heyeraas, K. J. and Berggreen, E. 1999. Interstitial fluid pressure in normal and inflamed pulp. *Crit Rev Oral Biol Med* 10(3), pp. 328-336.

Heyeraas, K. J. et al. 1994. Effect of electrical tooth stimulation on blood flow, interstitial fluid pressure and substance P and CGRP-immunoreactive nerve fibers in the low compliant cat dental pulp. *Microvasc Res* 47(3), pp. 329-343.

Heywood, B. R. and Appleton, J. 1984. The ultrastructure of the rat incisor odontoblast in organ culture. *Arch Oral Biol* 29(4), pp. 327-329.

Hilton, T. J. 2009. Keys to clinical success with pulp capping: a review of the literature. *Oper Dent* 34(5), pp. 615-625.

Hirao, K. et al. 2009. Roles of TLR2, TLR4, NOD2, and NOD1 in pulp fibroblasts. *J Dent Res* 88(8), pp. 762-767.

Hirata, M. et al. 2005. Expression of osteocalcin and Jun D in the early period during reactionary dentin formation after tooth preparation in rat molars. *Cell Tissue Res* 319(3), pp. 455-465.

Holst, D. 2005. Causes and prevention of dental caries: a perspective on cases and incidence. *Oral Health Prev Dent* 3(1), pp. 9-14.

Homer, K. A. et al. 1993. Chondroitin sulfate depolymerase and hyaluronidase activities of viridans streptococci determined by a sensitive spectrophotometric assay. *J Clin Microbiol* 31(6), pp. 1648-1651.

Hong, S. W. et al. 2014. Lipoteichoic acid of Streptococcus mutans interacts with Toll-like receptor 2 through the lipid moiety for induction of inflammatory mediators in murine macrophages. *Mol Immunol* 57(2), pp. 284-291.

Horst, O. V. et al. 2011. Caries induced cytokine network in the odontoblast layer of human teeth. *BMC Immunol* 12, p. 9.

Horton, W. A. et al. 1985. The cariogenicity of sucrose, glucose and maize starch in gnotobiotic rats mono-infected with strains of the bacteria Streptococcus mutans, Streptococcus salivarius and Streptococcus milleri. *Arch Oral Biol* 30(11-12), pp. 777-780.

Hsieh, S. C. et al. 2015. Static magnetic field attenuates lipopolysaccharide-induced inflammation in pulp cells by affecting cell membrane stability. *ScientificWorldJournal* 2015, p. 492683.

Hu, J. C. et al 2007. Enamel formation and Amelogenesis imperfecta. Cells Tissues Organs 186(1), pp. 78-85.

Huang, G. T. et al. 2006a. Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. *J Endod* 32(11), pp. 1066-1073.

Huang, G. T. et al. 2006b. In vitro characterization of human dental pulp cells: various isolation methods and culturing environments. *Cell Tissue Res* 324(2), pp. 225-236.

ICIS. 2008. Ciba defends antibacterial triclosan in soap. https://www.icis.com

Iejima, D. et al. 2007. Odontoblast marker gene expression is enhanced by a CC-chemokine family protein MIP-3alpha in human mesenchymal stem cells. *Arch Oral Biol* 52(10), pp. 924-931.

Inoue, T. et al. 1981. Bone morphogenesis induced by implantation of dentin and cortical bone matrices. *Bull Tokyo Dent Coll* 22(4), pp. 213-221.

Inoue, T. and Shimono, M. 1992. Repair dentinogenesis following transplantation into normal and germ-free animals. *Proc Finn Dent Soc* 88 Suppl 1, pp. 183-194.

Itzek, A. et al. 2010. Contribution of plasminogen activation towards the pathogenic potential of oral streptococci. *PLoS One* 5(11), p. e13826.

Izumi, T. et al. 1995. Immunohistochemical study on the immunocompetent cells of the pulp in human non-carious and carious teeth. *Arch Oral Biol* 40(7), pp. 609-614.

Jacobs, J. A. and Stobberingh, E. E. 1995. Hydrolytic enzymes of Streptococcus anginosus, Streptococcus constellatus and Streptococcus intermedius in relation to infection. *Eur J Clin Microbiol Infect Dis* 14(9), pp. 818-820.

Jacobsen, E. B. and Heyeraas, K. J. 1996. Effect of capsaicin treatment or inferior alveolar nerve resection on dentine formation and calcitonin gene-related peptide- and substance P-immunoreactive nerve fibres in rat molar pulp. *Arch Oral Biol* 41(12), pp. 1121-1131.

Jaiswal, R. K. et al. 2000. Adult human mesenchymal stem cell differentiation to the osteogenic or adipogenic lineage is regulated by mitogen-activated protein kinase. *J Biol Chem* 275(13), pp. 9645-9652.

Jang, J. H. et al. 2015. An Overview of Pathogen Recognition Receptors for Innate Immunity in Dental Pulp. *Mediators Inflamm* 2015, p. 794143.

Jegat, N. et al. 2007. Short-term effects of amelogenin gene splice products A+4 and A-4 implanted in the exposed rat molar pulp. *Head Face Med* 3, p. 40.

Jenkinson, H. F. and Demuth, D. R. 1997. Structure, function and immunogenicity of streptococcal antigen I/II polypeptides. *Mol Microbiol* 23(2), pp. 183-190.

Jenkinson, H. F. and Lamont, R. J. 1997. Streptococcal adhesion and colonization. *Crit Rev Oral Biol Med* 8(2), pp. 175-200.

Jenkinson, H. F. and Lamont, R. J. 2005. Oral microbial communities in sickness and in health. *Trends Microbiol* 13(12), pp. 589-595.

Jensen, A. et al. 2013. Taxonomy of the Anginosus group of the genus Streptococcus and description of Streptococcus anginosus subsp. whileyi subsp. nov. and Streptococcus constellatus subsp. viborgensis subsp. nov. *Int J Syst Evol Microbiol* 63(Pt 7), pp. 2506-2519.

Jiang, H. W. et al. 2008a. The expression of stromal cell-derived factor 1 (SDF-1) in inflamed human dental pulp. *J Endod* 34(11), pp. 1351-1354.

Jiang, L. et al. 2008b. The expression and role of stromal cell-derived factor-1alpha-CXCR4 axis in human dental pulp. *J Endod* 34(8), pp. 939-944.

Jontell, M. et al. 1998. Immune defense mechanisms of the dental pulp. *Crit Rev Oral Biol Med* 9(2), pp. 179-200.

Juarez, Z. E. and Stinson, M. W. 1999. An extracellular protease of Streptococcus gordonii hydrolyzes type IV collagen and collagen analogues. *Infect Immun* 67(1), pp. 271-278.

Jung, I. Y. et al. 2000. Molecular epidemiology and association of putative pathogens in root canal infection. *J Endod* 26(10), pp. 599-604.

Kakehashi, S. et al. 1965. the effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* 20, pp. 340-349.

Kanamori, S. et al. 2004. The role of the capsule of the Streptococcus milleri group in its pathogenicity. *J Infect Chemother* 10(2), pp. 105-109.

Kapoor, M. et al. 2004. Mutational analysis of the triclosan-binding region of enoyl-ACP (acyl-carrier protein) reductase from Plasmodium falciparum. *Biochem J* 381(Pt 3), pp. 735-741.

Kawai, T. and Akira, S. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* 21(4), pp. 317-337.

Kayaoglu, G. and Orstavik, D. 2004. Virulence factors of Enterococcus faecalis: relationship to endodontic disease. *Crit Rev Oral Biol Med* 15(5), pp. 308-320.

Keller, J. F. et al. 2010. Toll-like receptor 2 activation by lipoteichoic acid induces differential production of pro-inflammatory cytokines in human odontoblasts, dental pulp fibroblasts and immature dendritic cells. *Immunobiology* 215(1), pp. 53-59.

Keller, J. F. et al. 2011. Expression of NOD2 is increased in inflamed human dental pulps and lipoteichoic acid-stimulated odontoblast-like cells. *Innate Immun* 17(1), pp. 29-34.

Khan, S. et al. 2016. Chemical and morphological characteristics of mineral trioxide aggregate and Portland cements. *Dent Mater J* 35(1), pp. 112-117.

Kiatwateeratana, T. et al. 2009. Partial pulpotomy on caries-free teeth using enamel matrix derivative or calcium hydroxide: a randomized controlled trial. *Int Endod J* 42(7), pp. 584-592.

Kikuchi, N. et al. 2007. Formation of dentin-like particles in dentin defects above exposed pulp by controlled release of fibroblast growth factor 2 from gelatin hydrogels. *J Endod* 33(10), pp. 1198-1202.

Kim, S. et al. 1992. Effects of selected inflammatory mediators on blood flow and vascular permeability in the dental pulp. *Proc Finn Dent Soc* 88 Suppl 1, pp. 387-392.

Kitasako, Y. et al. 2008. Pulpal responses to bacterial contamination following dentin bridging beneath hard-setting calcium hydroxide and self-etching adhesive resin system. *Dent Traumatol* 24(2), pp. 201-206.

Koike, T. et al. 2014. Induction of reparative dentin formation on exposed dental pulp by dentin phosphophoryn/collagen composite. *Biomed Res Int* 2014, p. 745139.

Kolenbrander, P. E. et al. 2006. Bacterial interactions and successions during plaque development. *Periodontol 2000* 42, pp. 47-79.

Komiyama, E. Y. et al. 2016. Enterococcus Species in the Oral Cavity: Prevalence, Virulence Factors and Antimicrobial Susceptibility. *PLoS One* 11(9), p. e0163001.

Korkmaz, Y. et al. 2011. Irreversible inflammation is associated with decreased levels of the alpha1-, beta1-, and alpha2-subunits of sGC in human odontoblasts. *J Dent Res* 90(4), pp. 517-522.

Kraglund, F. 2014. Triclosan produces statistically significant reduction in plaque, gingivitis and caries but not clinically important benefit. *Evid Based Dent* 15(1), pp. 6-7.

Kuang, R. et al. 2016. Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp. *Acta Biomater* 33, pp. 225-234.

Kutllovci, T. et al. 2015. Bacteriological Identification of Selected Pathogens in Infected Primary and Young Permanent Teeth Associated with Clinical Symptoms. *Open Journal of Medical Microbiology* Vol.05No.02, p. 10.

Kuttler, Y. 1959. Classification of dentine into primary, secondary, and tertiary. *Oral Surg Oral Med Oral Pathol* 12(8), pp. 996-999.

Kuwana, M. et al. 2003. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. *J Leukoc Biol* 74(5), pp. 833-845.

Labro, M. T. 2005. Anti-inflammatory activity of ansamycins. *Expert Rev Anti Infect Ther* 3(1), pp. 91-103.

Landrygan-Bakri, J. et al. 2012. Real-time monitoring of the adherence of Streptococcus anginosus group bacteria to extracellular matrix decorin and biglycan proteoglycans in biofilm formation. *Res Microbiol* 163(6-7), pp. 436-447.

Lansdown, A. B. 2002. Calcium: a potential central regulator in wound healing in the skin. *Wound Repair Regen* 10(5), pp. 271-285.

Le Clerc, J. et al. 2013. Characterization of a programmed necrosis process in 3-dimensional cultures of dental pulp fibroblasts. *Int Endod J* 46(4), pp. 308-316.

Lee, D. H. et al. 2006. Effects of hydrogen peroxide (H2O2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biol Toxicol* 22(1), pp. 39-46.

Lee, S. I. et al. 2014. NOD2 Mediates Odontoblast Differentiation and RANKL Expression. *J Dent Res* 93(7), pp. 678-684.

Lee, Y. Y. et al. 2011. Up-regulation of nucleotide-binding oligomerization domain 1 in inflamed human dental pulp. *J Endod* 37(10), pp. 1370-1375.

Lesot, H. et al. 1992. The carboxy-terminal extension of the collagen binding domain of fibronectin mediates interaction with a 165 kDa membrane protein involved in odontoblast differentiation. *Differentiation* 49(2), pp. 109-118.

Lesot. H et al. 1993. Experimental Induction of Odontoblast Differentiation and Stimulation During Preparative Processes. *Cells and Materials,* 3(2), pp. 201-217.

Levin, L. G. et al. 2009. Identify and define all diagnostic terms for pulpal health and disease states. *J Endod* 35(12), pp. 1645-1657.

Levy, C. W. et al. 1999. Molecular basis of triclosan activity. *Nature* 398(6726), pp. 383-384.

Lewis, M. A. et al. 1990. A microbiological and clinical review of the acute dentoalveolar abscess. *Br J Oral Maxillofac Surg* 28(6), pp. 359-366.

Leye Benoist, F. et al. 2012. Evaluation of mineral trioxide aggregate (MTA) versus calcium hydroxide cement (Dycal((R))) in the formation of a dentine bridge: a randomised controlled trial. *Int Dent J* 62(1), pp. 33-39.

Li, F. et al. 2014. Porous chitosan bilayer membrane containing TGF-beta1 loaded microspheres for pulp capping and reparative dentin formation in a dog model. *Dent Mater* 30(2), pp. 172-181.

Li, J. G. et al. 2015. Melatonin attenuates inflammation of acute pulpitis subjected to dental pulp injury. *Am J Transl Res* 7(1), pp. 66-78.

Li, Y. et al 2011. odontoblast-like cell differentiation and dentin formation induced with TGF-beta1. *Arch Oral Biol* 56(11), pp. 1221-1229

Lima, M. et al. 1992. Protective effect of a T-cell-dependent immunosuppressive, B-cell-mitogenic protein (F3'EP-Si, or P90) produced by Streptococcus intermedius. *Infect Immun* 60(9), pp. 3571-3578.

Lin, L. M. et al. 2006. Histological study of periradicular tissue responses to uninfected and infected devitalized pulps in dogs. *J Endod* 32(1), pp. 34-38.

Lin, Z. M. et al. 2009. Expression of nucleotide-binding oligomerization domain 2 in normal human dental pulp cells and dental pulp tissues. *J Endod* 35(6), pp. 838-842.

Linde, A. 1985. The extracellular matrix of the dental pulp and dentin. *J Dent Res* 64 Spec No, pp. 523-529.

Linde, A. and Goldberg, M. 1993. Dentinogenesis. *Crit Rev Oral Biol Med* 4(5), pp. 679-728.

Lindl, T. ed. 2002. *Zell- und Gewebekultur.* 5th ed. Heidelberg: Oxford university press.

Liu, J. et al. 2005. In vitro differentiation and mineralization of human dental pulp cells induced by dentin extract. *In Vitro Cell Dev Biol Anim* 41(7), pp. 232-238.

Liu, S. et al. 2014. Immunohistochemical localization of NALP3 inflammasome in experimental periapical lesions. *Int Endod J* 47(10), pp. 949-957.

Liu, Y. et al. 2015. Necrosis-Induced Sterile Inflammation Mediated by Interleukin-1alpha in Retinal Pigment Epithelial Cells. *PLoS One* 10(12), p. e0144460.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4), pp. 402-408.

Love, R. M. and Jenkinson, H. F. 2002. Invasion of dentinal tubules by oral bacteria. *Crit Rev Oral Biol Med* 13(2), pp. 171-183.

Luheshi, N. M. et al. 2009. Nuclear retention of IL-1 alpha by necrotic cells: a mechanism to dampen sterile inflammation. *Eur J Immunol* 39(11), pp. 2973-2980.

Lumsden, A. G. 1988. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* 103 Suppl, pp. 155-169.

Ma, L. et al. 2015. Activation and dynamic expression of Notch signaling in dental pulp cells after injury in vitro and in vivo. *Int Endod J* 49(12), pp. 1165-1174.

Magloire, H. et al. 1996. An in vitro model of human dental pulp repair. *J Dent Res* 75(12), pp. 1971-1978.

Magloire, H. et al. 2001. Molecular regulation of odontoblast activity under dentin injury. *Adv Dent Res* 15, pp. 46-50.

Mangkornkarn, C. and Steiner, J. C. 1992. In vivo and in vitro glycosaminoglycans from human dental pulp. *J Endod* 18(7), pp. 327-331.

Maltz,M.et al.2007. Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study. Caries Res 41(6), pp. 493-496.

Mann, J. et al. 1996. Comparison of the clinical anticaries efficacy of a 1500 NaF silica-based dentifrice containing triclosan and a copolymer to a 1500 NaF silica-based dentifrice without those additional agents: a study on adults in Israel. *J Clin Dent* 7(4), pp. 90-95.

Mansour, M. H. and Cox, S. C. 2006. Patients presenting to the general practitioner with pain of dental origin. *Med J Aust* 185(2), pp. 64-67.

Marcet, B. et al. 2007. Extracellular nucleotides regulate CCL20 release from human primary airway epithelial cells, monocytes and monocyte-derived dendritic cells. *J Cell Physiol* 211(3), pp. 716-727.

Margosch, D. et al. 2005. Effect of High Pressure and Heat on Bacterial Toxins. *Food Technology and Biotechnology* 43(3), pp. 211-217.

Martin, F. E. et al. 2002. Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. *J Clin Microbiol* 40(5), pp. 1698-1704.

Massey, W. L. et al. 1993. The association of carious dentin microflora with tissue changes in human pulpitis. *Oral Microbiol Immunol* 8(1), pp. 30-35.

Matsuo, T. et al. 1996. A clinical study of direct pulp capping applied to carious-exposed pulps. *J Endod* 22(10), pp. 551-556.

McKnight, D. A. et al. 2008. A comprehensive analysis of normal variation and disease-causing mutations in the human DSPP gene. *Hum Mutat* 29(12), pp. 1392-1404.

McLachlan, J. L. et al. 2004. S100 and cytokine expression in caries. *Infect Immun* 72(7), pp. 4102-4108.

McMurry, L. M. et al. 1999. Genetic evidence that InhA of Mycobacterium smegmatis is a target for triclosan. *Antimicrob Agents Chemother* 43(3), pp. 711-713.

McMurry, L. M. et al. 1998. Triclosan targets lipid synthesis. *Nature* 394(6693), pp. 531-532.

Medzihradszky, K. F. et al. 2000. The characteristics of peptide collision-induced dissociation using a high-performance MALDI-TOF/TOF tandem mass spectrometer. *Anal Chem* 72(3), pp. 552-558.

Mei, Y. F. et al. 2007. Sequential expression of endothelial nitric oxide synthase, inducible nitric oxide synthase, and nitrotyrosine in odontoblasts and pulp cells during dentin repair after tooth preparation in rat molars. *Cell Tissue Res* 328(1), pp. 117-127.

Mejare, I. and Cvek, M. 1993. Partial pulpotomy in young permanent teeth with deep carious lesions. *Endod Dent Traumatol* 9(6), pp. 238-242.

Mejare, I. A. et al. 2012. Diagnosis of the condition of the dental pulp: a systematic review. *Int Endod J* 45(7), pp. 597-613.

Melin, M. et al. 2000. Effects of TGFbeta1 on dental pulp cells in cultured human tooth slices. *J Dent Res* 79(9), pp. 1689-1696.

Mendonca, M. L. et al. 2016. The sil Locus in Streptococcus Anginosus Group: Interspecies Competition and a Hotspot of Genetic Diversity. *Front Microbiol* 7, p. 2156.

Mendoza, D. d. and Schujman, G. E. 2009. *Encycopedia of Microbiology*. Third ed. Oxford: Elsevier.

Mente, J. et al. 2014. Treatment outcome of mineral trioxide aggregate or calcium hydroxide direct pulp capping: long-term results. *J Endod* 40(11), pp. 1746-1751.

Miller, R. J. et al. 2008. CXCR4 signaling in the regulation of stem cell migration and development. *J Neuroimmunol* 198(1-2), pp. 31-38.

Min, K. S. et al. 2008. Effect of mineral trioxide aggregate on dentin bridge formation and expression of dentin sialoprotein and heme oxygenase-1 in human dental pulp. *J Endod* 34(6), pp. 666-670.

Mitsiadis, T. A. and Rahiotis, C. 2004. Parallels between tooth development and repair: conserved molecular mechanisms following carious and dental injury. *J Dent Res* 83(12), pp. 896-902.

Mitsiadis, T. A. et al 2011. Dental pulp stem cells, niches, and notch signalling in tooth injury. *Adv* Dent Res 23(3), ppp 275-279.

Mizuno, M. and Banzai, Y. 2008. Calcium ion release from calcium hydroxide stimulated fibronectin gene expression in dental pulp cells and the differentiation of dental pulp cells to mineralized tissue forming cells by fibronectin. *Int Endod J* 41(11), pp. 933-938.

Moazzam, A. A. et al. 2015. Intracranial bacterial infections of oral origin. *J Clin Neurosci* 22(5), pp. 800-806.

Modeer, T. et al. 1996. Triclosan reduces prostaglandin biosynthesis in human gingival fibroblasts challenged with interleukin-1 in vitro. *J Clin Periodontol* 23(10), pp. 927-933.

Mohammadi, Z. et al. 2013. Microbial biofilms in endodontic infections: an update review. *Biomed J* 36(2), pp. 59-70.

Monteiro Bramante, C. et al. 2008. Presence of arsenic in different types of MTA and white and gray Portland cement. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 106(6), pp. 909-913.

Montuenga, L. M. et al. 1997. Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology* 138(1), pp. 440-451.

Morgan, M. J. and Liu, Z. G. 2011. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res* 21(1), pp. 103-115.

Moschioni, M. et al. 2010. Adhesion determinants of the Streptococcus species. *Microb Biotechnol* 3(4), pp. 370-388.

Mueller, S. N. et al. 1980. Cellular senescence in a cloned strain of bovine fetal aortic endothelial cells. *Science* 207(4433), pp. 889-891.

Munksgaard, E. C. et al. 1978. Biosynthesis of phosphoprotein by rat incisor odontoblasts in in-vitro culture. *Arch Oral Biol* 23(7), pp. 583-585.

Murdoch, C. 2000. CXCR4: chemokine receptor extraordinaire. *Immunol Rev* 177, pp. 175-184.

Murray, C. J. 1994. Quantifying the burden of disease: the technical basis for disability-adjusted life years. *Bull World Health Organ* 72(3), pp. 429-445.

Murray, C. J. and Lopez, A. D. 1994. Quantifying disability: data, methods and results. *Bull World Health Organ* 72(3), pp. 481-494.

Murray, P. E. et al. 2002. Cavity remaining dentin thickness and pulpal activity. *Am J Dent* 15(1), pp. 41-46.

Murray, P. E. et al. 2008. Comparison of operative procedure variables on pulpal viability in an ex vivo model. *Int Endod J* 41(5), pp. 389-400.

Musson, D. S. et al. 2010. Adrenomedullin is expressed during rodent dental tissue development and promotes cell growth and mineralization. *Biol Cell* 102(3), pp. 145-157.

Mustafa, M. et al. 2003. Uptake, distribution and release of 14C-triclosan in human gingival fibroblasts. *J Pharm Sci* 92(8), pp. 1648-1653.

Mustafa, M. et al. 1998. Effect of triclosan on interleukin-1 beta production in human gingival fibroblasts challenged with tumor necrosis factor alpha. *Eur J Oral Sci* 106(2 Pt 1), pp. 637-643.

Nagamune, H. et al. 2000. Distribution of the intermedilysin gene among the anginosus group streptococci and correlation between intermedilysin production and deep-seated infection with Streptococcus intermedius. *J Clin Microbiol* 38(1), pp. 220-226.

Nagata, S. et al. 2017. Anti-Inflammatory Effects of PEGylated Human Adrenomedullin in a Mouse DSS-Induced Colitis Model. *Drug Dev Res* 78(3-4), pp. 129-134.

Nair, P. N. et al. 2009. Histological, ultrastructural and quantitative investigations on the response of healthy human pulps to experimental capping with Mineral Trioxide Aggregate: a randomized controlled trial. 2008. *Int Endod J* 42(5), pp. 422-444.

Nakamura, Y. et al. 2001. Enamel matrix derivative promotes reparative processes in the dental pulp. *Adv Dent Res* 15, pp. 105-107.

Nakanishi, T. et al. 2005. Expression of macrophage inflammatory protein 3alpha in human inflamed dental pulp tissue. *J Endod* 31(2), pp. 84-87.

Nakashima, M. 1994. Induction of dentin formation on canine amputated pulp by recombinant human bone morphogenetic proteins (BMP)-2 and -4. *J Dent Res* 73(9), pp. 1515-1522.

Nakashima, M. et al. 1994. Regulatory role of transforming growth factor-beta, bone morphogenetic protein-2, and protein-4 on gene expression of extracellular matrix proteins and differentiation of dental pulp cells. *Dev Biol* 162(1), pp. 18-28.

Nakata, K. et al. 2000. Anaerobic bacterial extracts influence production of matrix metalloproteinases and their inhibitors by human dental pulp cells. *J Endod* 26(7), pp. 410-413.

Nallapareddy, S. R. et al. 2000. Enterococcus faecalis adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun* 68(9), pp. 5218-5224.

Nandlal, B. et al. 2016. Malodor reductions and improved oral hygiene by toothbrushing and mouthrinsing. *Indian J Dent Res* 27(1), pp. 42-47.

Nathan, C. 2002. Points of control in inflammation. *Nature* 420(6917), pp. 846-852.

Neumayr, A. et al. 2010. Multiple liver abscesses with isolation of Streptococcus intermedius related to a pyogenic dental infection in an immuno-competent patient. *Eur J Med Res* 15, pp. 319-322.

Ng, Y. L. et al. 2008. Outcome of secondary root canal treatment: a systematic review of the literature. *Int Endod J* 41(12), pp. 1026-1046.

Ng, Y. L. et al. 2010. Tooth survival following non-surgical root canal treatment: a systematic review of the literature. *Int Endod J* 43(3), pp. 171-189.

Ng, Y. L. et al. 2011. a prospective study of the factors affecting outcomes of nonsurgical root canal treatment: part 1: periapical health. *Int Endod J* 44(7), pp. 583-609.

Ng, Y. L. et al. 2007. Outcome of primary root canal treatment: systematic review of the literature - part 1. Effects of study characteristics on probability of success. *Int Endod J* 40(12), pp. 921-939.

Nishikawa, S. and Sasaki, F. 1999. Apoptosis of dental pulp cells and their elimination by macrophages and MHC class II-expressing dendritic cells. *J Histochem Cytochem* 47(3), pp. 303-312.

Nishio Ayre, W. et al. 2018. Enterococcus faecalis Demonstrates Pathogenicity through Increased Attachment in an Ex Vivo Polymicrobial Pulpal Infection. *Infect Immun* 86(5), pii: e00871-17.

O'Shea, J. J. et al. 2002. Cytokines and autoimmunity. *Nat Rev Immunol* 2(1), pp. 37-45.

Oh, M. and Nör, J. E. 2015. The Perivascular Niche and Self-Renewal of Stem Cells. Front Physiol 6 ,p. 367.

Ohshima, H. et al. 1995. Responses of immunocompetent cells to cavity preparation in rat molars: an immunohistochemical study using OX6-monoclonal antibody. *Connect Tissue Res* 32(1-4), pp. 303-311.

Okayama, H. et al. 2005. Experimental abscess formation caused by human dental plaque. *Microbiol Immunol* 49(5), pp. 399-405.

Okiji, T. et al. 1997. Structural and functional association between substance P- and calcitonin gene-related peptide-immunoreactive nerves and accessory cells in the rat dental pulp. *J Dent Res* 76(12), pp. 1818-1824.

Olatosi, O. O. et al. 2015. Effect of mineral trioxide aggregate and formocresol pulpotomy on vital primary teeth: a clinical and radiographic study. *Niger J Clin Pract* 18(2), pp. 292-296.

Olsen, I. and Yilmaz, O. 2016. Modulation of inflammasome activity by Porphyromonas gingivalis in periodontitis and associated systemic diseases. *J Oral Microbiol* 8, p. 30385.

Olson, A. B. et al. 2013. Phylogenetic relationship and virulence inference of Streptococcus Anginosus Group: curated annotation and whole-genome comparative analysis support distinct species designation. *BMC Genomics* 14, p. 895.

Orland, F. J. et al. 1954. Use of the germfree animal technic in the study of experimental dental caries. I. Basic observations on rats reared free of all microorganisms. *J Dent Res* 33(2), pp. 147-174.

Ørstavik, D. and Ford, T. P. 2008. *Essentials of Endodontology*. Oxford: Blackwell Munksgaard.

Paganelli, F. L. et al. 2012. Optimizing future treatment of enterococcal infections: attacking the biofilm? *Trends Microbiol* 20(1), pp. 40-49.

Panagakos, F. S. 1998. Transformation and preliminary characterization of primary human pulp cells. *J Endod* 24(3), pp. 171-175.

Panagakos, F. S. et al. 2005. Advanced oral antibacterial/anti-inflammatory technology: A comprehensive review of the clinical benefits of a triclosan/copolymer/fluoride dentifrice. *J Clin Dent* 16 Suppl, pp. S1-19.

Pancer, B. A. et al. 2016. Effects of triclosan on host response and microbial biomarkers during experimental gingivitis. *J Clin Periodontol* 43(5), pp. 435-444.

Parirokh, M. and Torabinejad, M. 2010a. Mineral trioxide aggregate: a comprehensive literature review--Part I: chemical, physical, and antibacterial properties. *J Endod* 36(1), pp. 16-27.

Parirokh, M. and Torabinejad, M. 2010b. Mineral trioxide aggregate: a comprehensive literature review--Part III: Clinical applications, drawbacks, and mechanism of action. *J Endod* 36(3), pp. 400-413.

Park, M. et al. 2015a. Effect of dentin treatment on proliferation and differentiation of human dental pulp stem cells. *Restor Dent Endod* 40(4), pp. 290-298.

Park, S. H. et al. 2015b. Inflammation of the Dental Pulp. *Mediators Inflamm* 2015, p. 980196.

Paster, B. J. et al. 2006. The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000* 42, pp. 80-87.

Paula-Silva, F. W. et al. 2009. TNF-alpha promotes an odontoblastic phenotype in dental pulp cells. *J Dent Res* 88(4), pp. 339-344.

Pereira, M. A. et al. 2017. No additional benefit of using a calcium hydroxide liner during stepwise caries removal: A randomized clinical trial. *J Am Dent Assoc* 148(6), pp. 369-376.

Perkins, D. N. et al. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20(18), pp. 3551-3567.

Peter, S. et al. 2004. Antiplaque and antigingivitis efficacy of toothpastes containing Triclosan and fluoride. *Int Dent J* 54(5 Suppl 1), pp. 299-303.

Peters, B. M. et al. 2012. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* 25(1), pp. 193-213.

Peterson, J. W. 1996. Bacterial Pathogenesis. In: Baron, S. ed. *Medical Microbiology*. Galveston TX: The University of Texas Medical Branch at Galveston.

Pinto, A. S. et al. 2006. Clinical and microbiological effect of calcium hydroxide protection in indirect pulp capping in primary teeth. *Am J Dent* 19(6), pp. 382-386.

Pleskanovskaia, N. V. et al. 2013. [Feasibility and assessment of combined anti-inflammatory, antibacterial and immunocorrective treatment in periodontal disease]. *Stomatologiia (Mosk)* 92(1), pp. 26-30.

Poole, P. M. and Wilson, G. 1979. Occurrence and cultural features of Streptococcus milleri in various body sites. *J Clin Pathol* 32(8), pp. 764-768.

Pourhajibagher, M. et al. 2017. Culture-dependent approaches to explore the prevalence of root canal pathogens from endodontic infections *Braz Oral Res* 31, p. e108.

Prasanth, T. and Saraswathi, T. 2012. Histopathological and radiographic evaluation of rat molar teeth after traumatic injury-a pilot study. *J Oral Maxillofac Pathol* 16(3), pp. 313-317.

Preza, D. et al. 2008. Bacterial profiles of root caries in elderly patients. *J Clin Microbiol* 46(6), pp. 2015-2021.

Qureshi, A. et al. 2014. Recent advances in pulp capping materials: an overview. *J Clin Diagn Res* 8(1), pp. 316-321.

Radeva, E. and Karayasheva, D. 2017. *Importance of Enterococci (Enterococcus faecalis) for Dental Medicine - Microbiological Characterization, Prevalence and Resistance* 6(7), pp. 1970-1975

Radman, I. K. et al. 2016. Microbiological Findings in Deep Caries Lesions. 63(1), p. 7.

Rajasekharan, S. et al. 2016. Efficacy of three different pulpotomy agents in primary molars - A randomised control trial. *Int Endod J* 50(3), pp. 215-228.

Rakita, R. M. et al. 1999. Enterococcus faecalis bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. *Infect Immun* 67(11), pp. 6067-6075.

Ramsey, M. M. et al. 2011. Metabolite cross-feeding enhances virulence in a model polymicrobial infection. *PLoS Pathog* 7(3), p. e1002012.

Randow, K. and Glantz, P.O. 1986. On Cantilever loading of vital and non-vital teeth. An experimental clinical study. *Acta Odontologica Scandinavica* 44(5), pp. 271-277.

Reeves, R. and Stanley, H. R. 1966. The relationship of bacterial penetration and pulpal pathosis in carious teeth. *Oral Surg Oral Med Oral Pathol* 22(1), pp. 59-65.

Regos, J. and Hitz, H. R. 1974. Investigations on the mode of action of Triclosan, a broad spectrum antimicrobial agent. *Zentralbl Bakteriol Orig A* 226(3), pp. 390-401.

Reit, C. P., K. 2010. *Textbook of endodontology*. 3nd ed. ed. Oxford: Wiley-Blackwell.

Renard, E. et al. 2016. Immune Cells and Molecular Networks in Experimentally Induced Pulpitis. *J Dent Res* 95(2), pp. 196-205.

Ricketts, D. et al. 2013. Operative caries management in adults and children. *Cochrane Database Syst Rev* (3), p. CD003808.

Ricketts, D. N. et al. 2006. Complete or ultraconservative removal of decayed tissue in unfilled teeth. *Cochrane Database Syst Rev* (3), p. CD003808.

Ricucci, D. et al. 2014. Correlation between clinical and histologic pulp diagnoses. *J Endod* 40(12), pp. 1932-1939.

Roberts, H. W. et al. 2008. Mineral trioxide aggregate material use in endodontic treatment: a review of the literature. *Dent Mater* 24(2), pp. 149-164.

Roberts, J. L. 2010. *Development of an ex-vivo co-culture system to model pulpal infection by Streptococcus anginosus group bacteria.*

Roberts, J. L. et al. 2013. Development of an ex vivo coculture system to model pulpal infection by Streptococcus anginosus group bacteria. *J Endod* 39(1), pp. 49-56.

Robertson, D. and Smith, A. J. 2009. The microbiology of the acute dental abscess. *J Med Microbiol* 58(Pt 2), pp. 155-162.

Rocas, I. N. et al. 2016. Microbiome of Deep Dentinal Caries Lesions in Teeth with Symptomatic Irreversible Pulpitis. *PLoS One* 11(5), p. e0154653.

Rocas, I. N. et al. 2015. Advanced Caries Microbiota in Teeth with Irreversible Pulpitis. *J Endod* 41(9), pp. 1450-1455.

Rocas, I. N. et al. 2004. Association of Enterococcus faecalis with different forms of periradicular diseases. *J Endod* 30(5), pp. 315-320.

Rocha, C. T. et al. 2008. Biofilm on the apical region of roots in primary teeth with vital and necrotic pulps with or without radiographically evident apical pathosis. *Int Endod J* 41(8), pp. 664-669.

Rodricks, J. V. et al. 2010. Triclosan: a critical review of the experimental data and development of margins of safety for consumer products. *Crit Rev Toxicol* 40(5), pp. 422-484.

Ruiz-Linares, M. et al. 2014. Antimicrobial activity of alexidine, chlorhexidine and cetrimide against Streptococcus mutans biofilm. *Ann Clin Microbiol Antimicrob* 13, p. 41.

Rutherford, R. B. and Gu, K. 2000. Treatment of inflamed ferret dental pulps with recombinant bone morphogenetic protein-7. *Eur J Oral Sci* 108(3), pp. 202-206.

Rutherford, R. B. et al. 1994. The time-course of the induction of reparative dentine formation in monkeys by recombinant human osteogenic protein-1. *Arch Oral Biol* 39(10), pp. 833-838.

Sadowski, M. C. et al. 2014. The fatty acid synthase inhibitor triclosan: repurposing an anti-microbial agent for targeting prostate cancer. *Oncotarget* 5(19), pp. 9362-9381.

Sagomonyants, K. et al. 2015. Enhanced Dentinogenesis of Pulp Progenitors by Early Exposure to FGF2. *J Dent Res* 94(11), pp. 1582-1590.

Saito, K. et al. 2011. The expression of GM-CSF and osteopontin in immunocompetent cells precedes the odontoblast differentiation following allogenic tooth transplantation in mice. *J Histochem Cytochem* 59(5), pp. 518-529.

Salah, R. et al. 2008. Prevalence of putative virulence factors and antimicrobial susceptibility of Enterococcus faecalis isolates from patients with dental Diseases. *BMC Oral Health* 8, p. 17.

Salehi, S. et al. 2016. Dentin matrix components extracted with phosphoric acid enhance cell proliferation and mineralization. *Dent Mater* 32(3), pp. 334-342.

Salvi, G. E. and Ramseier, C. A. 2015. Efficacy of patient-administered mechanical and/or chemical plaque control protocols in the management of peri-implant mucositis. A systematic review. *J Clin Periodontol* 42 Suppl 16, pp. S187-201.

Santos, A. D. et al. 2005. Physico-chemical properties of MTA and a novel experimental cement. *Int Endod J* 38(7), pp. 443-447.

Sanchez-Sanhueza, G. et al. 2018. Metagenomic study of bacterial microbiota in persistent endodontic infections using Next-generation sequencing. *Int Endod J*, pp.1-13

Sawicki, L. et al. 2008. Histological evaluation of mineral trioxide aggregate and calcium hydroxide in direct pulp capping of human immature permanent teeth. *Am J Dent* 21(4), pp. 262-266.

Schmid, M. B. and Kaplan, N. 2004. Reduced triclosan susceptibility in methicillin-resistant Staphylococcus epidermidis. *Antimicrob Agents Chemother* 48(4), pp. 1397-1399.

Schroder, U. 1985. Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. *J Dent Res* 64 Spec No, pp. 541-548.

Schumann, R. R. 2011. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. *Biochem Soc Trans* 39(4), pp. 989-993.

Schwarze, T. et al. 2002. Long-term cytocompatibility of various endodontic sealers using a new root canal model. *J Endod* 28(11), pp. 749-753.

Schwendicke, F. et al. 2013. Incomplete caries removal: a systematic review and meta-analysis. *J Dent Res* 92(4), pp. 306-314.

Sedgley, C. et al. 2006. Prevalence of Enterococcus faecalis at multiple oral sites in endodontic patients using culture and PCR. *J Endod* 32(2), pp. 104-109.

Seltzer, S. et al. 1963. The dynamics of pulp inflammation: correlations between diagnostic data and actual histologic findings in the pulp. *Oral Surg Oral Med Oral Pathol* 16, pp. 846-871.

Selwitz, R. H. et al. 2007. Dental caries. *Lancet* 369(9555), pp. 51-59.

Serhan, C. N. and Petasis, N. A. 2011. Resolvins and protectins in inflammation resolution. *Chem Rev* 111(10), pp. 5922-5943.

Services, D. o. H. 1994. Tropical antimicrobia. *Federal Register* 59(116), pp. 31402-31452.

Shevchenko, A. et al. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1(6), pp. 2856-2860.

Shiba, H. et al. 2003. Macrophage inflammatory protein-3alpha and beta-defensin-2 stimulate dentin sialophosphoprotein gene expression in human pulp cells. *Biochem Biophys Res Commun* 306(4), pp. 867-871.

Shibutani, T. et al. 1990. Immunohistochemical study of chondroitin sulfate and dermatan sulfate proteoglycan in dental pulp and dentin. *Gifu Shika Gakkai Zasshi* 17(1), pp. 124-130.

Shim, J. Y. et al. 2012. Antiplaque and antigingivitis effects of a mouthrinse containing cetylpyridinium chloride, triclosan and dipotassium glycyrrhizinate. *J Periodontal Implant Sci* 42(2), pp. 33-38.

Shweta and Prakash, S. K. 2013. Dental abscess: A microbiological review. *Dent Res J (Isfahan)* 10(5), pp. 585-591.

Silver, F. H. and Landis, W. J. 2011. Deposition of apatite in mineral vertebrate extracellular matrices: A model of possible nucleation sites on type I collagen. *Connect Tissue Res* 52(3), pp. 242-254.

Simon, S. et al. 2010. The MAP kinase pathway is involved in odontoblast stimulation via p38 phosphorylation. *J Endod* 36(2), pp. 256-259.

Simon, S. et al. 2009. Molecular characterization of young and mature odontoblasts. *Bone* 45(4), pp. 693-703.

Singh, K. V. et al. 2010. Importance of the collagen adhesin ace in pathogenesis and protection against Enterococcus faecalis experimental endocarditis. *PLoS Pathog* 6(1), p. e1000716.

Siqueira, J. F., Jr. and Sen, B. H. 2004. Fungi in endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 97(5), pp. 632-641

Siqueira, J. F., Jr. et al. 2008. Prevalence and clonal analysis of Porphyromonas gingivalis in primary endodontic infections. *J Endod* 34(11), pp. 1332-1336.

Six, N. et al. 2007. Dentonin, a MEPE fragment, initiates pulp-healing response to injury. *J Dent Res* 86(8), pp. 780-785.

Sloan, A. J. et al. 2000. Transforming growth factor-beta isoform expression in mature human healthy and carious molar teeth. *Histochem J* 32(4), pp. 247-252.

Sloan, A. J. et al. 1998. An in vitro approach for the study of dentinogenesis by organ culture of the dentine-pulp complex from rat incisor teeth. *Archives of oral biology* 43(6), pp. 421-430.

Sloan, A. J. and Smith, A. J. 1999. Stimulation of the dentine-pulp complex of rat incisor teeth by transforming growth factor-beta isoforms 1-3 in vitro. *Arch Oral Biol* 44(2), pp. 149-156.

Sloan, A. J. and Smith, A. J. 2007. Stem cells and the dental pulp: potential roles in dentine regeneration and repair. *Oral Dis* 13(2), pp. 151-157.

Smith, A. J. 2003. Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. *J Dent Educ* 67(6), pp. 678-689.

Smith, A. J. et al. 1995. Reactionary dentinogenesis. *Int J Dev Biol* 39(1), pp. 273-280.

Smith, A. J. et al. 2016. Exploiting the Bioactive Properties of the Dentin-Pulp Complex in Regenerative Endodontics. *J Endod* 42(1), pp. 47-56.

Smith, A. J. et al. 2001. Trans-dentinal stimulation of tertiary dentinogenesis. *Adv Dent Res* 15, pp. 51-54.

Smith, A. J. et al. 2012. Dentine as a bioactive extracellular matrix. *Arch Oral Biol* 57(2), pp. 109-121.

Smith, A. J. et al. 1994. Odontoblast stimulation in ferrets by dentine matrix components. *Arch Oral Biol* 39(1), pp. 13-22.

Smith, A. J. et al. 1990. In vivo morphogenetic activity of dentine matrix proteins. *J Biol Buccale* 18(2), pp. 123-129.

Smith, E. L. et al. 2010. An ex vivo rodent mandible culture model for bone repair. *Tissue Eng Part C Methods* 16(6), pp. 1287-1296.

Smith, R. F. and Willett, N. P. 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. *Appl Microbiol* 16(9), pp. 1434-1436.

Sowinski, J. A. et al. 2002. A clinical study to assess the anticalculus efficacy of a new dentifrice containing a special grade of silica (Colgate Total Plus Whitening Toothpaste): a clinical trial on adults. *J Clin Dent* 13(2), pp. 65-68.

Spellerberg, B. and Brandt, C. 2017. *Manual of Clinical Microbiology*. 9th ed. Washington, DC: ASM Press.

Splieth, C. H. and Tachou, A. 2013. Epidemiology of dentin hypersensitivity. *Clin Oral Investig* 17 Suppl 1, pp. S3-8.

Sreenivasan, P. K. et al. 2017. Reduction in bacteremia after brushing with a triclosan/copolymer dentifrice- A randomized clinical study. *J Clin Periodontol*.

Staquet, M. J. et al. 2011. Pattern-recognition receptors in pulp defense. *Adv Dent Res* 23(3), pp. 296-301.

Staquet, M. J. et al. 2008. Different roles of odontoblasts and fibroblasts in immunity. *J Dent Res* 87(3), pp. 256-261.

Stuart, C. H. et al. 2006. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J Endod* 32(2), pp. 93-98.

Suller, M. T. and Russell, A. D. 1999. Antibiotic and biocide resistance in methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococcus. *J Hosp Infect* 43(4), pp. 281-291.

Suller, M. T. and Russell, A. D. 2000. Triclosan and antibiotic resistance in Staphylococcus aureus. *J Antimicrob Chemother* 46(1), pp. 11-18.

Sun, J. and Song, X. 2011. Assessment of antimicrobial susceptibility of Enterococcus faecalis isolated from chronic periodontitis in biofilm versus planktonic phase. *J Periodontol* 82(4), pp. 626-631.

Sunwoo, B. Y. and Miller, W. T., Jr. 2014. Streptococcus anginosus infections: crossing tissue planes. *Chest* 146(4), pp. e121-e125.

Svatun, B. et al. 1993. The effects of three silica dentifrices containing Triclosan on supragingival plaque and calculus formation and on gingivitis. *Int Dent J* 43(4 Suppl 1), pp. 441-452.

Tadin, A. et al. 2014. Composite-induced toxicity in human gingival and pulp fibroblast cells. *Acta Odontol Scand* 72(4), pp. 304-311.

Taha, N. A. et al. 2017. Assessment of Mineral Trioxide Aggregate pulpotomy in mature permanent teeth with carious exposures. *Int Endod J* 50(2), pp. 117-125.

Takao, A. 2003. Cloning and expression of hyaluronate lyase genes of Streptococcus intermedius and Streptococcus constellatus subsp. constellatus(1). *FEMS Microbiol Lett* 219(1), pp. 143-150.

Takao, A. et al. 2010. Sialidase of Streptococcus intermedius: a putative virulence factor modifying sugar chains. *Microbiol Immunol* 54(10), pp. 584-595.

Tarsi, R. et al. 1998. Effect of low-molecular-weight chitosans on the adhesive properties of oral streptococci. *Oral Microbiol Immunol* 13(4), pp. 217-224.

Taub, D. D. and Oppenheim, J. J. 1994. Chemokines, inflammation and the immune system. *Ther Immunol* 1(4), pp. 229-246.

Tawil, P. Z. et al. 2015. Mineral trioxide aggregate (MTA): its history, composition, and clinical applications. *Compend Contin Educ Dent* 36(4), pp. 247-252; quiz 254, 264.

Tecles, O. et al. 2005. Activation of human dental pulp progenitor/stem cells in response to odontoblast injury. *Arch Oral Biol* 50(2), pp. 103-108.

Tennert, C, et al.2014. New bacterial composition in primary and persistent/secondary endodontic infections with respect to clinical and radiographic findings. J Endod 40(5), pp. 670-677.

Thesleff, I. 2003. Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J Cell Sci* 116(Pt 9), pp. 1647-1648.

Thompson, A. et al. 2005. The fate and removal of triclosan during wastewater treatment. *Water Environ Res* 77(1), pp. 63-67.

Thompson, V. et al. 2008. Treatment of deep carious lesions by complete excavation or partial removal: a critical review. *J Am Dent Assoc* 139(6), pp. 705-712.

Thurnheer, T. et al. 2001. Automated fluorescent in situ hybridization for the specific detection and quantification of oral streptococci in dental plaque. *J Microbiol Methods* 44(1), pp. 39-47.

Tjäderhane, L. et al. 2001. Human odontoblast culture method: the expression of collagen and matrix metalloproteinases (MMPs). *Adv Dent Res* 15, pp. 55-58.

Tjäderhane, L. et al. 1998. A novel organ culture method to study the function of human odontoblasts in vitro: gelatinase expression by odontoblasts is differentially regulated by TGF-beta1. *J Dent Res* 77(7), pp. 1486-1496.

Tobin, C. E. 1972. Correlation of vascularity with mineralization in human fetal teeth. *Anat Rec* 174(3), pp. 371-379.

Toledo-Arana, A. et al. 2001. The enterococcal surface protein, Esp, is involved in Enterococcus faecalis biofilm formation. *Appl Environ Microbiol* 67(10), pp. 4538-4545.

Tomson, P. L. et al. 2007. Dissolution of bio-active dentine matrix components by mineral trioxide aggregate. *J Dent* 35(8), pp. 636-642.

Tomson, P. L. et al. 2017. Growth factor release from dentine matrix by pulp-capping agents promotes pulp tissue repair-associated events. *Int Endod J* 50(3), pp. 281-292.

Torabinejad, M. et al. 1995. Physical and chemical properties of a new root-end filling material. *J Endod* 21(7), pp. 349-353.

Torabinejad, M. and Parirokh, M. 2010. Mineral trioxide aggregate: a comprehensive literature review--part II: leakage and biocompatibility investigations. *J Endod* 36(2), pp. 190-202.

Torabinejad, M. et al. 2018. Mineral trioxide aggregate and other bioactive endodontic cements: An updated overview- Part II: Other clinical applications and complications. *Int Endod J* 51(30, pp. 284-317.

Toyoda, K. et al. 1995. Pathogenicity of the Streptococcus milleri group in pulmonary infections--effect on phagocytic killing by human polymorphonuclear neutrophils. *Kansenshogaku Zasshi* 69(3), pp. 308-315.

Tran-Hung, L. et al. 2006. Role of human pulp fibroblasts in angiogenesis. *J Dent Res* 85(9), pp. 819-823.

Trantor, I. R. et al. 1995. The effects of neuropeptides (calcitonin gene-related peptide and substance P) on cultured human pulp cells. *J Dent Res* 74(4), pp. 1066-1071.

Trombelli, L. and Farina, R. 2013. Efficacy of triclosan-based toothpastes in the prevention and treatment of plaque-induced periodontal and peri-implant diseases. *Minerva Stomatol* 62(3), pp. 71-88.

Trope, M. 2008. Regenerative potential of dental pulp. *J Endod* 34(7 Suppl), pp. S13-17.

Trowbridge, H. O. 1981. Pathogenesis of pulpitis resulting from dental caries. *J Endod* 7(2), pp. 52-60.

Trowbridge, H. O. 2012. *Seltzer and Bender's dental pulp*. Chicago: Quintesssence Publish. Co,Inc.

Tsuji, T. et al. 1987. An experimental study on wound healing of surgically exposed dental pulps in germ-free rats. *Bull Tokyo Dent Coll* 28(1), pp. 35-38.

Tsunashima, H. et al. 2012. Organization of the capsule biosynthesis gene locus of the oral streptococcus Streptococcus anginosus. *J Biosci Bioeng* 113(3), pp. 271-278.

Tucker, A. and Sharpe, P. 2004. The cutting-edge of mammalian development; how the embryo makes teeth. *Nat Rev Genet* 5(7), pp. 499-508.

Tummers, M. and Thesleff, I. 2009. The importance of signal pathway modulation in all aspects of tooth development. *J Exp Zool B Mol Dev Evol* 312B(4), pp. 309-319.

Tziafas, D. 1995. Basic mechanisms of cytodifferentiation and dentinogenesis during dental pulp repair. *Int J Dev Biol* 39(1), pp. 281-290.

Tziafas, D. 2004. The future role of a molecular approach to pulp-dentinal regeneration. *Caries Res* 38(3), pp. 314-320.

Tziafas, D. et al. 1992a. Dentinogenic activity of allogenic plasma fibronectin on dog dental pulp. *J Dent Res* 71(5), pp. 1189-1195.

Tziafas, D. et al. 1998. Effects of recombinant basic fibroblast growth factor, insulin-like growth factor-II and transforming growth factor-beta 1 on dog dental pulp cells in vivo. *Arch Oral Biol* 43(6), pp. 431-444.

Tziafas, D. and Kolokuris, I. 1990. Inductive influences of demineralized dentin and bone matrix on pulp cells: an approach of secondary dentinogenesis. *J Dent Res* 69(1), pp. 75-81.

Tziafas, D. et al. 1992b. Short-term dentinogenic response of dog dental pulp tissue after its induction by demineralized or native dentine, or predentine. *Arch Oral Biol* 37(2), pp. 119-128.

Tziafas, D. et al. 2000. Designing new treatment strategies in vital pulp therapy. *J Dent* 28(2), pp. 77-92.

Uchida, T. et al. 1991. Immunocytochemical and immunochemical detection of a 32 kDa nonamelogenin and related proteins in porcine tooth germs. Arch Histol Cytol 54(5), pp.527-538.

Unsworth, P. F. 1989. Hyaluronidase production in Streptococcus milleri in relation to infection. *J Clin Pathol* 42(5), pp. 506-510.

van Amerongen, J. P. et al. 1983. The concentration, extractability and characterization of collagen in human dental pulp. *Arch Oral Biol* 28(4), pp. 339-345.

Van Hassel, H.J.1971. Physiology of the human dental pulp. *Oral Surg Oral Med Oral Patho*l 32(1), pp. 126-134

van Houte, J. et al. 1994. The predominant cultivable flora of sound and carious human root surfaces. *J Dent Res* 73(11), pp. 1727-1734.

Van Tyne, D. et al. 2013. Structure, function, and biology of the Enterococcus faecalis cytolysin. *Toxins (Basel)* 5(5), pp. 895-911.

Vandhana, S. et al. 2010. Evaluation of suitable solvents for testing the anti-proliferative activity of triclosan - a hydrophobic drug in cell culture. *Indian J Biochem Biophys* 47(3), pp. 166-171.

Veis, A. 1985. The role of dental pulp--thoughts on the session on pulp repair processes. *J Dent Res* 64 Spec No, pp. 552-554.

Veis, A. et al. 1989. A search for the osteogenic factor in dentin. Rat incisor dentin contains a factor stimulating rat muscle cells in vitro to incorporate sulfate into an altered proteoglycan. *Connect Tissue Res* 23(2-3), pp. 137-144.

Vermelin, L. et al. 1995. Effects of essential fatty acid deficiency on rat molar pulp cells. *Eur J Oral Sci* 103(4), pp. 219-224.

Villalain, J. et al. 2001. Membranotropic effects of the antibacterial agent Triclosan. *Arch Biochem Biophys* 390(1), pp. 128-136.

von Plocki, S. C. et al. 2012. Biodegradable sleeves for metal implants to prevent implant-associated infection: an experimental in vivo study in sheep. *Vet Surg* 41(3), pp. 410-421.

Vural, U. K. et al. 2017. Randomized Clinical Trial to Evaluate MTA Indirect Pulp Capping in Deep Caries Lesions After 24-Months. *Oper Dent* 42(5), pp. 470-477.

Waaler, S. M. et al. 1993. Effects of oral rinsing with triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. *Scand J Dent Res* 101(4), pp. 192-195.

Waddington, R. J. et al. 1993. The influence of fluoride on proteoglycan structure using a rat odontoblast in vitro system. *Calcif Tissue Int* 52(5), pp. 392-398.

Waddington, R. J. et al. 2013. Impact of bidirectional relationships between streptococcus anginosus group and host tissue matrix components on cellular activity: role in establishment of infection. *Microbiology Discovery* 1(1). pp. 4-15.

Wallet, M. A. et al. 2013. Triclosan alters antimicrobial and inflammatory responses of epithelial cells. *Oral Dis* 19(3), pp. 296-302.

Wanahita, A. et al. 2002. Interaction between human polymorphonuclear leukocytes and Streptococcus milleri group bacteria. *J Infect Dis* 185(1), pp. 85-90.

Washington, J.T.et al 2011. Biocompatibility and osteogenic potential of new generation endodontic material established by using primary osteoblasts, J Endod 37(8), pp.1166-1170.

Watabe, T. and Miyazono, K. 2009. Roles of TGF-beta family signaling in stem cell renewal and differentiation. *Cell Res* 19(1), pp. 103-115.

Watts, A. and Paterson, R. C. 1987. Bacterial contamination as a factor influencing the toxicity of materials to the exposed dental pulp. *Oral Surg Oral Med Oral Pathol* 64(4), pp. 466-474.

Whiley, R. A. and Beighton, D. 1991. Emended descriptions and recognition of Streptococcus constellatus, Streptococcus intermedius, and Streptococcus anginosus as distinct species. *Int J Syst Bacteriol* 41(1), pp. 1-5.

Whiley, R. A. and Beighton, D. 1998. Current classification of the oral streptococci. *Oral Microbiol Immunol* 13(4), pp. 195-216.

Whiley, R. A. et al. 1992. Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus (the Streptococcus milleri group): association with different body sites and clinical infections. *J Clin Microbiol* 30(1), pp. 243-244.

Whiley, R. A. et al. 1990. Phenotypic differentiation of Streptococcus intermedius, Streptococcus constellatus, and Streptococcus anginosus strains within the "Streptococcus milleri group". *J Clin Microbiol* 28(7), pp. 1497-1501.

Whiley, R. A. et al. 1999. A study of small-colony, beta-haemolytic, Lancefield group C streptococci within the anginosus group: description of Streptococcus constellatus subsp. pharyngis subsp. nov., associated with the human throat and pharyngitis. *Int J Syst Bacteriol* 49 Pt 4, pp. 1443-1449.

Whittaker, C. J. et al. 1996. Mechanisms of adhesion by oral bacteria. *Annu Rev Microbiol* 50, pp. 513-552.

Willcox, M. D. and Knox, K. W. 1990. Surface-associated properties of Streptococcus milleri group strains and their potential relation to pathogenesis. *J Med Microbiol* 31(4), pp. 259-270.

Willcox, M. D. et al. 1995. Degradative enzymes of oral streptococci. *Aust Dent J* 40(2), pp. 121-128.

Williams, D. C. et al. 1980. Mineralization and metabolic response in serially passaged adult rat bone cells. *Calcif Tissue Int* 30(3), pp. 233-246.

Wolters, W. J.et al.2017. Minimally invasive endodontics; a new diagnostic system for assessing pulpitis and subsequent treatment need. *Int Endod J* 50(9), pp. 825-829

Wu, S et al. 2018. Indirect pulp therapy for deciduous teeth with deep carious lesion. *Hua Xi Kou Qiang Yi Xue Za Zhi* 36(4), pp. 435-440.

Yamagishi, V. T. et al. 2011. Blockade of TLR2 inhibits Porphyromonas gingivalis suppression of mineralized matrix formation by human dental pulp stem cells. *J Endod* 37(6), pp. 812-818.

Yamaguchi, M. et al. 2004. Neuropeptides stimulate production of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in human dental pulp cells. *Inflamm Res* 53(5), pp. 199-204.

Yamakoshi, Y. 2009. Dentinogenesis and Dentin Sialophosphoprotein (DSPP). *J Oral Biosci* 51(3), p. 134.

Yan, L. et al. 2017. Insulin-like growth factor-1 promotes the proliferation and odontoblastic differentiation of human dental pulp cells under high glucose conditions. *Int J Mol Med* 40(4), pp. 1253-1260.

Yazid, F. B. et al. 2014. Comparison of immunodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. *Clin Oral Investig* 18(9), pp. 2103-2112.

Yoshiba, K. et al. 1996. Immunolocalization of fibronectin during repparative dentinogenesis in human teeth after pulp capping with calcium hydroxid. *Journal of Dental Research* 75(8), pp 1590-1597

Yoshiba, N. et al. 1994. A confocal laser scanning microscopic study of the immunofluorescent localization of fibronectin in the odontoblast layer of human teeth. *Arch Oral Biol* 39(5), pp. 395-400.

Yoshida, Y. et al. 2002. lcd from Streptococcus anginosus encodes a C-S lyase with alpha,beta-elimination activity that degrades L-cysteine. *Microbiology* 148(Pt 12), pp. 3961-3970.

Young, H. E. and Black, A. C., Jr. 2004. Adult stem cells. *Anat Rec A Discov Mol Cell Evol Biol* 276(1), pp. 75-102.

Young, K. A. et al. 1996. Interactions between Eikenella corrodens and 'Streptococcus milleri-group' organisms: possible mechanisms of pathogenicity in mixed infections. *Antonie Van Leeuwenhoek* 69(4), pp. 371-373.

Yu, C. and Abbott, P. V. 2007. An overview of the dental pulp: its functions and responses to injury. *Aust Dent J* 52(1 Suppl), pp. S4-16.

Zafar, A. B. et al. 1995. Use of 0.3% triclosan (Bacti-Stat) to eradicate an outbreak of methicillin-resistant Staphylococcus aureus in a neonatal nursery. *Am J Infect Control* 23(3), pp. 200-208.

Zander, H. A. and Glass, R. L. 2005. The healing of phenolized pulp exposures. 1949. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 100(2 Suppl), pp. S97-101.

Zeng, W. 2014. IL-1 in Th17 Differentiation and Inflammatory Tissue Damage. *Austin Clinial Journal of Immunology* 1(2), pp. 1-4.

Zhang, C. et al. 2015. Correlation between Enterococcus faecalis and Persistent Intraradicular Infection Compared with Primary Intraradicular Infection: A Systematic Review. *J Endod* 41(8), pp. 1207-1213.

Zhang, W. and Yelick, P. C. 2010. Vital pulp therapy-current progress of dental pulp regeneration and revascularization. *Int J Dent* 2010, p. 856087.

Zhu, C. et al. 2015. Clinical outcome of direct pulp capping with MTA or calcium hydroxide: a systematic review and meta-analysis. *Int J Clin Exp Med* 8(10), pp. 17055-17060.