Development of an *ex vivo* co-culture system to model pulpal infection by *Streptococcus anginosus* group bacteria

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"I won't sit down, and I won't shut up,
And most of all I will not grow up"

'Photosynthesise' by Frank Turner

For Mel, Steve and Grandad,
For their limitless love and support

and for Nana,
who would have been so proud.

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Development of a co-culture system to model infection of the pulp by Streptococcus anginosus group bacteria
J.L. ROBERTS, R.J. WADDINGTON, J.-Y. MAILLARD, and A.J. SLOAN

Summary

Streptococcus anginosus group (SAG) bacteria are opportunistic pathogens and a major cause of pulpal infection and subsequent abscess formation. The development of a suitable model was needed to illustrate the processes involved in pulpal infection by SAG bacteria.

The work presented in this thesis details the development of an *ex vivo* model system which allows co-culture of an organotypic tooth slice with SAG bacteria. This was made possible by the identification of novel media and conditions for use in the model. These defined culture conditions support growth of both SAG clinical isolates and rodent tooth slices and suggest that *S. intermedius* isolates have different nutritional requirements from other SAG species. SAG bacteria were shown to attach to the pulpal tissue in focal points causing disruption to the matrix and death of cells. Co-culture of tooth slices with SAG bacteria for 4, 8 or 24 hrs resulted in a significant decrease in healthy nuclei in a $50\mu m^2$ area. There was no significant difference in the effects caused by different SAG species. Expression levels of TNF-α and IL-1β were increased in tooth slices incubated with SAG bacteria, although IL-6 was not detectable in control or infected slices. Culture of tooth slices with SAG supernatants also resulted in a significant decrease in healthy nuclei in a $50\mu m^2$ area.

The efficacy of common oral biocides against SAG bacteria was also tested. Exposure to CHX at concentrations of 0.2% or above for 10 minutes resulted in complete eradication of SAG bacteria. Whilst exposure to triclosan was shown to reduce bacterial numbers, complete eradication could not be achieved after a contact time of 60 mins.

A reproducible model of infection of dental tissues by SAG bacteria has been produced which shows attachment patterns of bacteria and the effect on host tissues. This model can be used to further investigate processes involved in endodontic infections, including expression of virulence factors by bacteria and the host response from the dental tissues. It may also be used in the future for testing novel antimicrobials for use in treating pulpal disease.

List of Abbreviations

AMP – Antimicrobial peptide

ANOVA – Analysis of Variance

BHI – Brain heart infusion

BMP – Bone morphogenetic protein

CHX – Chlorhexidine

DMEM – Dulbecco's Modified Eagle Medium

DPP – Dentine phosphoprotein

DPSCs – Dental pulp stem cells

DSP – Dentine sialoprotein

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme linked immunosorbent assay

FAA – Fastidious anaerobe agar

FCS – Fetal calf serum

FDA – Fluorescein diacetate

FGF – Fibroblast growth factor

H&E – Haematoxylin and eosin

hBD – Human β-defensin

IFN – Interferon

IGF – Insulin-like growth factor

IL – Interleukin

LAP- Latency associated peptides

LPS - Lipopolysaccharide

MBC – Minimum bactericidal concentration

MIC – Minimum inhibitory concentration

MMLV – Moloney Murine Leukaemia Virus

MMP – Matrix metalloproteinase

ONC – Overnight culture

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PMNs – Polymorphonuclear neutrophils

RFV – Random field of view

RPM – Revolutions per minute

RT-PCR – Reverse transcriptase-polymerase chain reaction

***S.* MG – *Streptococcus* Milleri Group**

SAG – *Streptococcus anginosus* group

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

TBE – Trise-borate-EDTA

TGF – Transforming growth factor

TNF – Tumour necrosis factor

UV – Ultra-violet

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Chapter 1: General Introduction

1.0 Introduction

Pulpal disease, or pulpitis, is a common problem which can lead to loss of vitality of the tooth. Pulpal disease is separated into a number of different classifications of varying severity, which can be characterised by clinical symptoms (Pashley and Liewehr 2006). At all severity levels, pulpal disease is characterised by inflammation caused by an irritant. In most cases, this irritant is the presence of pathogenic bacteria or their products (Levin et al. 2009).

A wide variety of microbial species have been identified as playing a role in pulpitis, with the predominant species responsible for the infection shifting as the infection advances (Featherstone 2000). The focus of this study is the *Streptococcus anginosus* group (SAG) of bacteria, a group of bacteria present in the oral cavity but often neglected in dental research. SAG bacteria are generally considered part of the body's commensal flora but pathogenic forms of the bacteria have been associated with a number of infections (Hirai et al. 2005; Lamothe 1990). Infections of the pulpal chamber which are caused by SAG are a particular problem as they can lead to the formation of oral abscesses (Okayama et al. 2005). Abscesses result from bacteria infecting the pulpal chamber of the tooth and invading the surrounding periradicular tissue, stimulating a non-specific inflammatory response. Bacterial cells and metabolic by-products accumulate with inflammatory cells and their lysed contents, forming the purulent exudate of the abscess which results in swelling and pain. Bacteria from these sites have the capacity to spread from the pulpal chamber resulting in bacteraemia or abscesses at other sites in the body such as the liver and brain which can prove fatal (Verrall 1986; Whiley et al. 1992). SAG bacteria have also been identified as primary colonisers of the pulpal chamber which enable further infection by other bacterial species.

Treatment regimes for oral infections will vary from one patient to another, with the common aim of eradicating the infection and, where possible, preventing destruction of the pulp by the bacteria and their products. When an abscess has formed,

antibiotics are generally administered to the patient with the most commonly prescribed antibiotics being amoxicillin, clarithromycin and metronidazole (Pashley and Liewehr 2006). Prior to formation of an abscess, where pulpitis is treatable by removal of the infection, the pulp may be capped with an antimicrobial treatment such as calcium hydroxide (Desai and Chandler 2009). This is the most commonly used pulp capping treatment but in recent years there has been an increased interest in novel antimicrobials in for use in endodontic treatments and dentifrices. Due to the inflammatory nature of pulpitis there has been particular interest in antimicrobials that have also been identified as having anti-inflammatory properties, such as triclosan and certain naturally expressed proteins known as antimicrobial peptides (AMPs) (Mustafa et al. 1998; Yang et al. 2002).

Although triclosan has been incorporated into a number of commercially available dentifrices, there is no research to indicate the efficacy of triclosan against SAG bacteria, as the importance of these bacteria in pulpal infections is often overlooked. To further understand the mechanism of pulpal infection, bacterial activity during infection and how antimicrobials may be utilised as therapeutics, an appropriate model is needed. Whilst *in vivo* models are beneficial for studying the effects of antimicrobials, when substances are introduced into a complex *in vivo* system the presence of naturally expressed AMPs and a vast commensal flora introduces uncontrollable variables. In contrast, *in vitro* models are over simplified and do not allow for consideration of the complex interactions between the closely associated tissues within the tooth. The aim of this project is to develop an *ex vivo* model system which will allow the study of the bacterial interactions with the host tissues in an organotypic model which takes into account the importance of the close association between the pulp and dentine. An *ex vivo* model can study the tooth in isolation of inflammatory interference whilst preserving the important connections between the dental tissues. To study the infection of the pulp by SAG bacteria it will be necessary to manipulate conditions and media to successfully co-culture bacteria and mammalian cells. These conditions will then need to be validated to ensure that they are suitable for both cell types to survive and grow and to ensure that they do not result in different growth characteristics. Any changes that the novel conditions do incur will need to be accounted for before further experimentation can be carried out. Following the identification of suitable conditions the SAG bacteria will then be

cultured on the *ex vivo* tooth slice model and the effects of infection assessed through histological examination, vital dye staining and changes in cytokine expression. Ultimately the aim is that a model can be produced that will be suitable for testing the efficacy of novel antimicrobials for use in endodontic treatments.

As the dentine-pulp complex of the tooth has some natural ability to repair itself in response to injury and infection (Smith et al. 1995) it is important to consider the biological processes involved in this when developing new methods of treatment. It has already been shown that the success of this repair mechanism depends on a balance between cell necrosis through inflammation and the reparative abilities of the tissue (Bergenholtz 1981; Smith et al. 1999). In addition to this, the presence of different bacterial groups, different AMPs and the interactions between them will influence the reparative abilities of the tissues (Brown and Hancock 2006). It will be beneficial to limit the inflammatory response by limiting bacterial growth and, if possible, exploiting the anti-inflammatory properties of some antimicrobials. This introduction will therefore outline development of the dentine-pulp complex, the processes involved in dentinogenesis and pulpal repair, the involvement of SAG in pulpal disease, current treatment regimes and the scope for antimicrobial development.

1.1 Dental Anatomy

1.1.1 Tooth Development

Each tooth consists of mineralised tissues surrounding a core of connective tissue. The exterior layer which can be seen in the oral cavity is the hard mineralised tissue enamel, which is formed by the ameloblast cells. Enamel is approximately 96% mineral and as such is susceptible to erosion by acids from food, drink and bacterial metabolism. As it is a non-vital tissue there are no repair mechanisms to counteract this damage though some remineralisation may occur in favourable conditions (Amaechi and Higham 2001; Silverstone 1973). Enamel is supported by dentine which is a hard mineralised connective tissue which is tubular in structure. The mineral component of dentine is approximately 70%, compared to the 96% mineralisation of enamel. Unlike enamel, dentine is a vital tissue as it contains cell

processes which extend from the odontoblasts, the cells which form the dentine. Odontoblasts reside in the outer layer of cells of the soft connective tissue beneath the dentine, known as the dental pulp. The pulp contains blood vessels and so is capable of mounting inflammatory and immune responses. It is also densely innervated and so injury and inflammation results in pain. The cells in the pulpal chamber consist mainly of fibroblasts with the odontoblasts lining the periphery so the pulp is closely associated with the vital dentine matrix. These tissues function together in repairing damage which may occur during infection or injury. The tooth is attached to the bones of the maxillary or mandibular jaw by cementum, periodontal ligament and alveolar bone (Ten Cate 2003).

Teeth develop from two different tissues, the oral epithelium and the mesenchyme. The oral epithelium is of ectodermal origin and the oral mesenchyme originate from neural crest cells which forms from the nervous tissue of the developing embryo (Lumsden 1988). Initiation of tooth development is stimulated by interactions between the two cell types and is seen as the thickening of epithelial bands in the mouth that are roughly horseshoe shaped (Slavkin 1991) which will later develop into the dental arches of the maxillary and mandibular jaw. Neural crest cells migrate from the neural folds to the jaws and interact with the epithelial cells, inducing proliferation of the epithelium and formation of the dental lamina. There is a re-orientation of cells within the lamina and as a result as the cells proliferate they invaginate as a sheet into the underlying mesenchymal cells (Ruch 1985). This is the first sign of tooth development which then continues in distinguishable stages defined according to the shape of the epithelium which will eventually form the enamel of the tooth.

The initial bud stage of development is represented as the first invagination into the mesenchyme with rounded localised growth of epithelial cells. As the epithelial cells continue to proliferate it becomes possible to identify the formative elements of the tooth. This stage is referred to as the cap stage as the epithelial cells, now known as the enamel organ, form a cap shape above the mesenchymal cells which remain closely aggregated and form the dental papillae. The dental papillae will eventually give rise to the dentine-pulp complex. At this stage, epithelial cells and dental papillae are separated by a continuous basement membrane which is composed mainly of collagen and ground substance. It has been suggested that the basement membrane

plays an important role in terminal differentiation of cells (Ruch 1985) by immobilizing and activating bio-active molecules such as TGF-β which stimulate the odontoblast differentiation (Ruch 1995). Finally, at the bell stage of development, the enamel organ differentiates into four components. The outer enamel epithelium is on the inside of the enamel organ at the convex surface, the inner enamel epithelium borders the dental papillae, the stratum intermedium cells form a layer adjacent to the inner enamel epithelium and the stellate reticulum cells fill the remainder of the centre of the enamel organ. The inner epithelium cells differentiate into ameloblasts which function with the stratum intermedium cells to form the enamel. The outer enamel epithelial cells assist in bringing nutrition to the ameloblasts. The cells in the periphery of the dental papilla elongate and differentiate into odontoblasts which will form the dentine matrix (Ten Cate 2003).

Following the differentiation of the formative cells of the tooth the hard tissues of the tooth begin to develop. Dentine, which will eventually form the majority of the tooth, begins to form before enamel. Prior to the formation of the first layer of dentine, known as mantle dentine, enamel proteins are secreted by the ameloblasts but do not form an enamel layer until after the mantle dentine forms. It is thought that these proteins may play a role in the terminal differentiation of the odontoblasts which is required before they can begin to secrete the dentine matrix (Linde and Goldberg 1993).

The dentine matrix is formed by odontoblast cells which are post-mitotic cells differentiated from the mesenchymal cells of the dental papillae. Their differentiation is controlled by sequential interactions between the mesenchymal cells and epithelial cells of the oral environment (Yu et al. 2006). During the cell cycles before they terminally differentiate the cells, known at this stage as preodontoblasts, have two nuclei and exhibit an increase in cytoplasmic organelles which suggests they are about to withdraw from the cell cycle (Linde and Goldberg 1993; Ruch 1985). The final cell division before terminal differentiation then results in the formation of two daughter cells, only one of which is in contact with the basement membrane. It is only this latter cell which will differentiate into a functional odontoblast as specific tissue interactions must take place for differentiation to occur (Thesleff and Hurmerinta

1981). It has been suggested that the basement membrane is necessary for this to occur (Ruch 1985). It is possible that the basement membrance acts as a reservoir for bioactive molecules involved in the terminal differentiation of odontoblasts (Begue-Kirn et al. 1994; Lesot et al. 2001). A variety of such molecules have been identified that play an important role in the commitment of cells onto a differentiation pathway and include growth factors and extracellular matrix components. The growth factors involved include transforming-growth factors (TGFs), fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) (Thesleff and Aberg 1999; Tziafas et al. 2000). This terminal differentiation is characterised by elongation and polarization of the cells (Ruch 1985, 1998), with the nuclei located at the end of the cell which is furthest from the basement membrane (Linde, 1985). These cells line the periphery of the dental papilla which is now known as the pulp and begin dentine formation, a process known as dentinogenesis. The cells in the inner layer, adjacent to the odontoblasts do not differentiate and may form a cell-rich layer in the pulp of the developed tooth known as the layer of Hohl (Ten Cate 2003). Following the final mitotic division the odontoblasts then begin to secrete the extracellular matrix of the dentine.

1.1.2 Enamel

After a few micrometers of mantle dentine has been formed, the ameloblasts begin enamel formation, moving away from the dentinoenamel junction as they secrete the matrix. An initial matrix of non-collagenous proteins is secreted and then mineralized. Ameloblasts also develop a short cell process during their secretory phase, known as Tomes' processes. The majority of the matrix proteins secreted are low-molecular weight amelogenins and mineralization of the matrix begins immediately after they are deposited by the ameloblast. Initially, the matrix is approximately 30% mineralized and the remainder organic material and water (Robinson et al. 1978). At this stage there are small crystals rich in magnesium and carbonate. Crystal development involves a decrease of these minerals and an increase in amelogenin and albumin (Robinson et al. 1995). Once the full width of the enamel has been deposited the crystals increase in size and organic content and water are removed by the ameloblasts, causing mineral content to increase to 96%. This gives enamel a higher

mineral concentration than any other tissue in the body but also makes it brittle and susceptible to damage by acids and trauma (Ten Cate 2003).

1.1.3 Dentine

The presence of the odontoblast process makes dentine a vital tissue that is dynamic in its ability to respond to external stimulus. The dentinal tubules are tapered structures which extend through the dentine in an S-shaped path which follows that of the odontoblast during dentinogenesis. This tubular nature makes dentine unusually permeable for such a hard tissue and this is of particular significance to infection as it allows bacteria and their products to diffuse through the dentine to the pulp, where they can elicit an immune response (Love and Jenkinson 2002).

Primary dentinogenesis is the secretion of dentine during tooth development before the tooth is fully functional in the oral cavity. Primary dentine constitutes the majority of dentine in the tooth as it is produced at a relatively high rate but after initial development and eruption of the tooth, the rate of deposition is greatly reduced (Linde and Goldberg 1993). The process is then known as secondary dentinogenesis to distinguish between the two stages, although the physiology of the dentine is ultimately very similar. In some cases secondary dentine can be identified as being less regular in structure due to the overcrowding of the odontoblasts as they move further into the pulpal chamber.

Dentine is a mineralised connective tissue which is originally deposited as an unmineralised organic matrix consisting of collagenous and non-collagenous components (Ten Cate, 2003). The majority of collagen in dentine is type I, and the non-collagenous component consists mostly of proteoglycans including dentine phosphoprotein (DPP) and dentine sialoprotein (DSP) (MacDougall et al., 1997). A minor part of the organic matrix is made up of lipids such as phospholipids, cholesterol and triacylglycerols (Linde and Goldberg 1993). The collagenous components make up the majority of the dentine (approximately 90%) and are particularly important when considering the establishment of pulpal infections, as they provide an anchor for invading bacteria to attach to and grow (Yamakoshi 2009).

The main inorganic component of the dentine matrix is hydroxyapatite. The initial mineral formation is initiated through interactions of reactive groups of macromolecules in the matrix, causing calcium and phosphate ions to be attracted and form crystals, mineralizing the matrix (Linde, 1985). This is thought to be mediated by DPP and DSP (Amaechi and Higham 2001). The unmineralised matrix is known as predentine and the advancing area of mineralisation is known as the mineralisation front.

DPP and DSP are translated as a single protein, dentine sialophosphoprotein, before being cleaved into the two separate proteins by bone morphogenetic protein-1 (BMP-1) (Feng et al. 1998). DPP is involved in dentine mineralization by binding to collagen and initiating formation of apaptite crystals. It is then involved in the regulation of the growth of these crystals (Butler and Ritchie 1995) . DPP has a high affinity for binding calcium (Zanetti et al. 1981) which may contribute to the role it plays in crystal formation at the mineralization front. The exact function of DSP remains unclear, although it is expressed in odontoblasts during pre-dentine formation before mineralization (D'Souza et al. 1992). Studies using DPP knockout mice have indicated that DSP regulates the initiation of mineralization (Suzuki et al. 2009).

In addition to dentine matrix proteins the dentine contains a variety of growth factors such as bone borphogenetic protein (BMP), transforming growth factor β (TGF-β) (Cassidy et al. 1997; Sloan et al. 2000) and insulin-like growth factors (IGF) I and II (Finkelman et al. 1990). These growth factors are incorporated into the matrix during synthesis and, as previously outlined, are also involved in the differentiation of odontoblasts (Ruch et al. 1995) when they may be associated with the basement membrane (Begue-Kirn et al. 1994) and in stimulating matrix secretion (Begue-Kirn et al. 1992). The presence of these proteins in the dentine is important as they may play a role in repair when they are released by the action of bacterial acids on the tissue (Goldberg et al. 2008). During tissue repair the processes that occur mimic those seen in development, highlighting their relevance to studies concerned with bacterial destruction of host tissues.

The mineralised component the dentine is comprised of intertubular dentine which constitutes the majority of the dentine and the intratubular (peritubular) dentine which is a hypermineralised ring of dentine that forms tubules within the matrix (Ten Cate 2003). Within the tubules formed by the intratubular dentine is a cell process extending from the odontoblast in the pulpal chamber. There is evidence to suggest that this process extends through the entire thickness of the dentine (Sigal et al. 1984) although there is still some debate regarding this and other studies suggest the odontoblast process only extends through a portion of the dentinal tubules (Weber and Zaki 1986). It is the presence of this process that makes the dentine a vital tissue and which forms the connection between the dentine and the pulp. These are also the first cells of the tooth to come into contact with bacteria and their products during the progression of a carious lesion or following trauma to the tooth. Using immunohistochemistry, it has been demonstrated that certain Streptococcal species are able to advance through carious lesions and attach to the odontoblast process (Ackermans et al. 1981). As these cells are the first to detect an injury or infection they are the first line of defence of the host tissues and have been shown to produce immunoglobulins which assist in fighting an incoming infection (Okamura et al. 1980). However, bacteria and their products are often able to overcome host defences and advance further into the dentinal tubules, resulting in destruction of the odontoblasts, which elicits a further immune response in the pulp.

1.1.4 Pulp

The pulp is an unmineralised connective tissue consisting mostly of fibroblasts. It resides in the centre of the tooth in the space known as the pulpal chamber with the odontoblasts lining the periphery. A cell free-zone separates the odontoblast layer and the area which is particularly abundant in fibroblasts known as the cell-rich layer of Höhl. This layer also contains a number of immune cells such as macrophages and lymphocytes. The fibroblasts within the pulp secrete an extracellular matrix consisting mainly of collagens (Shuttleworth et al. 1980), the most abundant of which are type I and III (Shuttleworth et al. 1978), and glycoproteins (Shuttleworth et al. 1982) such as chondroitin sulphate and hyaluronic acid (Linde 1973). This extracellular matrix (ECM) acts as support for the fibroblasts and also mediates many of the cellular

interactions, as cell metabolites, nutrients and waste pass through the matrix between the different cell types present in the pulp. In addition, the matrix acts as a source of nutrients for invading bacteria, whilst the collagen components provide an anchor to which many bacteria can attach, making the matrix composition an important point for consideration when studying pulpal disease. As previously mentioned (1.1.3), Streptococcal species have been shown to express adhesins which allow their attachment to collagen in dental tissues. This enables these bacteria to adhere to the pulpal tissues and initiate infection, where they proceed to produce other proteins such as hyaluronidase and chondrotin sulphatase which are able to break down the other components of the pulpal matrix.

In addition to the role of the pulpal matrix components in infection, they also give the pulp its unique gelatinous form. It is unclear if this has any effect on bacterial attachment and infection *in vivo* but it should be taken into consideration when developing culture systems to model pulpal infection. Bacteria have been shown to behave differently when grown on different substrates, in particular in some conditions they are able to form biofilms which infer a level of protection to the bacteria, whilst in other growth mediums they will grow only in suspension. Whilst pulpal cells that are grown *in vitro* have been shown to produce extracellular matrix proteins, this does not always result in a gelatinous matrix as seen *in vivo* (Martinez 2003). As such, the bacterial growth on pulpal cells grown *in vitro* may not accurately represent the growth characteristics of bacteria which infect the pulp *in vivo*.

In addition to the pulpal fibroblasts and the ECM, the pulp also contains blood vessels, nerves and cells of the immune system including lymphocytes (Hahn et al. 1989), mast cells (Walsh 2003) and macrophages (Pashley and Liewehr 2006). The presence of immune cells, and blood vessels through which further immune cells can reach the pulp, are essential in the host defence against infecting bacteria from the oral cavity.

Recently, a population of clonogenic, highly proliferative cells has been identified in the pulp which it has been suggested are dental pulp stem cells and may be the cells from which a new generation of odontoblasts differentiate during reparative dentinogenesis (Gronthos et al. 2000). Whilst these stem cells remain quiescent in the

pulp when the tissue is healthy, death of the odontoblasts caused by invading bacteria or other injury stimulates a complex cascade of signals which cause the stem cells to proliferate and differentiate. The cells then form odontoblast-like cells to replace those which have been damaged by the invading bacteria (Sloan and Waddington 2009). These cells may then secrete dentine to repair the damage that has occurred, providing that conditions within the tooth are favourable with limited inflammation (Sloan and Smith 2007).

As secondary dentinogenesis continues throughout the life of the tooth, the pulpal chamber decreases in size and cell density decreases. This also occurs during repair processes where dentine secretion is up-regulated as a response to injury or infection. This decrease in cell number then affects the ability to respond to further stimulus (Murray et al. 2002). As the pulp loses its ability to repair damage and fight infection it is more likely that an unresolved inflammatory response will occur, resulting in pulpal necrosis and an increased chance of tooth loss.

1.2 Dentine-Pulp Complex

1.2.1 Dynamic nature of the dentine-pulp complex

Dentine and pulp are mesenchymal in origin, both forming from the dental papillae which develops during the cap stage of development (Ten Cate 2003). As has been mentioned in the previous section (**1.1**), the physical interactions and the functional coupling of these tissues result in them being considered together as the dentine-pulp complex.

The complex nature of these tissues can be seen during an infection of the dentine when inflammation in the pulp is observed. This is due to the diffusion of bacterial products through the dentinal tubules and eliciting an immune response in the absence of bacterial cells (Bergenholtz 1990). The physiological events that occur in the pulp as a result of such infection or injury can also directly affect the dentine and the odontoblasts which are secreting the dentine matrix as they reside within the pulpal chamber.

The dentine-pulp complex is dynamic in nature and reacts to changes in its environment. If it is exposed to the oral cavity through trauma to the enamel or the formation of a carious lesion it can become infected, causing damage to the tissues. The dentine-pulp complex shows an extensive capacity for repair in response to such injury and infection. The type of repair that occurs is determined by the severity of the damage to the tissues and involves the secretion of a matrix termed tertiary dentine. Tertiary dentine differs from secondary and primary dentine in that is only produced as a result of an external stimulus (Smith et al. 1994, 1995). Also, tertiary dentine is produced only by the odontoblasts which are in direct contact with the stimulus, leading to specific foci of deposition (Sloan and Smith 1999; Smith et al. 1994).

There are two types of tertiary dentine which have been defined by Smith *et al.* (1995) as:

- Reactionary dentine which is a tertiary dentine matrix secreted by surviving post-mitotic odontoblast cells in response to an appropriate stimulus.
- Reparative dentine which is a tertiary dentine matrix secreted by a new generation of odontoblast-like cells in response to an appropriate stimulus, after the death of the original post-mitotic odontoblasts responsible for primary and physiological secondary dentine secretion.

The result of both forms of dentinogenesis is the rapid secretion of a dentine matrix adjacent to the pulpal chamber which increases the distance between the pulp and the advancing bacterial front. However the biological processes involved are different in each case and as such they can be considered separately.

1.2.2 Reactionary Dentinogenesis

To confirm whether tertiary dentine is reactionary or reparative, chronological information on the event following damage to the tissues is required. This makes it possible to determine whether the dentine has been secreted by an existing generation of odontoblasts or a newly differentiated population of odontoblast-like cells (Lesot *et al.* 1993). It has also been suggested that reactionary dentine shows tubular continuity with secondary dentine which is not commonly seen in reparative dentine (Mjör, 1983). However, it is possible that both kinds of tertiary dentine may be present

beneath a carious lesion although they may not be able to be distinguished on morphology alone (Smith et al. 1995). This can occur when a carious lesion initially stimulates a reactionary response as it is a relatively mild stimulus. If it is then left untreated it may cause more damage as it progresses and exposes the dentine. As this allows direct infection of the dentine, which is a much stronger stimulus, this can then result in a reparative response.

Reactionary dentinogenesis involves an up-regulation of the secretion of the matrix that occurs during secondary dentinogenesis which continues throughout adult life after the tooth has erupted. For this up-regulation to occur there must be an interaction between the odontoblast which secretes the matrix and a molecular stimulus (Begue-Kirn et al. 1992; Smith et al. 1990) which results from or is the cause of tissue damage. It has been shown in an *in vivo* study that when components of isolated dentine matrix are implanted into prepared cavities in ferrets, existing post-mitotic odontoblasts are stimulated to secrete reactionary dentine (Smith et al. 1994). This suggests that the molecular stimulus for reactionary dentine formation is bound within the dentine and is released during infection of the tissue. This study also showed that distance between the implanted dentine matrix components and the odontoblast had an effect on the intensity of the response, from which the authors concluded that molecular diffusion down the dentinal tubules was involved. Further studies have suggested that members of the Transforming Growth Factor-β (TGF-β) family may be involved in stimulating the odontoblasts to up-regulate secretion. Smith *et al* (1995) used affinity chromatography to purify the EDTA-soluble fraction of dentine matrix and implanted the fraction containing TGF-β1 into unexposed cavities in ferrets. The results of this study showed that reactionary dentine was deposited at the pulp-dentine interface in areas where the dentinal tubules were in contact with the cavity. In addition to implicating the role of TGF-β1 in reactionary dentinogenesis this also supports earlier suggestions that molecules diffuse through the tubules to stimulate odontoblasts. A rodent tooth slice model has also been used to show that TGF-β1 and 3 are able to stimulate reactionary dentinogenesis (Sloan and Smith 1999). This was achieved by direct application of the bioactive molecules to the odontoblast layer of the tooth slice using agarose beads which had been soaked in TGF-β solutions. The results showed a localised increase in reactionary dentine

deposition which was associated with the bead that had been soaked in growth factors. In addition, this study showed that TGF-β2 had a minimal effect on the tissues.

TGF-β isoforms 1, 2 and 3 are all expressed by odontoblasts (Sloan et al. 2000) and TGF-β1 has been shown to be sequestrated within the dentine matrix (Cassidy et al. 1997). Latency-associated peptides (LAPs) influence the activity of TGF-βs as they form an association with TGF-β which must be cleaved before it can become active (Munger et al. 1997). LAPs have been found to be expressed in the pulpal cells and predentine of both carious and healthy teeth but not in mineralized dentine. This suggests that TGF-β is present in this matrix in its active form (Sloan et al. 2002). Therefore, it is possible that bacterial acids produced by organisms within a lesion of caries can release these molecules as they dissolve the dentine matrix, allowing diffusion down the dentinal tubules and stimulation of the odontoblasts to react to the invading pathogens. As previously mentioned (**1.1.3**) dentine also contains other growth factors such as BMPs and IGF I and II. It is likely that these are also released along with TGF-β and that there is a cocktail of growth factors which are involved in stimulating reactionary dentinogenesis (Begue-Kirn et al. 1994).

1.2.3 Reparative Dentinogenesis

Reparative dentinogenesis occurs when there is more severe damage to the dentine-pulp complex which results in the death of the post-mitotic odontoblasts. Therefore, for a reparative dentine matrix to be secreted it is necessary for a new generation of odontoblast-like cells to differentiate, a process which involves cell division, chemotaxis, cell migration, cell adhesion and cytodifferentiation. This is a more complex cascade of events than those required for the production of reactionary dentine, which involves only the up-regulation of odontoblast activity.

The formation of reparative dentine is dependent on conditions in the pulp being favourable for tissue healing which is defined as the absence of inflammation and a sufficient supply of oxygen (Tziafas 1995). Under such conditions reparative dentine commonly forms as a tubular matrix which is secreted by columnar, polarized

odontoblast-like cells. When conditions are not favourable for tissue healing the matrix may be atubular with cellular inclusions and is secreted by cells which are cuboidal or spindle-shaped (Tziafas 1995). The cellular inclusions may contribute to further pulpal inflammation as they degrade and the resulting degeneration products act as inflammatory stimuli (Trowbridge 1981). The formation of an irregular matrix by cuboidal cells is known as fibrodentinogenesis and though it may be a non-specific wound healing response it has also been suggested that the formation of this initial matrix results in the induction of differentiation of odontoblast-like cells (Baume 1980), possibly by providing a substrate for signalling molecules to be immobilised on (Smith 2002). However, direct induction of odontoblast-like cell differentiation by dentine matrix implantation has also been shown (Tziafas 2004) so it is possible that there are different mechanisms involved in reparative dentinogenesis which may explain the heterogeneity of the resulting matrix.

There has been much debate regarding the derivation of the cells which give rise to the odontoblast-like cells which secrete the reparative dentine matrix. The cell-rich layer of the pulp, perivascular cells, undifferentiated mesenchymal cells and pulpal fibroblasts have all been suggested as the possible source (Ruch 1998) and more recently a small population of clonogenic, highly proliferative cells has been identified in the pulp (Gronthos et al. 2000). These cells have been termed dental pulp stem cells (DPSCs) as they are capable of self renewal and the formation of a dentine-like structure *in vivo* and are similar to other stem cell populations in possessing the ability to develop into developmentally diverse phenotypes (Gronthos et al. 2002; Sloan and Smith 2007). Following death of the odontoblasts due to bacterial invasion or tooth injury these cells may proliferate and differentiate into odontoblast-like cells which are able to secrete a dentine matrix to repair damage to the existing matrix (Sloan and Waddington 2009).

Little is known about the processes involved in recruitment and cytodifferentiation of these progenitor cells for reparative dentinogenesis, although EDTA-soluble fractions of dentine have been shown to induce the formation of reparative dentine (Smith et al. 1990) which suggests that, as in reactionary dentinogenesis, there is an involvement of bio-active molecules contained in the matrix. It has been shown that members of the TGF-β superfamily, including TGF-β1, BMP-4 and BMP-2, may be involved in

the initial differentiation of the DPSCs and the subsequent stimulation of these cells to secrete the extracellular matrix (Nakashima et al. 1994). The release of these molecules from the primary dentine matrix and possible immobilisation on fibrodentine may allow odontoblast differentiation in the absence of a basement membrane.

Both types of repair mechanisms outlined here are important processes in tissue regeneration and overcoming infections as they facilitate protection of the pulp. This is necessary if the tooth is to remain viable as if the pulp is destroyed or has to be removed the tooth may be lost as damage is more likely to occur to the mineralised tissues which the pulp supports and the healing capacity is reduced. An understanding of the molecular and biochemical basis of repair in both reparative and reactionary dentinogenesis is important if pulp therapies are to be developed which can utilise the innate ability of the dentine-pulp complex to regenerate and heal and so research in this area is of particular importance. In addition to understanding what drives repair it is also important to understand the factors which inhibit repair such as inflammation.

1.2.4 Pulpal inflammation

Inflammation occurs as a response to an advancing lesion of caries and the infiltration of bacteria and their products into the pulp. This pulpal inflammation is known as pulpitis and it can be reversible or irreversible, depending on the severity of the damage that has been occurred in the vital tissues of the tooth. Reversible pulpitis has been defined as mild inflammation that is capable of healing if the irritant is removed. Irreversible pulpitis is characterized by a more severe degeneration of the tissues which will not heal and will lead to necrosis of the pulp if left untreated. This type of pulpitis requires endodontic treatment which involves the removal of some or all of the dental pulp to eradicate the bacterial infection from the tooth and prevent progression of the infection and necrosis of the pulp (Levin et al. 2009). It is also accepted that there is a difference in cytokine expression in reversible and irreversible pulps. An example of this is the expression of the cytokine TNF-α in irreversible pulpitis which does not occur in less severe infections (Kokkas et al. 2007).

Pulpitis occurs as part of the host response which is orchestrated by immune cells that are found within the pulp and are recruited through the blood vessels of the pulpal chamber. In the initial pulpal response, polymorphonuclear lymphocytes (PMNs) and monocytes are recruited. These cells are part of the non-specific innate immune response and are stimulated by the detection of generalised pathogen-associated molecules such as lipopolysaccharides and lipotechoic acid components of the bacterial cell wall, flagellin, peptidoglycans and lipoproteins (Cooper et al. 2010). The main role of the PMNs is to actively phagocytose microbes, whilst the monocytes differentiate to become macrophages. The macrophages also have a role in the phagocytosis of microbes in addition to stimulating other immune cells to respond to the bacterial invasion.

The activation of macrophages and the continuation of the immune response is co-ordinated by the secretion of cytokines. These are proteins which are secreted by cells of the immune system, allowing interaction between the different cell types as they bind to the receptors of target cells. Cytokines are divided into different groups depending on their function with the most important and extensively researched being the interleukins (IL), tumour necrosis factors (TNF) and interferons (IFN).

Macrophages produce a number of cyokines, including IL-1β, which plays a central role in pulpal inflammation. The stimulation of its production in response to bacterial products has been well documented by using enzyme-linked immunosorbent assays (ELISAs) and reverse-transcriptase-polymerase chain reaction (RT-PCR). Comparison of the cytokine levels seen from pulpal fibroblasts cultured in the presence and absence of bacterial lipopolysaccharide (LPS) showed an increased expression of IL-1β in those cells exposed to bacterial products (Bletsa et al. 2006; Silva et al. 2009), confirming its roles in inflammation as a response to bacterial invasion. Similar patterns of expression have been detected for IL-1α, IL-6 and TNF-α through use of ProteoPlex antibody arrays to compare cytokine expression in healthy and diseased pulp (Cooper et al. 2010). The upregulation of these cytokines in disease states is probably due to induction by IL-1β rather than a direct response to bacterial products, as IL-1β has been shown to stimulate the production of TNF-α in oral fibroblasts (Agarwal et al. 1995).

In addition to the direct role of bacterial products in stimulating an inflammatory response, it has also been demonstrated that dentine matrix extracts are able to induce recruitment of inflammatory cells and the production of IL-1β and TNF-α (Lara et al. 2003). This suggests that as the bacterial acids dissolve the dentine, components of the matrix diffuse into the pulp along with the bacterial products and are involved in stimulating the innate immune response.

The production of these cytokines by the host cells promotes the phagocytosis of bacteria and infected host cells by macrophages and PMNs in an attempt to eliminate the bacterial infection. The subsequent pulpal necrosis that may result from this if the inflammation is not resolved may also act as a defence mechanism as it provides a harsh environment for the survival of invading bacteria due to the lack of available oxygen and nutrients in necrotic tissues (Stashenko et al. 1998).

Inflammation is of particular significance to the success of vital pulp therapy as it can result in the destruction of host tissues if the inflammatory reaction is unresolved, therefore negating the innate repair mechanisms of the tooth. However, recent research has shown that whilst severe inflammation will degrade the pulpal tissues and may prevent regeneration, a low-grade level of inflammation may be advantageous for the dental repair processes (Cooper et al. 2010). In particular, it has been suggested that TNF-α may promote the differentiation of pulp cells towards an odontoblastic phenotype (Paula-Silva et al. 2009) and increases dentine mineralisation along with the expression of DPP and DSP, therefore aiding the repair mechanisms involved in tertiary dentinogenesis.

1.3 Oral Microbiology

1.3.1 Commensals of the oral flora

Bacteria colonise all animal surfaces with specific bacterial species being associated with specific environments in their host. In humans, *Escherichia coli* is a species associated with the digestive tract and *Staphylococcus aureus* is found on the skin of a large proportion of the population (Liljemark and Bloomquist 1994). The oral cavity

contains a particularly large number of bacteria, approximately 10^{10} organisms from more than 500 species (Paster et al. 2001). The majority of these bacteria are Streptococci and *Actinomyces* species (Tlaskalova-Hogenova et al. 2004). These bacterial species are categorised as commensal or transient based on their prevalence in the oral cavity. Commensal species are almost always present and are divided into those bacteria which are present in high numbers as a dominant population and are considered indigenous and those which are present only in low numbers which are known as supplemental species. Transient species are only found occasionally and may be introduced into the oral cavity by food or drink. These species rarely colonise the cavity due to the competition with normal species but some pathogens such as *Porphyromonas gingivalis* and *Streptococcus mutans* (Hahn et al. 1991; Macarthur and Jacques 2003) are able to pass quickly from the transient stage to being the predominant bacterial population if conditions are favourable (Loesche 1994).

Commensal bacteria of the oral cavity are predominantly anaerobic species with an optimal growth temperature of approximately 37°C. Many have complex nutritional requirements which are only met by growing on animal tissues. Commensals are considered to have entered into a stable relationship with the host and generally do not cause damage to the host tissues. However, it is possible for some species which are considered commensals, such as *Streptococcus oralis*, to cause infections when certain environmental changes occur (Byers et al. 1999). These are known as opportunistic pathogens (Cole and Arnold 1982).

1.3.2 Plaque and Dental Caries

Bacteria in the oral cavity will adhere to the exposed tooth surfaces and form plaque. This adherence is possible due to the expression of cell-surface adhesins by primary colonisers of the tooth such as *Streptococcus gordonii* (Heddle et al. 2003). Bacteria initially adhere to an organic film known as the pellicle which forms over the teeth from glycoprotein precipitated from the saliva. The bacteria form a biofilm and the species within this change over time. This microbial succession is possible as bacteria such as *Streptococcus mutans* adhere to other species in the plaque, including

Streptococcus sanguis and *Actinomyces viscosus.* Although this mechanism is not fully understood it has been shown that it is enhanced by salivary agglutinin (Lamont et al. 1991).

The primary colonisers of the pellicle consist mostly of streptococci but as the plaque formation progresses the bacterial composition changes to a mixed flora of cocci, rods and filaments (Jenkinson and Lamont 2005). These bacteria produce organic acids when they metabolise fermentable carbohydrates found in food and drink (Loesche 1986) and these acids can dissolve the mineral component of enamel and eventually dentine, a process known as demineralisation (Featherstone 2000). Demineralisation increases the porosity of the enamel, enabling increased diffusion of bacterial acids through the tissue and thus further damage can occur deeper in the enamel (Kidd and Joyston-Bechal 1998). This demineralisation, if left untreated, leads to dental caries and cavity formation. Dental caries first appears as an area of demineralisation beneath the dental plaque known as a "white spot" lesion. In the most advanced area of this lesion up to 50% demineralisation may have occurred but the surface remains intact due to remineralisation (Silverstone 1973). If bacterial plaque is removed at this point the demineralisation can be arrested and some degree of lesion regression may occur (Thylstrup et al. 1994). This remineralisation is possible as the surface of the tooth is exposed to the saliva which has a neutralising effect on the bacterial acids and provides minerals to replace those which have been lost (Amaechi and Higham 2001). Therefore, caries is a dynamic disease process which can be reversed if conditions are favourable. For this to occur the lesion of caries must not have progressed into the dentine and the bacterial plaque must be removed from the enamel. This then allows neutralisation of the bacterial acids and remineralisation of the enamel. However if the plaque is not removed and there are conditions which favour progression of the carious lesion, a cavity will form and caries will progress into the dentine, which is irreversible without clinical intervention. Cavity formation also results in the exposure the dentine-pulp complex to the oral cavity and the vast number of bacteria which colonise it (Featherstone 2004). The exposure of the dentine provides an environment for bacterial growth which is more protected than the tooth surface, allowing opportunistic bacteria to thrive. The microaerophilic nature of bacteria such as those in the *Streptococcus anginosus* group gives these species an ecological advantage in these conditions (Facklam 2002).

1.3.3 Dentine infection

Dentine, as discussed previously, is a vital tissue containing the odontoblast processes and it is capable of initiating defence mechanisms against invading bacteria. As outlined previously, dentine is highly permeable due to its tubular nature and once it is exposed to the oral cavity and the bacteria in the advancing carious lesion it is possible for bacterial products to diffuse through to the pulp. Therefore, an initial response is to up-regulate the deposition of peritubular dentine, causing the dentinal tubules to become occluded and block the tubules, reducing permeability and producing sclerotic dentine (Kidd and Joyston- Bechal 1998). If this is unsuccessful and bacterial products diffuse through to the pulp, repair mechanisms will be initiated, producing tertiary dentine (Smith et al. 1995). In addition to this, an immune response will be stimulated. This inflammatory response may be acute or chronic and this will differ depending on how rapidly the carious lesion is progressing (Trowbridge 2002). In acute inflammation the response is rapid and involves innate immunity which does not recognise antigens of specific species but reacts to highly conserved antigens present in a large number of bacterial species such as LPS (Kidd and Joyston- Bechal 1998). A chronic inflammatory response occurs over a longer time period than an acute response and involves lymphocytes, macrophages and T-helper cells which are part of the adaptive immune system (Trowbridge and Stevens 1992). In other areas of the body it is common for chronic inflammation to follow an acute response if the infection is not resolved. However, pulpal inflammation that occurs as a result of caries is characteristically chronic in nature, since caries generally progresses gradually and the bacterial products diffuse through the tissue and illicit an immune response prior to the bacteria reaching the pulpal chamber and infecting the pulp (Trowbridge 2002).

Streptococcus mutans and lactobacilli are widely considered to be the main bacterial species involved in caries (Loesche 1986; Munson et al. 2004) but, as with plaque formation, the dominant bacterial species will change as the cavity advances deeper into the tissues as there will be environmental changes such as the availability of nutrients and oxygen (Marsh 2006). Two types of deep lesion of caries have been

characterised; those with a high number of lactobacilli (where more than 90% of the flora are lactobacilli) and those with low numbers. In both types of deep lesion, the number of *S. mutans* is much lower than in shallow lesions of caries. In those lesions classified as having low numbers of lactobacilli there was found to be a diverse flora with the predominant species being *Bacteroides,* Gram positive cocci, Gram positive non-branching rods and branching rods (Hahn et al. 1991).

1.3.4 Pulpal and Periapical Infection

Following infection and necrosis of the pulp, it is possible for a periapical lesion to develop which can be in the form of an abscess or granuloma, depending on the types of bacteria present in the root canal. Whilst it is possible for a number of bacterial species to form abscesses and it has been shown they are often polymicrobial in nature (Lewis et al. 1990), there are certain species which are more commonly associated with periapical infections. When abscesses were formed using dental plaque as an inoculum it was found that the majority of isolates from the abscess were from the *Streptococcus anginosus* group of bacteria, despite their usual low number in plaque (Okayama et al. 2005). These results have been supported by further clinical studies where 16 of 45 patient samples taken contained SAG bacteria, the majority of which were *Streptococcus anginosus* (Fisher and Russell 1993). Although these are not the only abscess-forming bacteria it is evident that they play an important role. Infection with the SAG bacteria, along with other pyogenic organisms such as *Prevotella intermedia* and *Porphyromonas endodontalis,* is more likely to lead to abscess formation than infection by less virulent organisms which may result in a granuloma (Trowbridge and Stevens 1992).

In addition to their role in abscess formation, it has also been suggested that SAG bacteria may be involved in the primary colonisation of the pulp (Love and Jenkinson 2002). Following the attachment of SAG bacteria to the pulp, other bacterial species may then be able to attach to them, allowing for further infection of the pulp.

The formation of abscesses greatly increases the chance of a tooth being lost as the bacterial infection and resultant inflammation cause necrosis of the supportive

periodontal tissues and may result in resorption of the bone surrounding the affected tooth, causing it to become loose (Herrera et al. 2000). Long term studies of patients with periodontal abscesses have shown that a tooth that has had an abscess associated with it is unlikely to survive and as a result of this many clinicians may choose to extract the tooth as part of the treatment regime to avoid further complications (Becker et al. 1984). There is also evidence to suggest that infection can spread from periradicular tissues to other sites in the body (Wagner et al. 2006) particularly in individuals with a compromised host defence system such as cancer patients (Manian 1997). As SAG bacteria have been indicated as important pathogens in abscess formation and the initiation of pulpal infection, it is necessary to have a clearer understanding of their prevalence and virulence to aid treatment and control of such infections.

1.4 Streptococcus anginosus Group Bacteria

1.4.1 Taxonomy

There has been much controversy regarding the taxonomy and nomenclature of the bacteria which have previously been referred to as *Streptococcus milleri, S. anginosus-constellatus* and *S.* MG-*intermedius,* amongst other terms. It was then shown that what had previously been grouped as one species consisted of three distinct genetic groups (Whiley and Hardie 1989). These species were then termed the *Streptococcus milleri* group but this has now been changed to the *Streptococcus anginosus* group (SAG). The SAG is now considered to be Gram positive bacteria from the species *S. anginosus, S. intermedius* and *S. constellatus.* They are generally regarded as being microaerophilic but show enhanced growth in the presence of CO_2 and are generally grown in laboratory conditions under anaerobic conditions on blood agar or fastidious anaerobe agar (Facklam 2002). They are part of the group known as viridans streptococci which originally referred to oral bacteria which were α-haemolytic. The term is now used to describe all oral streptococci, despite the heterogeneity that is observed amongst the species. Streptococci can be grouped according to their haemolytic ability. α-haemolytic strains cause partial lysis of red blood cells whilst β-haemolytic strains cause complete lysis of red blood cells which is seen on blood agar plates as a clear halo around colonies. α-haemolysis results in

the formation of a greenish halo around colonies. Within the SAG there are α-haemolytic and both β-haemolytic and non-β-haemolytic strains of each species (Facklam 2002). β-haemolytic strains can be further characterised based on the presence of specific carbohydrates in the cell wall, known as Lancefield group antigens. SAG bacteria may possess one of four Lancefield group antigens or the antigen may be absent (Whiley and Beighton 1998).

The three species may be distinguished from each other using biochemical tests as SAG show variation in their glycosidase activity which refers to their ability to metabolise sugars. *S. intermedius* produces β-galactosidase, β-glucosidase, β-fucosidase, neuraminidase, N-acetyl-β-D-glucosaminadase and N-acetyl-β-D-galactosaminadase and sialidase (Whiley et al. 1990) and is the only species that has mannosidase activity (Homer et al. 1993). This allows it to be distinguished from the other two species. *S. constellatus* has α-glucosidase and hyaluronidase activity whilst *S. anginosus* shows limited α-glucosidase activity and no hyaluronidase activity but does produce β-glucosidase (Whiley et al. 1990). This gives a distinct profile for the three species using simple tests although there is evidence of strain variations within a species (Willcox et al. 1995).

Hyaluronidase is an enzyme produced by some bacteria which catalyses the hydrolysis of hyaluronic acid, causing an increase in tissue permeability and so enhancing the ability of the bacteria to invade the tissue. Studies have confirmed that SAG bacteria exhibit hyaluronidase activity (Whiley et al. 1990) and that it tends to be present more frequently in *S. intermedius* and *S. constellatus* than in *S. anginosus* (Jacobs and Stobberingh 1995). This study also showed a similar profile for the enzyme chondroitin sulphatase which also facilitates bacterial spread (Jacobs and Stobberingh 1995). However, some studies found that only *S. intermedius* produced chondroitin sulphatase, mainly the strains isolated from liver and brain abscesses (Homer et al. 1993). These findings may be affected by the variation that can occur between strains of a species. The production of hyaluronidase and chondroitin sulphatase by SAG bacteria is of particular significance when considering pulpal infection as the pulpal matrix is rich in the substrates of these enzymes, suggesting that SAG may have a prominent effect on matrix degradation.

1.4.2 Virulence

Little is known about the pathogenicity and specific virulence factors of the SAG bacteria. However, it has been shown that *S. intermedius* produces the toxin intermedilysin, a human-specific cytolysin that can directly damage host cells (Nagamune et al. 1996). Intermedilysin is classified as a cholesterol-dependent cytolysin as its ability to form pores in host cell membranes is dependent on the presence of cholesterol, although its ability to bind to the cell is not (Polekhina et al. 2005) PCR and Southern blot hybridisation for the intermedilysin gene in all SAG bacteria has demonstrated that *Streptococcus intermedius* is the only species in the group to produce the toxin (Nagamune et al. 2000). This study also shows a correlation between higher expression of the intermedilysin gene and abscess formation in the liver and brain (Jacobs et al. 2000), indicating that this may be an important virulence factor in purulent infection. Intermedilysin has also been shown to reduce the numbers of fully functional PMNs during an infection, possibly through pore formation and subsequent cell lysis, although this has not been confirmed (Macey et al. 2001). The production of hyaluronidase and chondroitin sulphatase may also contribute to pathogenicity of some strains by facilitating bacterial spread and enabling bacteria to liberate nutrients from host tissues (Shain et al. 1996). In contrast to intermedilysin expression, there is no clear correlation between enzymatic activity and severity of infection (Nagamune et al. 2000).

Another SAG product which may increase virulence is antigen I/II, which is found on the surface of SAG and is involved in adherence to the pulpal matrix. This antigen has also been implicated in protecting the bacteria from the host immune system due to its antibody suppressive traits (Jenkinson and Demuth 1997). There is also evidence to suggest that certain SAG strains are able to produce an extracellular protein (Ep-Si) which assists the bacteria in evading the host immune system by reducing the efficacy of B and T lymphocytes. This non-cytotoxic protein was discovered in 1978 and was found to suppress the fibroblast formation and lymphocyte response *in vivo* (Higerd et al. 1978). More recently, the protein has been re-named protein P90 and studies have

shown that mice treated with high levels of P90 were 50 times more susceptible to infection by *S. intermedius* strains (Lima et al. 1992). This was demonstrated to be a result of B cells being unable to respond normally to the antigens of invading bacteria in the presence of P90.

There is some evidence to suggest that the presence of a capsule in SAG strains may also contribute towards pathogenicity by inhibiting the function of PMNs as encapsulated strains are more resistant to phagocytosis and phagocytic killing by PMNs (Kanamori et al. 2004). As evasion of the host response is particularly important in abscess formation, encapsulated SAG bacteria are likely to play an important role. This has been demonstrated by injecting mice with both encapsulated and non-encapsulated forms of SAG bacteria, which showed that whilst the encapsulated forms were capable of forming abscesses those without a capsule were less likely to do so. However, in the presence of encapsulated isolates SAG bacteria which do not have a capsule were also isolated from the resulting abscess that formed (Brook and Walker 1985), indicating that the presence of the capsule is needed for abscess formation.

Abscesses are polymicrobial in nature (Lewis et al. 1990) and there is some evidence to suggest that pathogenicity of SAG bacteria may be enhanced by co-infection with certain other species. A study using a murine model showed that co-infection of SAG with *Fusobacterium nucleatum* caused an increase in abscess size and in the number of bacteria of each species isolated from the abscess compared to mono-inoculation (Nagashima et al. 1999). The strongest synergistic effect was seen with *S. constellatus* and *F. nucleatum*, and this has been confirmed in further studies which found co-inoculation with these species killed all test mice whilst none died from mono-inoculation (Kuriyama et al. 2000). The mechanism of this synergy is not clear although it has been suggested that the co-infection increases the growth of SAG or inhibits the bactericidal activity of the host (Shinzato and Saito 1995).

1.4.3 Infections caused by SAG

The confusion regarding taxonomy and nomenclature has made it difficult to fully determine incidence of infections caused by the group and as such it is estimated that their pathogenicity has previously been greatly underestimated. It is now accepted though that whilst SAG bacteria can be harmless commensal organisms they are also associated with a number of infections, including meningitis, endocarditis, brain, liver and oral abscesses (Lamothe 1990) and pneumonia, pulmonary abscesses and thoracic empyema (Shinzato and Saito 1995). It has also been shown that certain species may be more often associated with certain infections than others (Whiley et al. 1992). In this study *S. anginosus* was the most commonly isolated species.

An association of SAG with head and neck infections has also been shown in a study by Fisher and Russell (1993) in which 45 samples were taken from periapical abscesses. 37% were found to contain strains from the SAG with the vast majority being *S. anginosus* strains. It has also been shown, using Amplified Fragment Length Polymorphism analysis, that SAG isolated from the blood and oral abscesses in the same patient are identical, suggesting that infection may be capable of spreading from oral abscesses to other sites in the body (Jacobs et al. 2003). The importance of pulpal infections to patient health may be underestimated but studies such as this emphasise the need for effective treatments to limit bacterial spread. Abscesses that can then form in the liver and brain can be life-threatening (Wagner et al. 2006).

SAG bacteria have been found to associated with abscesses as part of a polymicrobial infection in the liver and spleen (Brook and Frazier 1998) and in oral abscesses. As abscesses tend to be polymicrobial in nature there generally is not one antibiotic which will work against all infections. In the treatment of oral abscesses, it is most likely that a penicillin would be given as an initial treatment and in the event of its failure, administration of a broad-spectrum antibiotic such as metronidazole (Lewis et al. 1990). *In vitro* tests have shown the most common causal agents of oral abscesses to have approximately 85-90% susceptibility to penicillin and 45% susceptibility to metronidazole (Baumgartner and Xia 2003; Lewis et al. 1989).

The use of broad spectrum antibiotics creates an environment which selects for the growth of species carrying resistance. In addition to this, the natural defensive flora is reduced so these antibiotic resistant bacteria can become pathogenic (Madigan 2005). Therefore, it would be beneficial to find alternative therapies to combat pulpal infection and to prevent the formation of abscesses with more effective methods for limiting pulpal inflammation. This is important due to the increased chance of tooth loss in those teeth which have been affected by an abscess (Herrera et al. 2000) and due to the ability of oral infections to cause more serious infections at other sites in the body (Jacobs et al. 2003).

1.5 Pulpal infection and current treatment regimes

Current treatments for pulpal infections vary from administration of antibiotics to more invasive surgical procedures. The aim of any of these treatments is to eradicate the bacterial infection to leave the pulpal chamber sterile and prevent recurrent infection. As previously mentioned, the use of antibiotics has many disadvantages and surgical options, known as endodontics, are often preferred.

Endodontics is a branch of dentistry which is concerned with the treatment of pain caused by inflammation in the pulpal and periapical tissues and eliminating the source of inflammation (Reit et al. 2010). Endodontics can often result in the pulp being removed from the tooth to prevent further infection or spread of the bacteria within the pulp. The loss of the vital pulp can leave a tooth more susceptible to fracture and consequent removal (Tang et al. 2010).

The use of vital pulp therapies which aim to reverse pulpal injury whilst maintaining pulp vitality and function are now being proposed as an alternative to other treatments which involve the removal of the pulp and lead to an increased incidence of tooth loss (Tziafas 2004; Tziafas et al. 2000). For vital pulp therapies to be successful it is crucial that the tissues which have been exposed to the oral flora are treated to prevent recurring infection in the pulp (Siqueira and Rocas 2008). Calcium hydroxide is the more commonly used treatment for pulpal wounds as its high pH is thought to have a general antibacterial effect whilst encouraging pulpal repair (Desai and Chandler

2009; El Karim et al. 2007). However, since the introduction of calcium hydroxide in the early 20th century, many other compounds with more specific antimicrobial and anti-inflammatory properties have been developed (Tervit et al. 2009). These include substances such as antimicrobial peptides and triclosan and may be of clinical significance. However, a suitable model system is required for testing their efficacy in a clinical situation, highlighting the importance of the development of the model in this study.

1.6 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are proteins which have a broad range of antimicrobial activity against Gram positive and negative bacteria, fungi and viruses and are expressed by all complex species. Over 700 have been identified to date and they are known to play an important role in innate immunity. They are defined as peptides of 12-50 amino acids which are amphipathic and have an excess of basic amino acids, giving them a net positive charge (Hancock and Diamond 2000). They are generally constitutively expressed although a small number are induced shortly after microbial infection.

AMPs have a direct antimicrobial killing effect against a variety of bacterial pathogens *in vitro* (Hancock and Diamond 2000; Zasloff 2002). These direct killing effects are due to an interaction with the bacterial membrane which results in disruption of the bilayer, although the exact mechanism of this interaction is yet to be confirmed. Two models have been proposed in which the amphipathic structure of the peptides play an important role:

- Barrel-stave model
  - involves the binding of peptide monomers and insertion into the membrane to form transmembrane pores consisting of bundles of α helices, dissipating the transmembrane potential.
- Carpet model
  - peptides bind to the surface of the membrane and cover it in a carpet-like manner. They orientate themselves so that the hydrophilic surface faces the

phospholipids head groups. When the peptides reach a critical concentration they permeate the membrane and cause disruption of the bilayer (Shai 2002).

AMPs are seen to bind preferentially to bacterial rather than mammalian membranes (Reddy et al. 2004). This is due to the electrostatic charges between the positively charged AMPs and the negatively charged headgroups of the phosopholipids in the bacterial membrane (Zasloff 2002), whilst the phospholipid headgroups of mammals tend to carry no net charge.

In addition to membrane associated methods of direct killing, some AMPs have been seen to have intracellular killing mechanisms. The proteins are internalised into cells and can have a variety of effects depending on the specific peptide, such as inhibition of enzymatic activity, inhibition of nucleic acid synthesis and inhibition of cell wall synthesis (Brogden 2005).

Although direct antimicrobial killing mechanisms have been demonstrated *in vitro* this is generally when they are applied at concentrations that are significantly higher than they would be found *in vivo.* It is likely that the majority of AMPs act by alternative means *in vivo* because their direct activity is antagonised by physiological concentrations of ions such as sodium and magnesium (Bowdish et al. 2005a). It is thought that they have a broad range of functions relating to innate immunity by stimulating or inhibiting a variety of biological events and as such they are often referred to as host defence peptides (Brown and Hancock 2006; Finlay and Hancock 2004).

The exact effect that each peptide has on the host immune system will differ and can result in immune activation, immune suppression or immune enhancement (Brown and Hancock 2006). Some effects which have been observed include promoting recruitment of cells involved in adaptive immunity to sites of infection by increasing secretion of chemokines and cytokines (Bowdish et al. 2005b). Others function by promoting phagocytosis and stimulating apoptosis of macrophages and activated lymphocytes (Hancock and Diamond 2000). These processes all contribute to clearance of bacterial cells without direct killing and may involve pro-inflammatory processes. In contrast to this, some AMPs have an important anti-inflammatory effect

by suppressing cytokine production (Finlay and Hancock 2004; Marta Guarna et al. 2006). This allows natural tissue regeneration processes to occur which may be enhanced by certain defence proteins which stimulate fibroblast growth and inhibit certain proteases (Hancock and Diamond 2000; Hancock and Sahl 2006). If such properties can be harnessed for therapeutic use this would be particularly useful in treatment of pulpal disease where inflammation has been shown to be a major a problem and where there is a great potential for healing if inflammation can be controlled.

To develop AMPs as a novel therapeutic for treatment of pulpal inflammation it is important to consider the AMPs that are being expressed naturally in the oral cavity which may effect treatment.

hBD-1 and -2 are found to widely expressed in healthy oral tissues with hBD-1 being more frequently detected than hBD-2. Whilst both hBD-1 and -2 were found to be expressed by oral keratinocytes, neither were found to be expressed by fibroblasts, suggesting that their expression is confined to the epithelial compartment of the oral cavity (Dunsche et al. 2001). However, there are some reports that RT-PCR on extracted pulp cells showed expression of hBD-1 and 2 (Dommisch et al. 2005). Immunohistochemical analysis showed that this expression occurs in the cytoplasm of the odontoblasts. These AMPs may therefore also be important in defence of the pulp as these are the first cells invading bacteria will encounter (Dommisch et al. 2005). hBD-1 is expressed constitutively by the oral mucosa and salivary glands whilst hBD-2 expression can be induced by LPS found in the bacterial cell wall (Mathews et al. 1999). The more recently discovered hBD-3 has a similar expression profile to hBD-1 and -2 as it is also widely expressed in oral tissues but absent in fibroblasts (Dunsche et al. 2002).

In addition to its antimicrobial and immunomodulatory effects it has been suggested that a synthetic β-defensin-2 may also have the ability to stimulate the differentiation of pulpal fibroblasts into odontoblasts. This is thought to be regulated by the chemokine receptor CCR6 (Shiba et al. 2003). This is an important discovery as differentiation of pulpal fibroblasts into odontoblasts is required for reparative

dentinogenesis. Therefore, this may be a target for development as a therapy for pulpitis.

A number of oral streptococci, including all SAG bacteria, have been shown to have some degree of susceptibility to hBD-2 (Landrygan-Bakri 2006). However, there is variation between strains, with some strains of *S. anginosus* having an MIC of more than 100μg/ml (Nishimura et al. 2004). This is an important consideration when developing novel therapeutics as high concentrations of AMP may incur toxic effects to host cells.

1.7 Triclosan

Triclosan is a broad-spectrum antimicrobial agent that has been used for over 25 years in a wide range of dermatological preparations such as deodorants, soaps and cosmetics (Bhargava and Leonard 1996) and has become particularly popular in recent years due to its efficacy in handwashes against methicillin-resistant *Staphylococcus aureus* (Zafar et al. 1995).

At high concentrations triclosan acts against most susceptible bacteria, including *Streptococcus* species, by disrupting the phospholipid membrane of bacteria and interfere with its normal functions (Cottell et al. 2009; Villalain et al. 2001). Leakage titration experiments have been used to study the effects of triclosan on the bacterial membrane of the oral bacteria *Pseudomonas ginigivalis* and *Streptococcus sobrinus,* which showed that whilst the bacterial growth was inhibited there was negligible cell leakage. This indicates that when triclosan acts upon the cell membrane it destabilises the phospholipid structure in a way which compromises its functional integrity, but that it does not induce cell lysis (Villalain et al. 2001).

Triclosan is also effective against *E. coli* and some other bacterial species at lower concentrations by specifically inhibiting the enoyl-acyl carrier protein reductase enzyme FabI (Escalada et al. 2005; Zhang et al. 2004). FabI has an essential role in the type II fatty acid synthase system, so its inhibition results in bacterial cell death. Some *E. coli* strains which have been selected for their resistance to triclosan have

been shown to have mutations in the FabI gene which result in an overproduction of the enzyme or the production of an insensitive form (Russell 2004). More recent studies have shown there are also other mechanisms which may infer triclosan resistance, including efflux mechanisms which pump the triclosan out of the cell, capture of the triclosan to prevent it affecting the cell and over expression of important enzymes and metabolic regulators (Yu et al. 2010).

In recent years triclosan has been incorporated into a number of oral health care products such as toothpastes and mouthwashes (Bhargava and Leonard 1996; Binney et al. 1995; Blinkhorn et al. 2009) with the aim of reducing bacterial plaque numbers and protecting against periodontal disease. However there have been mixed views on the efficacy of these products, with some studies showing a significant inhibition of plaque growth (Haraszthy et al. 2010; Vered et al. 2009) whilst others indicate that there is no significant advantage of triclosan containing products over regular toothpastes (Andrade Acevedo et al. 2009). There has also been increasing debate regarding the cross-resistance of triclosan and antibiotics, particularly in drug-resistant strains of *Staphylococcus aureus* (Bayston et al. 2007; Brenwald and Fraise 2003; Seaman et al. 2007). Some research suggests that bacteria which develop a resistance to triclosan through efflux mechanisms may also be able to pump out antibiotics, or that modifications to the target enzyme of triclosan may also infer resistance to antibiotics that act on a similar enzyme (Schweizer 2001). However, there are also suggestions that whilst bacteria may be shown to have an increased resistance to triclosan following *in vitro* exposure, this may not relate to a resistance to the 'in-use' concentrations of triclosan found in widely used disinfectants and dentifrices (Maillard 2007)

One of the important factors when considering triclosan as a potential therapeutic in pulpal disease is its role as an anti-inflammatory, in addition to its antimicrobial properties. As previously outlined, low-grade inflammation may be beneficial for promoting natural repair mechanisms of the tooth, whilst severe inflammation will result in pulpal necrosis. Therefore, antimicrobials that can downplay the inflammatory response may be advantageous for use in endodontic treatments and pulp capping to promote host repair processes whilst eradicating the bacterial infection.

To investigate the effect of antimicrobials such as triclosan and AMPs on bacterial and mammalian cells, and to study how these antimicrobials effect the interactions between the two cell types, it is necessary to develop an effective model. *In vivo* studies can be problematic due to the presence of commensal bacteria and naturally expressed antimicrobials which may make it difficult to obtain clear results. Whilst *in vitro* studies may overcome some of these problems and provide a more easily manipulated model, they are unable to take into consideration the dynamic nature of the dentine-pulp complex. Therefore an *ex vivo* model system may provide the benefits of studying whole tissues rather than single cell populations whilst providing a model which is easier to manipulate.

1.8 Ex-vivo model systems

As discussed in this introduction, the processes involved in pulpal disease and the resulting inflammation that occurs are complex and varied, with a large number of host and bacterial proteins present in the pulpal chamber. To elucidate the events that occur during pulpal infection, a wide array of *in vivo* and *in vitro* models have been used. Whilst these have led to many advances in the treatment of pulpal disease in recent years there are drawbacks in all currently available models. *In vitro* models using pulpal fibroblast cells grown in culture are an over-simplified representation of the multi-cellular dentine-pulp complex. In particular, the lack of spatial arrangement of the cells as they would be found within the tooth is a disadvantage as this is an important consideration when modelling events within the tooth. It has also been shown that odontoblasts must remain in contact with the dentine matrix in order to maintain the phenotypic and secretory activity that would be seen *in vivo* (Munksgaard et al. 1978). In addition to the limitations of *in vitro* mammalian models, the majority of experiments which study the antimicrobial efficacy of biocides such as triclosan are also tested on bacteria *in vitro,* which involves the bacteria growing in suspension. Whilst this may represent how the bacteria grow within the fluid of the dentinal tubules, the majority of bacteria found in the tooth will be attached to cells of the pulp, the odontoblast processes or the walls of the dentinal tubules (Love et al. 1997; Perez et al. 1993). When an infection becomes established biofilms may also

form, which have been shown to have an increased resistance to biocides and may also express different proteins from those expressed when grown in suspension (Aslam 2008). As such, *in vitro* models may represent an oversimplified version of events and may not take into account the effect of bacterial attachment to a multi-layered tissue such as the pulp.

Whilst one of the main disadvantages of *in vitro* models is an oversimplification, *in vivo* models have the opposite problem in that the presence of systemic influences may make it difficult to obtain clear data. This is particularly true in the case of microbiological models as it is difficult to obtain animals which do not have some degree of microbial contamination. Although germ-free animals are available, this will further increase the extensive expenses associated with whole animal models. Such models require a number of extra considerations, including the maintenance of the animal housing, ethical approval and will generally require a large number of animals as each animal represents only one experiment.

In an attempt to overcome some of the problems associated with *in vitro* and *in vivo* models, a number of *ex-vivo* models have been developed. These models allow cells to be cultured in the spatial arrangement they would be found in *in vivo* whilst removing the systemic influences that are associated with animal models. Existing *ex-vivo* models have been used to study a wide variety of developmental, physiological and pathological conditions. One such model that has been established is the *ex vivo* tooth slices organ culture model, which has been used to study the secretion of growth factors in the dentine-pulp complex, dentinogenesis and the effect of mechanical strain on the dentine-pulp complex (Dhopatkar et al. 2005; Sloan et al. 1998; Sloan and Smith 1999). Modification of the existing tooth slice model has also led to the development of a mandible model which has been used to study the processes involved in bone repair (Smith et al. 2010). These models have shown that tooth slices taken from 28 day old Wistar rats can be cultured for up to 14 days at the liquid-gas interface with no detrimental effect on the tissues (Sloan et al. 1998) and have provided an alternative to the existing *in vivo* models which use live rats, rabbits and dogs. Research to date has used the development of these models to study development and repair in healthy tissues and the introduction to microbes to mimic a diseased tooth state has not been a focal point of these investigations. Currently,

models of microbial infection of the pulp are focussed on *in vivo* models (Balto et al. 2002; Kurland et al. 2006). Modification of the existing *ex vivo* models of the dentine-pulp complex may provide a model for co-culture of bacteria involved in pulpal infection and abscess formation which would reduce the numbers of animals needed in research, in addition to reducing experimental costs. Such a model would also provide a system for studying one or two bacterial species in isolation without the complications of commensal organisms. A further benefit of the existing models is that there is no blood system associated with the model, meaning that inflammatory responses would be limited to those mediated by the cells found within the pulp.

1.9 Aims and Objectives.

The aim of this project is to:

- develop the existing tooth slice system to model infection of the pulp by SAG bacteria, focussing on the interactions that occur between bacterial and mammalian cells

This will provide information on how bacteria interact with the tissues of the dentine-pulp complex and the effect that SAG bacteria have on the pulpal matrix and the cells within it.

As there has been very limited investigation into the effect of SAG bacteria on the pulpal matrix and such a model has not been previously established, the main focus of this work is to establish appropriate co-culture conditions which will support the growth of both mammalian and bacterial cells. Validation of the model will be required to assess the effect of these co-culture conditions on both cell types and to ensure that they do not have deleterious effects on normal cell growth. The model will then be used to:

- identify any patterns of bacterial attachment to the tooth slices
- assess the effect of co-culture on the viability of cells of the tooth slice and the structural integrity of the pulpal matrix
- assess the effect of the bacterial supernatants on the cells of the tooth slice and the pulpal matrix
- investigate the efficacy of currently available biocides on SAG bacteria

Ultimately, the aim of this study is to develop an *ex vivo* co-culture model which will allow further investigation into the process of pulp infection by SAG bacteria so that this model may be used in future work for development of novel antimicrobials for use in endodontics. This may make it possible to improve bacterial elimination from the infected root canal, providing an environment in which natural tissue regeneration of the dentine-pulp complex can occur, and it may be possible to reduce pulpal

inflammation and necrosis. Consequently, infections of the pulp by SAG are less likely to result in the formation of abscesses and their associated complications.

Chapter 2: Characterisation of SAG clinical isolates and validation of the culture conditions for the co-culture system

2.1 Introduction

As outlined previously, SAG bacteria have the ability to infect various sites within the human body and have a predisposition to causing abscesses (Ruoff 1988). During a pulpal infection in which SAG bacteria may be involved, there is increased inflammation in the pulpal chamber causing a rise in pressure which may result in tooth pain. If this inflammation is not resolved by the host responses of the tooth then pulpal necrosis may occur. It is under such instances that an acute dentoalveolar abscess may form (Trowbridge 2002). These are the most commonly occurring orofacial bacterial infections and SAG bacteria are often isolated from them, indicating a role in their formation (Lewis et al. 1990). The formation of such an abscess results in the patient having to have treatment to drain the abscess, extirpate the pulp or remove the tooth, all of which can be expensive and painful. In the event that there is evidence of systemic spread of the bacteria, antimicrobial treatments will also be required (Marsh 2009).

The development of a system to model the infection of pulpal tissues by SAG bacteria is needed to provide information on the processes that occur prior to abscess formation. Currently, very little is known as to the role these bacteria play in the progression from carious lesion to abscess formation except that when plaque bacteria is used as an infective agent, these bacteria are isolated from the majority of the resulting abscesses in high numbers, despite being present in the plaque at relatively low numbers (Okayama et al. 2005; Thurnheer et al. 2001). A model is required that enables the bacteria to interact with host tissues not just as a planktonic suspension but also as a biofilm, as the characteristics of bacteria have been found to be greatly altered in these different growth states, in particular biofilms have an increased resistance to antimicrobials (Aslam 2008; Fux et al. 2005). As the dentine and pulp of the tooth are closely associated *in vivo* it is also important that any model that is to be developed accounts for this. Other models may focus on pulp fibroblasts grown in culture and such isolated growth without the structural organisation of cells found

within the tooth may not accurately represent how the cells behave *in vivo.* Similarly, there are limitations with *in vivo* models as they are expensive, parameters are more difficult to define and they pose ethical problems.

For these reasons, an established *ex vivo* organotypic tooth slice culture system, where the dentine-pulp complex is cultured *in situ* (Sloan et al. 1998), will provide the basis for the development of a model for SAG infection. This allows observation of bacterial growth on the tissues as would be found *in vivo,* without the normally associated complications such as host inflammation and the presence of commensal oral microflora. Although such a model addresses a number of problems faced with *in vivo* and *in vitro* models there is still a major challenge to be faced in its development, that of identifying the culture conditions that will support the growth of both the bacterial and mammalian cells.

SAG bacteria are normally grown under anaerobic conditions in nutrient rich media such as BHI broth (Facklam 2002). The mammalian tooth slices need to be grown in supplemented cell culture medium and incubated at 5% CO_2. Although SAG bacteria are generally incubated anaerobically they are facultative aerobes and have been found to have enriched growth when grown at 5% CO_2 (Pulliam et al. 1980). However a culture medium that enables growth of SAG bacteria and maintains culture of the tooth slice needs to be developed for use in the model. Any effects this media has on the components of the model must also be assessed.

In addition to the generalised effect that different culture conditions have on the bacterial group as a whole, there are also differences between strains. Expression of virulence factors may be upregulated in some strains within the group or they may have characteristics that enable them to infect the host more easily. SAG bacteria are found as commensals in the oral cavity (Poole and Wilson 1979) but the strains isolated from infected sites are known as pathogenic strains as they are found to be actively causing infection. The bacteria used in this study are pathogenic clinical strains that have been isolated from various infected sites in the body and as such their idenitification needs to be confirmed before their use in the study. Purification and positive identification of strains is of particular importance when using SAG bacteria as there have been a number of changes in the classification and nomenclature of

these bacteria in recent years, leading to confusion regarding their role in abscess formation (Facklam 2002) .

2.2 Methods

2.2.1 Preparation of bacterial stocks

The bacterial species to be studied were selected from the culture collection of the Oral Microbiology Unit, Cardiff School of Dentistry. These strains were genotypically and phenotypically characterised in previous work and are listed in Table 2.1. Bacteria were grown on fastidious anaerobe agar (FAA) (Lab M™ International Diagnostic Group plc, Bury, UK) supplemented with 5% (v/v) defibrinated sheep blood (TCS Bioscience Ltd., Buckingham, UK).

Brain-heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) was inoculated from colonies on FAA plates to prepare bacterial suspensions. Bacteria were incubated in an anaerobic cabinet (10% v/v CO_2, 20% v/v H_2, 70% v/v N_2), at 37°C (Don Whitley Scientific Ltd., Shipley, UK). Gram stains were performed on colonies from FAA plates. A pure sample of the microbe was taken from the plate using a sterile loop and smeared on a glass slide with a drop of PBS on it. The slide was air-dried prior to heat-fixation in a Bunsen flame. The slide was flooded with crystal violet stain for 30 s, washed under running water and flooded with iodine for 5 s. The slide was decolourised by rinsing with acetone before the counterstain, fuchsin, was applied for 30 s. The slide was then rinsed and air-dried before being viewed by oil-immersion microscopy.

Species	**Reference**	**Clinical source**
S. anginosus	39/2/14A	Unknown
S. anginosus	670/95	Dentoalveolar abscess
S. anginosus	447/95	Dentoalveolar abscess
S. constellatus	45386	High vaginal swab
S. constellatus	350/96	Dentoalveolar abscess
S. constellatus	322/95	Dentoalveolar abscess
S. intermedius	127/95	Dentoalveolar abscess
S. intermedius	HW13	Dentoalveolar abscess

Table 2.1 Identity and source of clinical isolates used

2.2.2 Identification of Bacterial Strains

2.2.2.1 API Rapid ID 32 Strep bacterial identification strips

API rapid ID 32 Strep (bioMerieux, Marcy-l'Etoile, France) is a micro-method designed to identify streptococcal species using standardised enzymatic tests. The system consists of 32 test cupules which contain a dehydrated enzymatic substrate (see Table 2.2). Collating the results from each cupule allows an enzymatic profile of each bacterial strain to be complied, enabling species identification.

A bacterial suspension was prepared by using a sterile loop to select colonies grown on fastidious anaerobe agar for 48 h and transferring to distilled water until a turbity of 4 MacFarland was reached. The enzyme strip was then assembled according to manufacturer's instructions. Fifty-five µl of suspension was added to each cupule and incubated aerobically for 4 h at 37°C. Following incubation, 1 drop of VP A and 1 drop of VP B reagents (supplied in kit) were added to cupule 0.0 (see table 2.2), 1 drop of FB reagent (supplied in kit) was added to cupules 0.1 to 0.5 and 1 drop of NIN reagent (supplied in kit) was added to cupule 0.6. No further reagents are needed for colour to develop in the other cupules. Following the addition of the required reagents, colour was allowed to develop for 5 min before reading the strip using the table provided. Collation of the results generates an identification code relating to a species which can be identified using the Strep 32 ID reference catalogue.

2.2.2.2 16S rDNA sequencing

2.2.2.2.1 Extraction and amplification of 16S rDNA from SAG clinical isolates

DNA was extracted from suspensions of each pure bacterial isolate using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, USA), following the "DNA Isolation from 1ml Gram-positive Bacteria Culture Medium" protocol (http://www.gentra.com/technical_assistance/protocols.asp).

16S rDNA genes were PCR amplified using the D88/E94 primers at a working concentration of 0.5 µM. PCR was performed in a total volume of 50 µl using 3 µl of DNA template and 25 µl GoTaq Green (Promega) which contains *Taq* DNA polymerase, nucleotides and buffer. Total volume was made up using nuclease free water. A PCR protocol was used in which denaturation was performed at 95°C for 8

Cupule	Reaction	Negative Result	Positive Result
1.0	Arginine dihydrolase	Yellow	Red/Orange-red
1.1	β-glucosidase	Pale orange	Pink/Red-orange
1.2	β-galactosidase	Orange	Pink/Red-orange
1.3	β-glucuronidase		
1.4	α-galactosidase	Colourless	Yellow
1.5	Alkaline phosphatase	Colourless/Very pale yellow	Yellow
1.6	Ribose (acidification)	Red/Red-orange	Yellow/Orange
1.7	Mannitol (acidification)		
1.8	Sorbitol (acidification)		
1.9	Lactose (acidification)		
1.A	Trehalose (acidification)		
1.B	Raffinose (acidification)		
1.C	Sucrose (acidification)		
1.D	L-arabinose (acidification)		
1.E	D-arabitol (acidification)		
1.F	Cyclodextrin (acidification)		
0.0	Acetoin production	Colourless	Pink
0.1	Alanine-phenylalanine-proline arylamidase	Colourless/Pale orange	Orange
0.2	β-galactosidase	Colourless/Pale orange/Pale purple	Purple
0.3	Pyroglutamic acid arylamidase	Colourless/Pale orange	Orange
0.4	N-acetyl-β glucosaminidase	Colourless/Pale orange/Pale purple	Purple
0.5	Glycyl-tryptophane arylamidase	Colourless/Pale orange	Orange
0.6	Hydolysis of hippurate	Colourless	Blue
0.7	Glycogen (acidification)	Red/Red-orange	Yellow/Orange
0.8	Pullulan (acidification)		
0.9	Maltose (acidification)		
0.A	Melibiose (acidification)		
0.B	Melezitose (acidification)		
0.C	Methyl-B-D Glucopyranoside (acidification)		
0.D	Tagatose (acidification)		
0.E	β-manosidase	Colourless	Yellow
0.F	Urease	Yellow/Beige-pink	Pink/Red-violet

Table 2.2 Rapid ID 32 Strep reading table

min, primer annealing was performed at 60°C for 1 min and elongation was performed at 72°C for 1 min 45 s (single cycle). 30 cycles were then performed with further denaturing steps lasting 45 s and each elongation step increasing by 5 s. A final elongation step runs for 10 min before the PCR products were stored at 4°C before purification for sequencing.

PCR products were purified by precipitation and washing with ethanol. Firstly, a solution of equal volumes of 40% polyethylene glycol (Mol. Wt 8000; Sigma-Aldrich, Poole, UK) and 5 M NaCl (Sigma) was prepared which enables DNA precipitation by preferentially suspending proteins. This was added to the PCR product at a volume that is half that of the final PCR reaction and mixed by pipetting prior to centrifugation (13,000 rpm, 15 min). The resulting supernatant was discarded and the pellet re-suspended in 200 µl chilled ethanol prior to further centrifugation (13,000 rpm, 15 min). This centrifugation and ethanol washing step was repeated prior to a final centrifugation step after which the PCR products were air-dried under a fume hood overnight. Products were re-supsended in 30 µl nuclease-free water and stored at -20°C.

2.2.2.2.2 Sequencing of amplified 16S rDNA genes

PCR products were sequenced using ABI Prism BigDye terminator cycle sequencing ready reaction kits (Applied Biosystems, Warrington, UK). BigDye Terminator Reaction premix and Sequencing Buffer were added to PCR tubes containing approximately 5-20 ng of PCR product. Sequencing was performed using the primers 357F and 1492R (Invitrogen) at a concentration of 0.5 µM to give a sequence of at least 1200 nucleotides. Primer sequences are listed in table 2.3. Reactions comprised of 1 min of denaturation at 95°C followers by 30 cycles of denaturation at 95°C (30 s), annealing at 50°C (15 s) and elongation at 60°C (5 min).

Extension products were purified by addition of 1µl sodium acetate (3M, Sigma), 1µl EDTA (0.5M, Sigma) and 80µl 100% chilled ethanol. Products were then centrifuged (13, 000rpm, 15 mins) and supernatant was aspirated and replaced with 70% chilled ethanol. The centrifugation step was then repeated and products air-dried overnight in a fume hood before being resuspended in formamide (30µl) and run on an automated

DNA sequencer (ABI PRISM 3100 Genetic Analyser; Applied Biosystems). This resulted in sequences being obtained that could then be compared to those in the public databases.

Primer	Sequences 5'-3'	Source
D88	F: GAGAGTTTGATYMTGGCTCAG	Paster et al. 2001
E94	R: GAAGGAGGTGWTCCARCCGCA	Paster et al. 2001

Table 2.3 Primer sequences for 16S rDNA sequencing

2.2.2.2.3 Identification of clinical SAG isolates by 16S rDNA sequence analysis

Sequences that were obtained were identified by comparison to the GenBank DNA sequence database using the FASTA sequence homology search (http://www.ebi.ac.uk/services/index.html). 16S sequences were compared to those of the type strain for *S. intermedius, S.anginosus* and *S. constellatus.* A homology of >99% was the criterion used to identify an isolate to the species level. If there were no definitive matches to any of the reference strains the isolate was identified using the results of the indiscriminant GenBank search.

2.2.2.3 Expression of intermedilysin gene

Bacterial DNA was extracted and amplified as previously described (**2.2.2.2.1**). The intermedilysin gene was PCR amplified using the ILY-NFw/CBw primer pair, as listed in table 2.4 (0.5μM of each, Invitrogen). A PCR protocol was used in which denaturation was performed at 95°C for 5 min, primer annealing was performed at 55°C for 1 min and elongation then performed at 72°C for 2 min. Thirty cycles were performed with further denaturing steps lasting 45 s and each elongation step increasing by 5 s. A final elongation step for 10 min was carried out and the PCR products stored at 4°C before purification for sequencing. PCR products were run on 1.5% (w/v) agarose gels containing 125 ng/ml ethidium bromide (Sigma) and visualised under UV light using a GelDoc system (Bio-Rad Laboratoried Ltd, Hemel Hempstead, UK).

Primer	Sequences 5'-3'	Source
ILY-NFw	F: AACACCTACCAAACCAAAAGCAGC	Nagamune, H. et al. 2000
CBw	R: ACTGTGGATGAAGGGTTGTTCCCC	Nagamune, H. et al. 2000

Table 2.4 Primer sequences for PCR of intermedilysin gene

2.2.3 Bacterial growth in various culture media

2.2.3.1 Growth at 5% CO2

Bacterial suspensions were prepared as previously described (2.1) in BHI broth. Suspensions were then incubated overnight in gas jars at 37°C, 5% CO_2.

2.2.3.2 Bacterial growth under mammalian cell culture conditions

Protocol 1 – Bacterial growth in DMEM

Bacterial suspensions were prepared as previously described in Dulbecco's Modified Eagle's Medium (DMEM) without phenol red (Sigma 41965-047) supplemented with 10% heat inactivated foetal calf serum and 0.15 mg/ml vitamin C. Suspensions were incubated overnight in gas jars at 37°C, 5% CO_2.

Protocol 2 – Bacterial growth in DMEM supplemented with BHI

Bacterial suspensions were prepared in DMEM without phenol red (Sigma) supplemented as in protocol 1. BHI was also added at concentrations of 10, 20, 30 and 40%. Suspensions were incubated as above.

Protocol 3 – Bacterial growth in DMEM supplemented with BHI and haemin

Suspensions of *S. intermedius* 127/95 and HW13 were prepared in DMEM without phenol red (Sigma) supplemented as in protocol 1. Media was further supplemented with 10% BHI and 2 μM haemin, 10% BHI and 4 μM haemin, 20% BHI and 2 μM haemin and 30% BHI and 2 μM haemin. Suspensions were incubated as above.

2.2.4 Bacterial growth characteristics under modified culture conditions

Bacterial suspensions of 45386, 39/2/14A and HW13 were prepared in both BHI broth and DMEM+10% BHI and incubated overnight at 37°C, 5% CO_2. 1 ml of each

overnight culture (ONC) was transferred to 9 ml of fresh broth and incubated under the same conditions. A sample was taken every hour and the absorbance measured at 550 nm.

2.2.5 Tooth Slice Culture under standard conditions

Upper and lower incisor teeth were dissected from freshly sacrificed 28 day old male Wistar rats by removing the bone from around the teeth using a sterile scalpel. The teeth were then placed in sterile DMEM prior to being cut into 2mm thick transverse sections on a diamond edged rotary saw (TAAB, Berkshire, UK). The blade was sterilised using 70% ethanol and was kept cool with sterile DMEM in a well at the base of the blade. The cut sections were immediately transferred to fresh sterile supplemented DMEM for no more than 20 min before being cultured in 2ml of supplemented DMEM at 37°C, 5% CO_2. Storage of the dissected incisors and resulting tooth slices in supplemented DMEM before culture is vital to maintain maximum viability of the cells within the slices. Tooth slices were cultured for 7 and 14 days with fresh media provided every 2 days.

2.2.6 Histological, fixation, processing and staining methods

2.2.6.1 Fixation and demineralisation

Following culture, incisor slices were removed from culture using sterile forceps and transferred to 10% (v/v) neutral-buffered formalin solution for 24 h for fixation of tissues to occur. The slices were then transferred to a minimum of 5 ml 10% (v/v) formic acid for 48 – 72 h with constant agitation to allow for demineralization.

2.2.6.2 Processing of tissue

Protocol 1: Automated processing

Tooth slices were transferred to individual biopsy cassettes and processed through a series of graded alcohols on an automatic tissue processor as shown below:

1. 70% ethanol, 1 h

2. 90% ethanol, 1 h
3. 100% ethanol, 1.5 h
4. 100% ethanol, 1.5 h
5. 100% ethanol, 1.5 h
6. 100% ethanol, 1.5 h
7. Xylene, 1.75 h
8. Xylene, 1.75 h
9. Xylene, 1.5 hr
10. Molten wax, 2.5 h, 60-65°C
11. Molten wax, 2.5 h, 60-65°C

Protocol 2: Manual processing

Tooth slices were transferred to individual biopsy cassettes and processed through graded chemicals as shown below:

1. 50% ethanol, 2 h
2. 70% ethanol, 2 h
3. 95% ethanol, overnight
4. 100% ethanol, 2 h
5. 100% ethanol, 2 h
6. 100% ethanol, 2h
7. 100% ethanol overnight
8. 100% ethanol 1h
9. Methyl salicylate, 1h
10. Methyl salicylate, 1h
11. 0.5% necloidine in methyl salicylate, 1 h
12. 0.5% necloidine in methyl salicylate, 1 h
13. 1% necloidine in methyl salicylate, 1 h
14. 1% necloidine in methyl salicylate, 1 h
15. Molten wax, 60°C, 1h
16. Molten wax, 60°C, 1h
17. Molten wax, 60°C, 1h
18. Molten wax, 60°C, overnight

Following both methods of processing the tissues were embedded in paraffin wax using a processing machine (Shandon Pathcentre, Thermo Scientific, Surrey, UK) and aligned so that transverse sections of the slice can be cut. Fig. 2.1 demonstrates the orientation of the tooth slice from which the section is cut, relative to its position in the incisor.

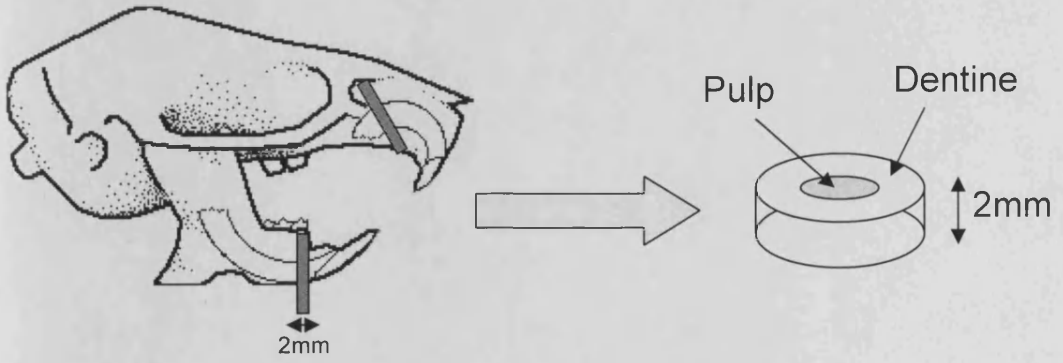

Fig. 2.1. Schematic diagram of rat skull showing position of incisors. Blue areas on schematic represent 2mm sections that are cut along the length of the incisors. The right-hand side of the diagram shows how the tooth slices appear after being cut and how the tooth slice is orientated in relation to its previous position in the tooth.

2.2.6.3 Sectioning of tooth slices

Sections of 7μm thickness were cut from the wax blocks using a Leitz 1400 microtome (Leica, Buckinghamshire, UK). The embedded sections were floated on warm water (40°C) and mounted on glass slides. These were then placed in a slide drying oven (60°C) before staining to improve adhesion of the section to the slide.

2.2.6.4 Staining histological sections

Histological sections were mounted on uncoated microscope slides and placed on an automated tissue stainer. The slides were taken through xylene, alcohol and water washes followed by haemotoxylin and eosin (H&E) stains. They were then taken through a further series of alcohol washes before finally being immersed in xylene. The slides were then removed and a coverslip glued over the stained section for viewing by an Olympus AX40 light microscope.

2.2.6.5 Vital dye staining

A working solution of acridine orange/ethidium bromide was prepared by mixing 1ml of a 0.25% acridine orange solution to 0.25 ml of ethidium bromide at 10 mg/ml. This was then made up to 25 ml using DMEM working media to give a final concentration of both acridine orange and ethidium bromide of 100 μg/ml. At the end of a culture period the tooth slice was removed from the embedding media and placed into the well of a culture dish containing 4 ml of the acridine orange/ethidium bromide for 10 s. The slice was then removed and washed in 4ml of phosphate buffered saline (PBS) and placed on a microscope slide so the cut surface of the slice could be viewed. The slice was then immediately viewed on an Olympus Ax40 fluorescence microscope under UV light.

2.2.7 Tooth slice culture under modified conditions

Upper and lower incisors were dissected from 28 day old male Wistar rats and cultured as previously described (**2.2.5**) for 5 days. Tooth slices were then transferred to DMEM+10% BHI for 4, 8 and 24 h prior to fixation and processing for H&E staining. Image Pro-Plus analysis was used to count the number of nuclei in a 50 μm^2 area. For each time point sections were cut from 5 tooth slices. 5 random fields of view (RFV) were taken within each section and the nuclei counted in five 50 μm^2 areas in each RFV to obtain an average nuclei number for each time point.

2.3 Results

2.3.1 Bacterial identification

Identification of the bacterial strains was initially performed using Strep 32 ID test strips. A number of the clinical isolates tested did not generate a code that had an exact match and so the species was taken as that having the most closely matched code number. The identification of each clinical isolate was confirmed using 16S rDNA sequencing, as shown in table 2.5.

PCR of the intermedilysin gene resulted in bands produced by strains HW13 and 127/95, indicating the presence of the intermedilysin gene (Figure 2.2).

2.3.2 Bacterial growth in various culture media

The growth of clinical isolates in the various culture media tested is summarised in table 2.6. All clinical isolates showed normal growth when cultured in BHI at 5% CO_2, which was defined as an absorbance of above 1.0 at 550nm after overnight incubation. A minimum absorbance of 0.5 is required as this relates to a cell number of 10^8 cfu/ml and therefore provides adequate bacterial load for further experiments. However, growth was variable between the different isolates when they were inoculated in DMEM. The overnight absorbance varied from 0.001 to 0.133 but as none of the isolates reached the minimum absorbance level required, this medium was concluded to be inadequate for supporting bacterial growth. An addition of a minimum of 10% BHI to the DMEM resulted in overnight absorbance increasing back to levels that were comparable to growth in undiluted BHI for *S. anginosus* and *S. constellatus* strains, indicating that this may be a possible growth media mix for use in the model. However, *S. intermedius* strains failed to reach sufficient growth levels in any of the concentrations of BHI tested.

As *S. intermedius* growth can be limited by iron-deficient culture medium, haemin was added in various concentrations to the media. However this also failed to produce sufficient and reproducible growth of all *S. intermedius* clinical isolates (Table 2.7).

2.3.3 Bacterial growth characteristics under modified culture conditions.

SAG isolates 45386, 39/2/14A and HW13 were used as representative strains for each species as these were found to give higher bacterial yields than others within the species or, as in the case of HW13, they provided more reproducible growth results, with absorbance levels being similar each time the growth experiments were carried out. The isolates showed standard bacterial growth characteristics when grown at 37°C and 5% CO_2 in both BHI and DMEM+10% BHI, shown in their growth curves as an initial lag followed by exponential growth until the nutrients are limited and stationery phase is reached. However there were differences in growth rate and yield between the different species and this was also effected by the media the bacteria are grown in (Figure 2.3). *S. intermedius* HW13 showed very slow growth in BHI with absorbance levels remaining below 0.2 after 7 h compared to 0.8 and 1.1 for *S. constellatus* 45386 and *S. anginosus* 39/2/14A. Growth of *S. intermedius* HW13 was further impaired when grown in DMEM+BHI with no measurable growth after 7 h. Similarly, growth of *S. anginosus* 45386 occurs at a slower rate when grown in DMEM+10% BHI compared to BHI alone and the total growth after 7 h is lower. Only *S. constellatus* 39/2/14A does not follow this trend, with growth rate and total bacterial yield being increased when grown in DMEM+10% BHI.

2.3.4 Tooth slice culture under standard conditions

Tooth slices were processed either automatically or manually prior to sectioning and staining with H&E. Following both processing methods the general morphology of the tissues could be observed with the dentine, odontoblasts and pulp clearly identifiable. Blood vessels could also be observed in the centre of the pulpal chamber (Fig. 2.4a). However it was also seen that the tissue architecture had been affected by the automatic processing as the pulp shrank and became separated from the dentine (Fig. 2.4a and b). Tears could also be seen in the dentine layer (Fig. 2.4c). As the odontoblast processes extend through the dentinal tubules these structures are required to remain in contact following culture and processing.

During manual processing tissues were dehydrated gradually using a series of graded alcohols. In these slices tissue architecture was maintained and the odontoblasts remained associated with the dentine matrix. Areas of dentine and predentine could be

seen with a clear mineralisation front and the tubular structure of the dentine was observed. The odontoblasts were seen as tall columnar cells with darkly stained nuclei at the basal end. The cell rich layer of Höhl could be seen below the odontoblast layer. The pulpal fibroblasts appeared as spindle shaped cells with darkly stained nuclei (Fig. 2.5). Upon extension of the culture period to 14 days, a similar level of tissue maintenance and cell viability was seen (Fig. 2.6).

The vitality of tooth slices was confirmed by acridine orange and ethidium bromide staining. Fig 2.7 shows the majority of cells fluorescing bright green, indicating that ethidium bromide was being actively extruded from these viable cells. Non-viable cells are unable to remove ethidium bromide from the cells and the resultant fluorescence appears orange, as seen in the control, where only a small number of viable cells were present and fluorescing green.

2.3.5 Tooth slice culture under modified culture conditions

Tooth slices cultured in DMEM+10% BHI were processed manually to avoid shrinkage caused by automatic processing. As is seen under standard culture conditions, tissue architecture and morphology is maintained for up to 24 hr culture in DMEM+10% BHI (Fig 2.8). Areas of both dentine and predentine can be observed with odontoblasts remaining as tall columnar cells, the viability of which can be seen from the darkly stained purple nuclei. Healthy pulp fibroblasts are seen as spindle shaped cells with dark purple nuclei. At higher magnifications, the densely packed odontoblasts are easily distinguishable from a healthy cell rich zone and staining of the matrix can be seen between the cells, all of which are indicators that the tooth slices are surviving in the modified medium with no deleterious effects (Fig. 2.9). This was confirmed using cell counts on the pulp and odontoblast layer which were performed using Image Pro-Plus software. Five counts were performed over a $50\mu m^2$ area for each section with five sections being studied for each time point. ANOVA tests showed no significant decrease in cell number at 4, 8 or 24 hr incubation in DMEM+10% BHI compared to a control (Fig 2.10).

Strain number	Identification by API	Identification by 16s rDNA sequencing	% homology to type strain
45386	*S. anginosus*	*S. constellatus*	94.1
350/96	*S. constellatus*	*S. constellatus*	99.9
322/95	*S. constellatus*	*S. constellatus*	99.9
447/95	*S. intermedius*	*S. anginosus*	99.7
670/95	*S. anginosus*	*S. anginosus*	99.7
39/2/14A	*S. anginosus*	*S. anginosus*	99.9
127/95	*S. intermedius*	*S. intermedius*	71.2
HW13	*S. intermedius*	*S. intermedius*	99.9

Table 2.5 Identification of clinical isolates by API and 16s rDNA sequencing. Inconsistencies indicate importance of 16s rDNA sequencing.

	BHI	DMEM	DMEM + 10% BHI	DMEM + 20% BHI	DMEM + 30% BHI	DMEM + 40% BHI
S. anginosus						
670/95	1.365	0.035	1.121	1.164	1.298	1.334
39/2/14A	1.023	0.133	1.166	1.276	1.289	1.275
447/95	1.125	0.067	0.837	1.091	1.195	1.201
S. constellatus						
350/96	1.132	0.078	0.835	0.965	1.023	1.069
322/95	1.65	0.013	0.596	0.863	1.135	1.163
45386	1.523	0.103	1.200	1.275	1.076	1.2039
S. intermedius						
HW13	1.203	0.001	0.001	0.061	0.027	0.056
127/95	1.032	0.007	0.013	0.079	0.086	0.090

Table 2.6 Bacterial growth in different media after overnight culture, measured by absorbance at 550nm.

Media	HW13 growth	127/95 growth
DMEM+10% BHI 2μM haemin	0.177	0.137
DMEM+10% BHI 4μM haemin	0.050	0.060
DMEM+20% BHI 2μM haemin	0.180	0.070
DMEM+30% BHI 2μM haemin	0.090	0.660

Table 2.7 Effect of addition of various concentrations of haemin to media on growth of *S. intermedius* isolates, measured by absorbance at 550nm.

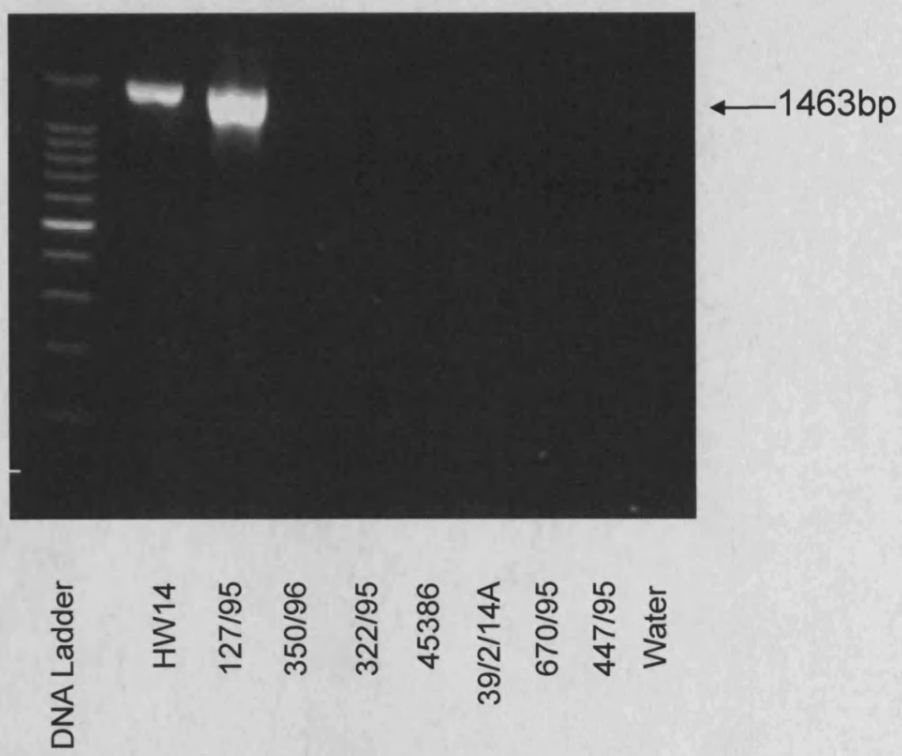

Fig. 2.2 PCR of intermedilysin gene in SAG clinical isolates. PCR confirmed presence of the gene encoding intermedilysin toxin only in isolates HW13 and 127/95 which belong to the *S. intermedius* species. No bands were visible for other strains or negative control lane.

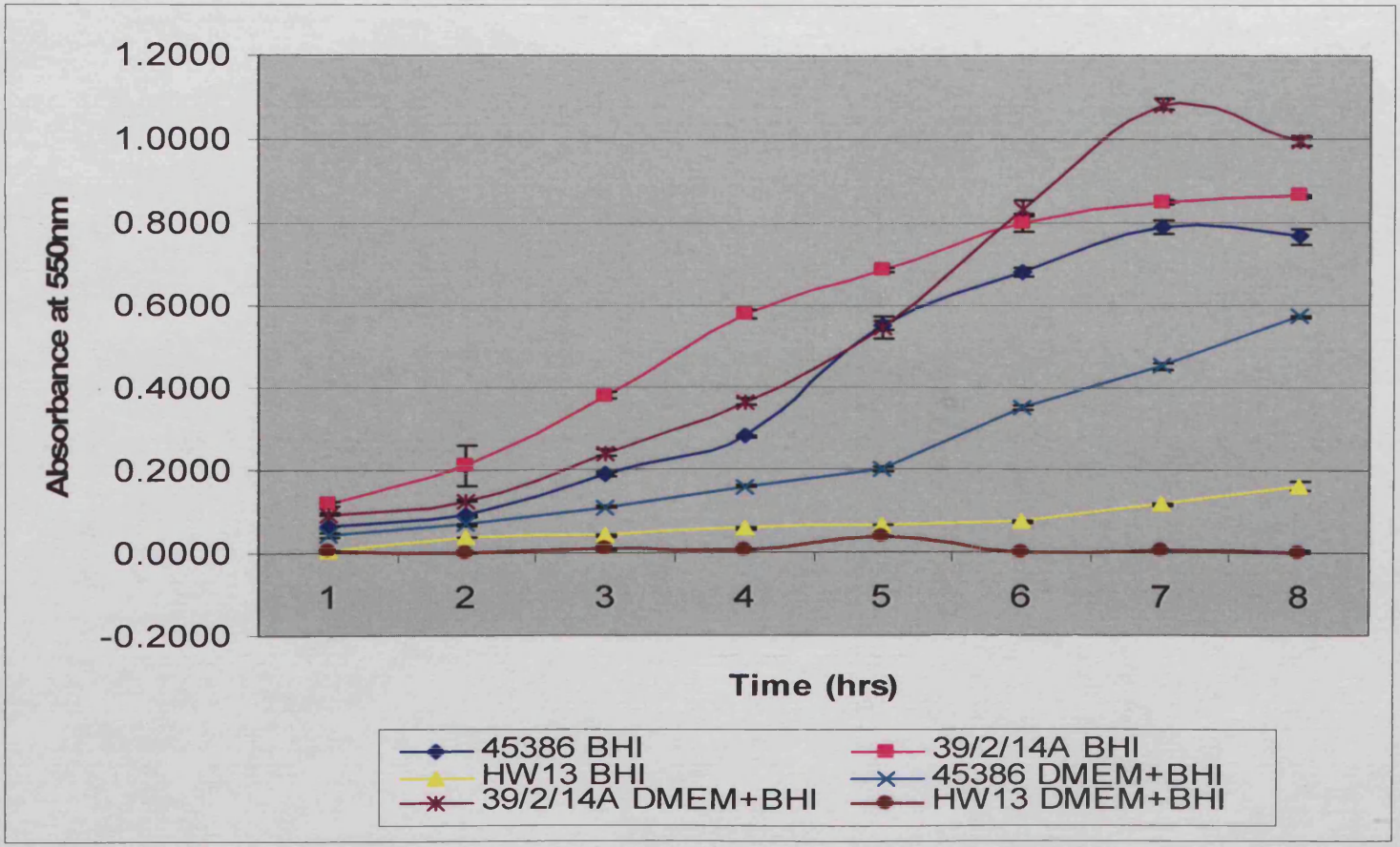

Figure 2.3 SAG isolates growth curve. Absorbance of bacterial suspension grown in BHI and DMEM+10% BHI, showing bacterial growth rates and total growth of isolates after 7 hours incubation at 37°C, 5% CO2. Error bars represent standard error.

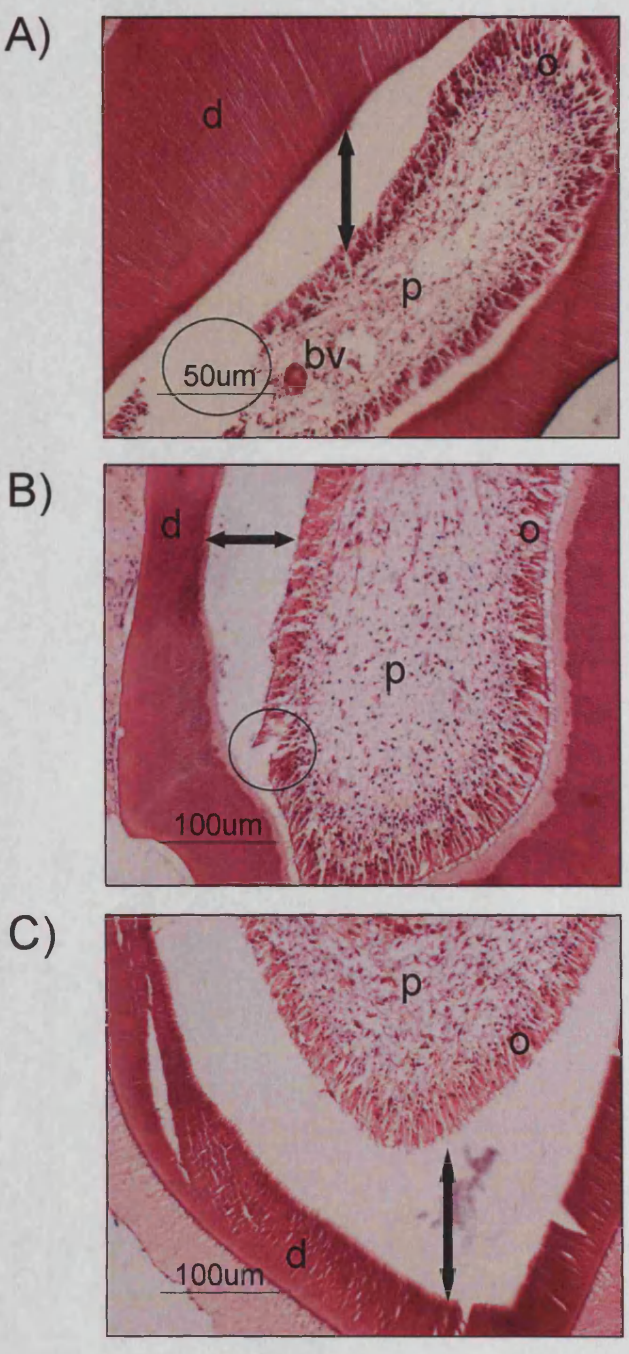

Fig 2.4 Tooth slice following automatic processing showing pulpal shrinkage and separation from dentine. A) 7 day cultured tooth slice x 20 mag. B) 7 day cultured tooth slice x 10 mag. C) 14 day cultured tooth slice x 10 mag. d = dentine, o = odontoblasts, p = pulpal cells, bv = blood vessel. Arrows indicate pulpal shrinkage away from dentine layer. Circled areas indicate tearing of the odontoblast layer.

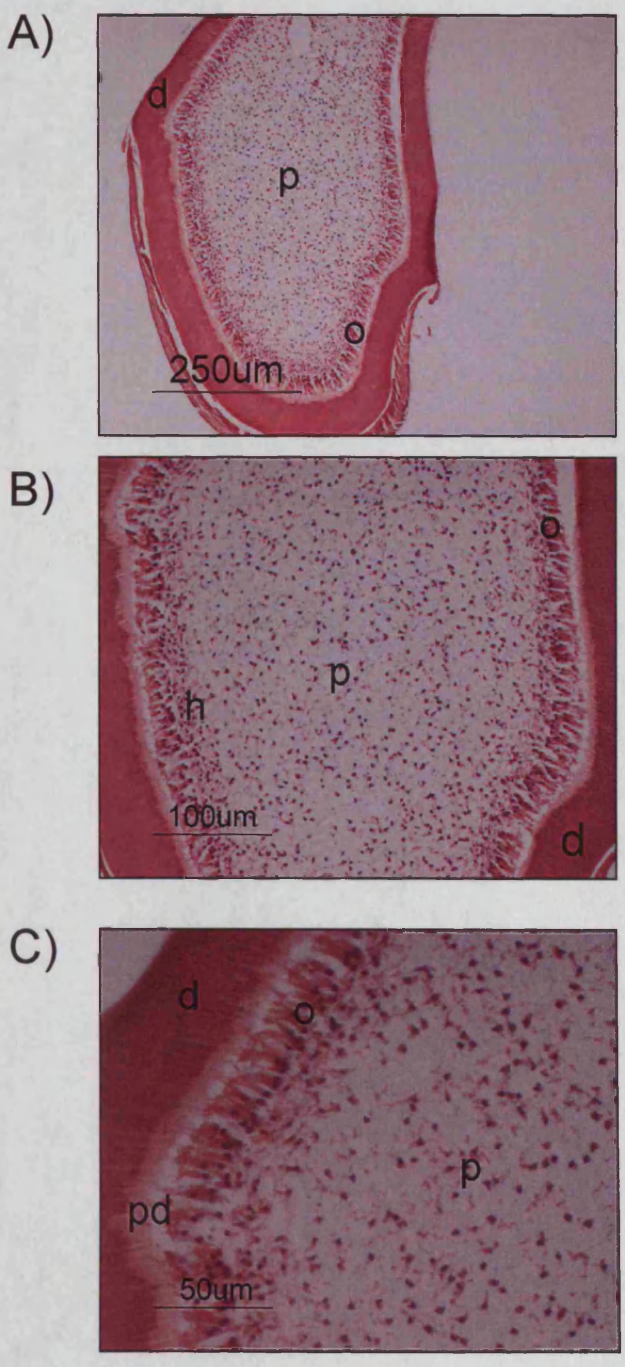

Fig 2.5 7 day cultured tooth slices following manual processing, showing maintenance of tissue architecture. A) x 10 mag. B) x 20 mag. C) x 40 mag. d = dentine, pd = predentine, o = odontoblasts, h = cell rich layer of Höhl, p = pulpal cells.

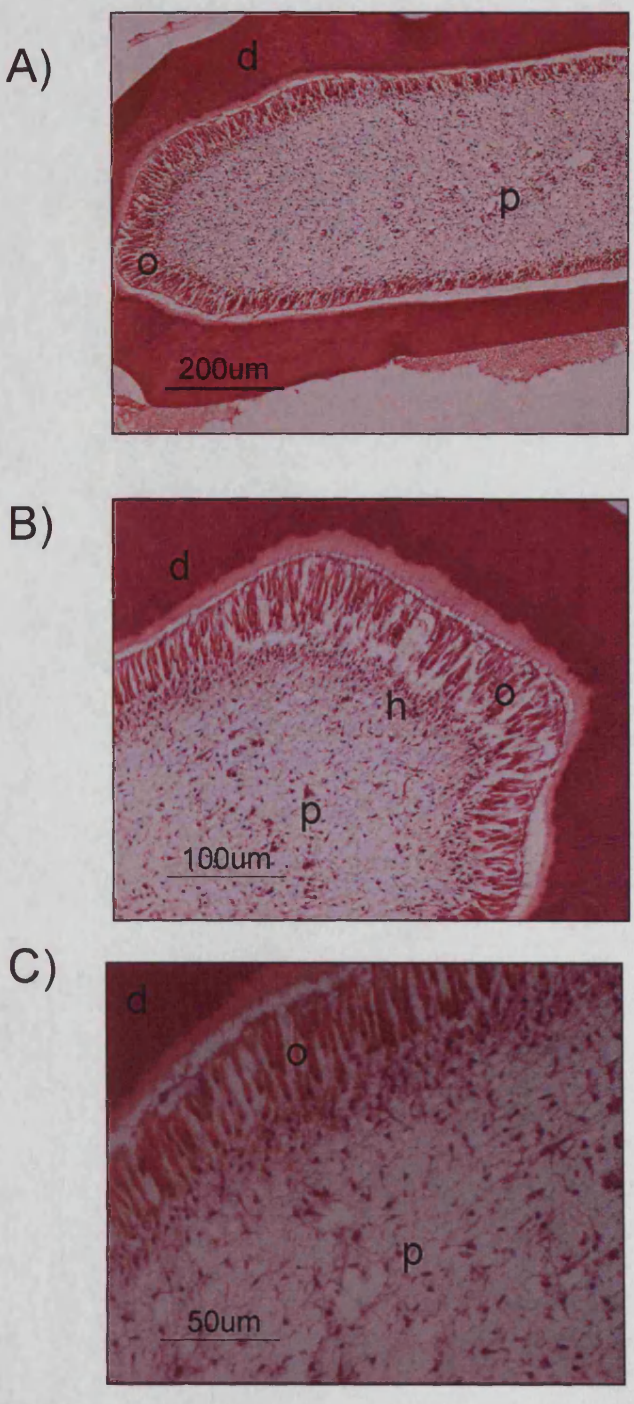

Fig 2.6 14 day cultured tooth slices following manual processing, showing maintenance of tissue architecture. A) 14 day cultured tooth slice x10 mag. B) 14 day cultured tooth slice x 20 mag. C) 14 day cultured tooth slice x40 mag. d = dentine, o = odontoblasts, p = pulpal cells.

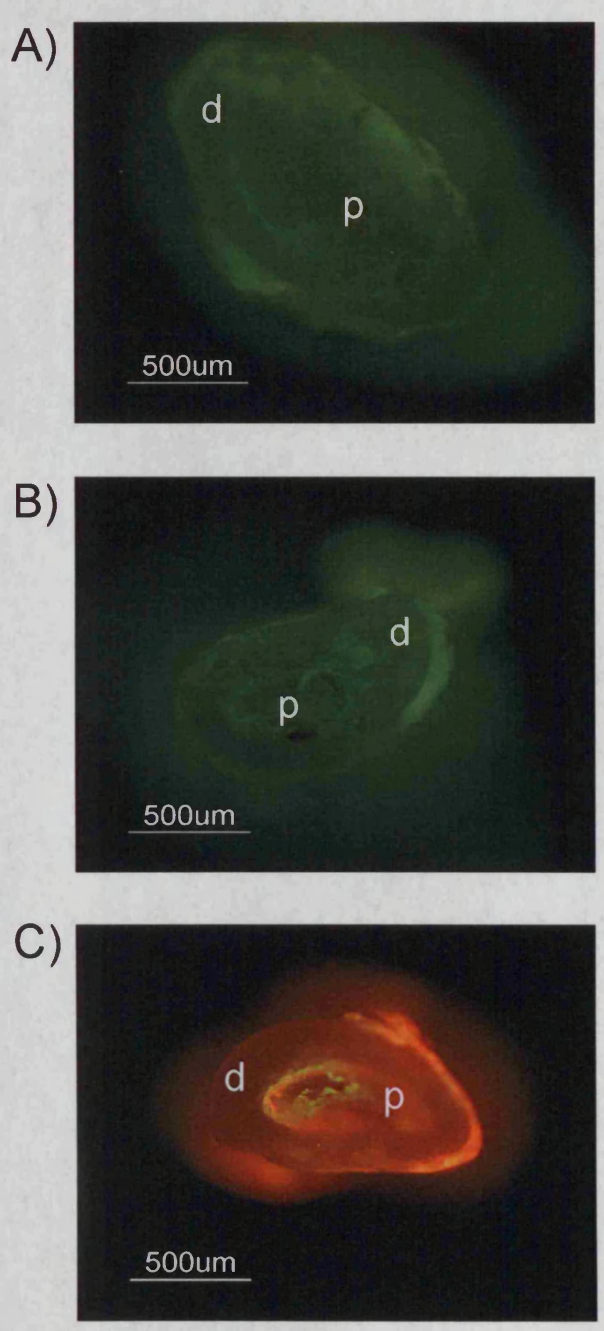

Fig. 2.7 Vital dye staining of tooth slices cultured for 7 and 14 days. Staining with acridine orange and ethidium bromide demonstrates tooth slice viability following culture under standard conditions. Green staining = live cells, red staining = dead cells. A) 7 days x 4 mag. B) 14 days x 4 mag. C) Control tooth slice represents the appearance of dead tissues and confirms presence of ethidium bromide in the stain x 4 mag. d=dentine, p=pulp.

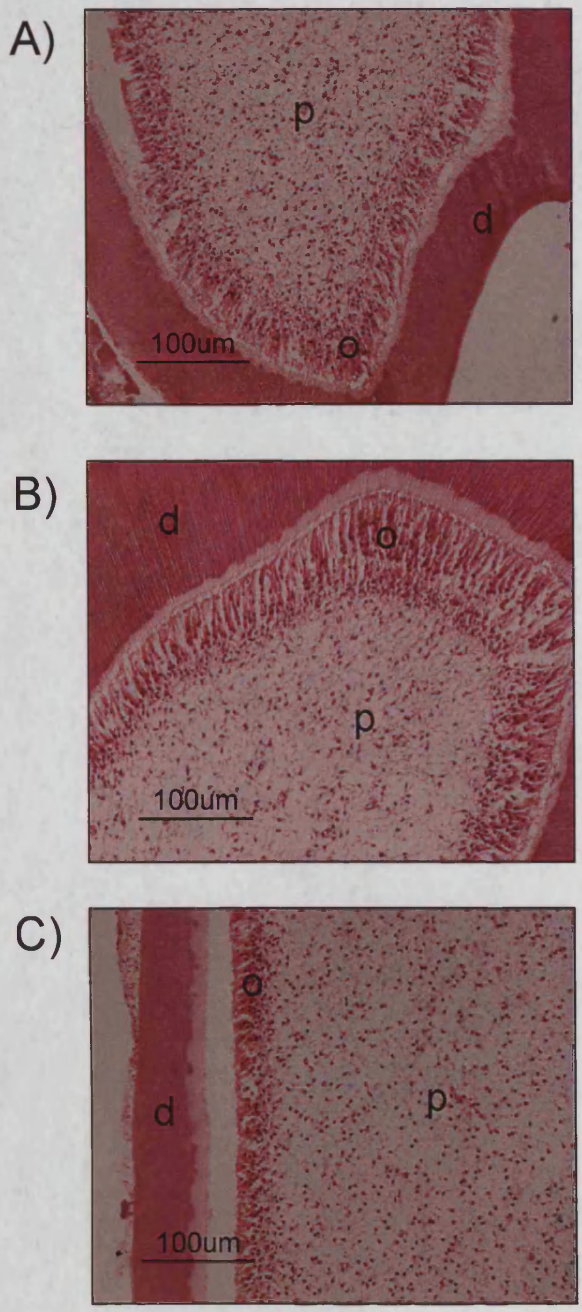

Fig. 2.8 Tooth slices cultured in DMEM+10% BHI show no significant cell death or altered tissue morphology. A) 4 hr incubation x 20 mag. B) 8 hr incubation x 20 mag. C) 24 hr incubation x 20 mag. O = odontoblasts, d = dentine, p = pulp.

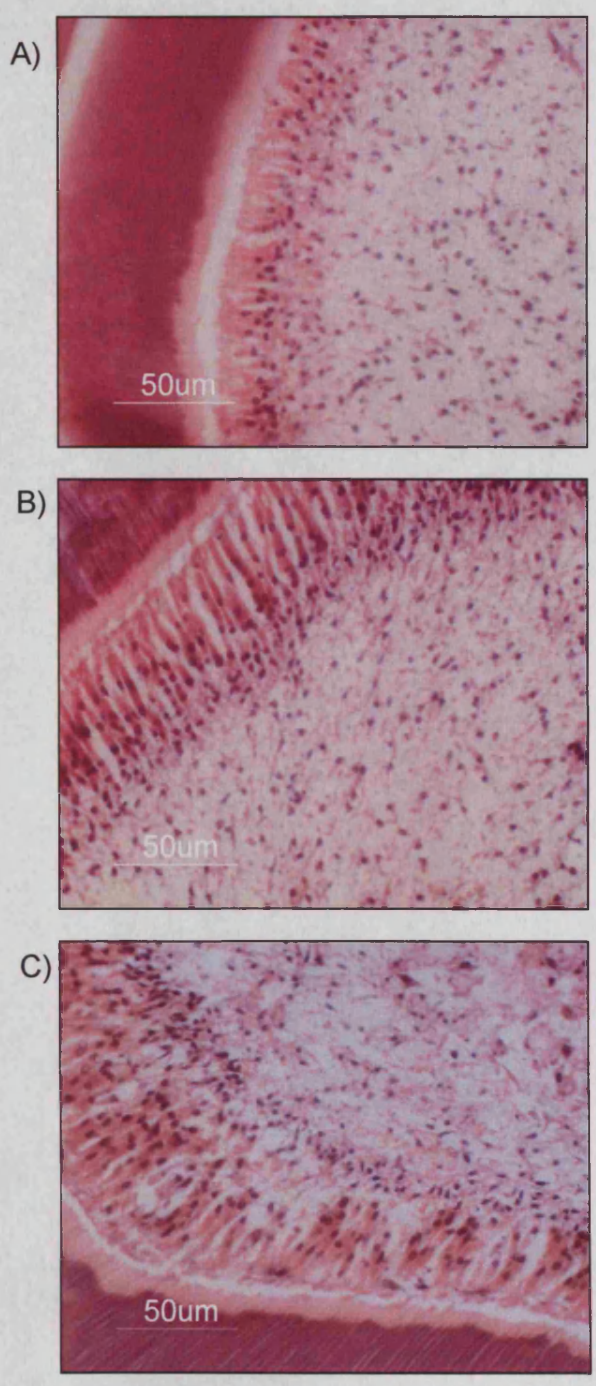

Fig. 2.9 High magnification H&E stained sections from tooth slices cultured in DMEM+10% BHI (x40 mag.). Odontoblasts are densely packed and columnar and pulpal fibroblasts are densely stained. Some thinning of the collagen matrix can be observed in the 24 hr image. A) 4 hr incubation B) 8 hr incubation C) 24 hr incubation.

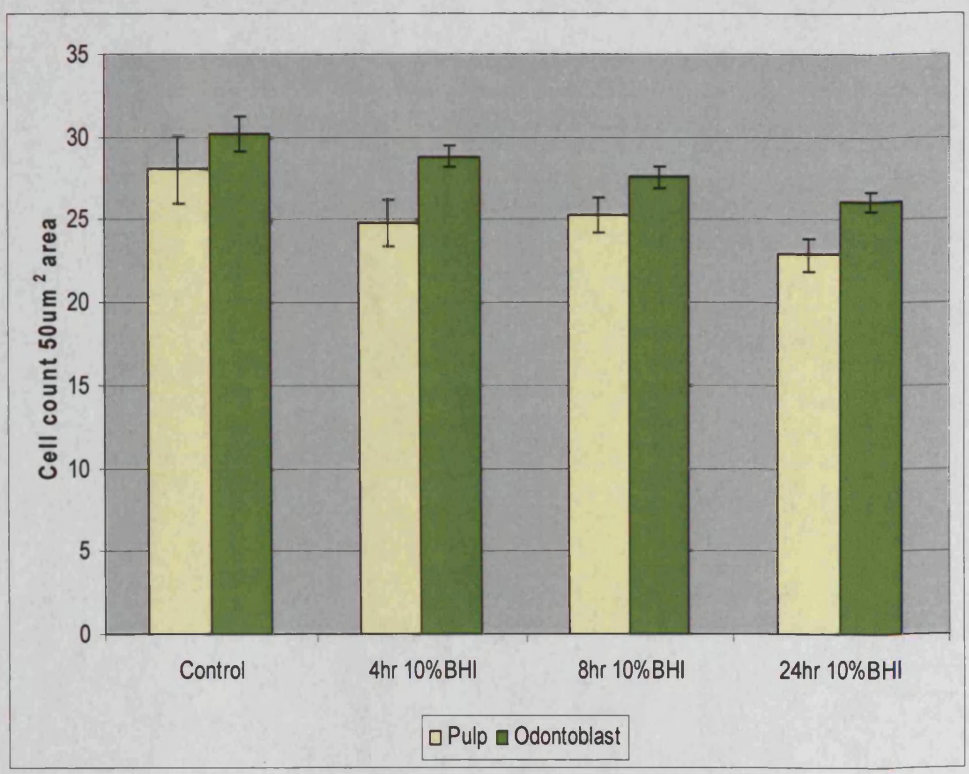

Fig 2.10 Cell count for tooth slices cultured in DMEM+10% BHI. Nuclear counts were performed to provide an average cell number in a 50μm^2 area of the pulp and the odontoblast layer. Counts indicate that culture of tooth slices in DMEM+10% BHI for up to 24hrs has no significant effect on cell number. Error bars represent standard error.

2.4 Discussion

Confirming the identity of the clinical isolates used in this study is particularly important as SAG bacteria have been incorrectly classified in the past, resulting in confusion regarding their role in oral infections. Due to changes in nomenclature and classification guidelines SAG isolates are often incorrectly classified when using Strep 32 ID alone (Facklam 2002). For this reason, 16S rDNA sequencing was used in addition to Strep32 ID. 16S rDNA of bacteria contains highly conserved regions of DNA present in all bacteria alongside highly variable regions which allow identification of different species. A universal primer which recognises the conserved regions is used to amplify the DNA which can then be identified by comparing homology with a known species (Lane et al. 1985). A homology of >99% indicates a positive species match. Identification is generally confirmed by comparison with the species type strain but due to previously mentioned problems in SAG classification, homology to the type strain may be less than 99%. This occurred in the case of strain 127/95 which showed only 71.2% homology to the type strain *S. intermedius* ATCC 27335 (GenBank accession number AF104671). However this homology was higher than that with the type strains of *S. anginosus* or *S. constellatus* and homology of 99.8% was found with sequence data from another closely related strain, *S. intermedius* (AF104672). This study also used PCR to confirm that the only SAG bacteria that produce the intermedilysin toxin are classified as *S. intermedius* strains, which concurs with results found in previous studies (Nagamune et al. 2000).

Following culture of the tooth slices under standard conditions, one of two tissue processing methods was used to prepare the tooth slices for histological sectioning and examination. Previously published work with similar model systems used only routine histological processing, taking the samples rapidly through graded alcohols and xylene (Dhopatkar et al. 2005; Smith et al. 2010). However, results from this model show that there is significant pulpal shrinkage caused by the rapid dehydration involved in standard histological processing. As the odontoblasts are shown to be healthy and viable it can be concluded that this shrinkage and subsequent separation of the pulp from the dentine occurs after fixation and is not a result of preparation or culture of the tooth slices. In order to conclude that any effects seen in later co-

cultures are caused only by the presence of bacteria it was important to eliminate these processing artefacts. As such, a second processing method was used where alcohol dehydration is performed more gradually and xylene is replaced with methyl salicylate and necloidine as these clearing agents have been found to cause less tissue shrinkage (Bucher et al. 2000). This successfully eliminated problems with pulpal shrinkage, allowing any effects observed in co-culture to be confidently attributed to the presence of SAG bacteria.

In order to develop the co-culture model it was necessary to identify conditions under which both the mammalian and bacterial cells could be successfully cultured. The gaseous environment used in standard mammalian culture conditions was introduced into bacterial incubation first, as SAG bacteria have previously been shown to respond favourably to increased carbon dioxide levels in their environment (Ruoff 1988). As expected this had no detrimental effect on bacterial growth when cultured in BHI media, as absorbance of overnight cultures remained at similar levels when measured at 550nm. However, culture of mammalian tooth slices was not possible in BHI as it does not contain the appropriate serum components and sugars to support cell growth, making it necessary to develop a media which could support the bacterial growth without disrupting the delicate balance of nutrients required to successfully culture the tooth slices. There has been no history of SAG bacteria being co-cultured with mammalian cells, the main focus of such co-culture work has centred on *Escherichia coli* and *Psudomonas aeruginosa* and the infection of monolayers rather than organotypic cultures as used in this study (Claesson and Gotthardsson 1988; Hirakata et al. 2010; Kim et al. 2010). As such, the production of an appropriate medium was particularly time consuming due to the 'trial and error' nature of its development. The process was further complicated by the differences in nutritional requirements between the different bacterial species. As there are different types of mammalian cells in the tooth slice system, having more complex nutritional requirements than the bacterial cells, the decision was taken to maintain the balances found in supplemented DMEM used in tissue culture and to adapt it for co-culture by further supplementing it with the minimum possible amount of bacterial medium. This concept was developed under the assumption that the less the medium was changed, the more successful it would be in maintaining vitality of the complex

organotypic tooth slice cultures and that this could be confirmed with further experiments when a suitable growth medium had been defined.

As an addition of 10% BHI to the DMEM was found to support the growth of the majority of the SAG clinical isolates, this medium was carried through into the next stage of experiments to assess the effect on bacterial growth rates and characteristics. Growth curves and absorbance levels of overnight cultures confirmed that it had no significant effect on growth of *S. anginosus* or *S. constellatus* species, although *S. intermedius* strains showed no growth. Culture medium was further supplemented with haemin as available iron in the culture medium is essential for the growth of *S. intermedius* and it has been shown that growth may be induced upon addition of an iron source to a deficient media (Brochu 1998). Failure of *S. intermedius* strains to grow following the addition of haemin indicates that iron is not limited in the media and that this species has different growth requirements from other SAG bacteria. For this reason, *S. intermedius* strains were omitted from further study due to complications in finding a suitable growth medium. However, the differences in growth requirements of SAG bacteria is of interest and warrants further study as this may play an important role in their ability to cause purulent infection and form abscesses *in vivo*.

The success of culturing the tooth slice in DMEM+10% BHI was assessed using Image ProPlus software to enumerate live pulpal cells after a defined time period. This involves defining parameters that represent a healthy cell and selecting an area in which to identify cells which match the characteristics set. For each time period 5 tooth slices were analysed with five 5μm sections taken from each slice. Counts were performed in 5 random fields of view covering a $50\mu m^2$ area for each section and the total counts averaged for each time period. Prior to culture in DMEM+10% BHI, tooth slices were cultured as normal in supplemented DMEM under standard conditions for 5 days. This was necessary due to damage that occurs to cells on the surface of the tooth slice during the cutting process. By day 5 of culture these cells are able to slough off, leaving the healthy tissue underneath. It is important that this period of culture is allowed before exposing the tooth slices to any other external factors that may affect tissue health in order to confidently assess the influence these factors may have. In addition, tooth slices were cultured for up to 14 days when under

standard conditions with both H&E and vital dye staining, showing no significant cell death or alteration of tissue architecture. Whilst such a long culture period is not required when incubating the bacteria with the tooth slices, this indicates that effects of culture period up to 14 days are negligible and that any changes seen when incubated with bacteria are due to their activity.

Chapter 3: Development of a Bacterial and Mammalian Co-Culture System

3.1 Introduction

During a pulpal infection, tissue destruction arises as a result of inflammation (Baume 1980; Marsh 2009). This manifests itself as clinical symptoms such as pain and swelling and if the inflammation remains unresolved it can lead to pulpal necrosis and abscess formation. Although these are widespread clinical problems the underlying mechanisms of bacterial infection are poorly understood due to a lack of adequate model systems, resulting in fewer developments in new treatment regimes.

SAG bacteria are thought to be involved in the initial infection of the pulp and may play an important role in the environmental changes that occur in the pulpal chamber, allowing further colonisation by other bacterial species that are prevalent during pulpal infection and abscess formation (Sundqvist 1992; Sundqvist et al. 2003). SAG bacteria and other facultative anaerobes predominantly ferment carbohydrates to obtain energy. As the infection advances further into the root canal such nutrients become less easily available as the direct contact with the oral cavity is lost. In addition to the change in nutrients available, oxygen availability also decreases as the infection advances, creating perfect conditions for anaerobic pathogens to thrive. Targeting antimicrobials towards SAG bacteria may enable the clinician to limit further infection by other bacterial species. Therefore these bacteria were chosen for use in the model system developed in this study. As the model comprises dental tissues *in situ* and is sterile prior to the introduction of SAG bacteria, it is representative of an initial pulpal exposure to the oral cavity whilst having the advantage of studying the effects of an individual species.

As SAG bacteria have not been studied in such a model before it was important to first establish how the bacteria interacted with the pulpal tissues; whether they attached to the pulp or dentine or simply secreted proteins that affected the tissues. Bacteria can adhere to the surfaces of cells and tissues and interact directly with them directly, which is common when bacteria move into the dentinal tubules of the tooth and when they attach to the enamel surface (Love et al. 1997; Yamaguchi 2004).

However, as the dentinal tubules and pulpal chamber are filled with fluid around the cells and their processes it is possible for the bacteria to release endotoxins into the fluid which diffuse through the pulp and cause damage to cells that have no direct contact with the bacteria (Love and Jenkinson 2002). To effectively target oral pathogens it is important to first elucidate their mode of action. This was achieved through use of both fluorescence and histological staining on vital and devitalised pulp tissue.

Although it is known that SAG and other bacteria cause generalised inflammation and subsequent tissue destruction within the pulp, nothing is known about the specific patterns of this infection process. For the validation of this model and its future use in testing antimicrobials it is important to know how the bacterial and mammalian cells interact. The response of the mammalian tissues to infection by SAG must be assessed in order to evaluate the effect of bacterial attachment on cell death and expression of inflammatory markers such as TNF-α, IL-1β and IL-6. These are pro-inflammatory cytokines which are expressed by mammalian cells in response to the presence of bacterial antigens. TNF-α and IL-1β are produced by activated monocytes to recruit other cells of the immune system to the site of infection to fight the bacterial invasion (Hahn and Liewehr 2007a, b; Stashenko et al. 1998), IL-6 is involved in the production of neutrophils from the bone marrow which then may be recruited to the site of infection (Hahn and Liewehr 2007b). These and other cytokines play an important role in pulpitis and are sometimes used to establish the clinical severity of an infection (Kokkas et al. 2007). Measuring their expression in the model enables data to be extrapolated to the clinical condition and to determine how effective treatments may be on limiting inflammation caused by bacterial invasion.

The effect of the bacterial infection on the tissues of the tooth slice was visualised through histological and vital dye staining which also enabled patterns of bacterial attachment to be observed. RT-PCR was used to investigate the expression of pro-inflammatory cytokines in pulpal cells from both sterile and infected tooth slices. This gives an indication of the processes which occur in the host tissues as a response to bacterial invasion into the pulpal chamber.

3.2 Methods

3.2.1 Co-culture of SAG and devitalised pulpal matrix

3.2.1.1 SAG growth on devitalised pulpal matrix

Protocol 1 – Co-culture of SAG bacteria and unsterilised pulpal matrix

Upper and lower incisors were dissected from 28 day old male Wistar rats and pulp was extirpated using a sterile 21 gauge disposable needle. Three pulps were pooled and placed into the bottom of a six well tissue culture dish prior to three freeze-thaw cycles (-80°C overnight, room temperature 30 min) to devitalise the pulpal matrix. An overnight culture of *S. anginosus* 39/2/14A was prepared in BHI broth. One ml of this culture was transferred to 9ml of fresh broth and further incubated for 4 h at 37°C, 5% CO_2. The suspension was then diluted to Ab_{550nm}=0.5 which had been previously calculated to contain 10^8 cfu/ml bacteria. The suspension was further serially diluted to 10^2 cfu/ml before being incubated with the devitalised matrix for 24 h at 37°C, 5% CO_2, 60 rpm.

Due to the problems associated with extracting large amounts of pulp from the rat teeth only one SAG isolate was used for this experiment. *S. anginosus* 39/2/14A was chosen as this isolate had been shown to have the most successful growth in BHI (Fig. 2.2).

Following incubation, the bacterial suspension was aspirated and pulpal matrix transferred to a glass slide for staining with BacLight LIVE/DEAD. Samples were viewed immediately after staining by UV microscopy. The aspirated bacterial suspension was plated out on FAA and incubated aerobically and anaerobically.

Protocol 2 – Co-culture of SAG bacteria and sterilised pulpal matrix

As previously described in protocol 1, pulp was extirpated from the incisors of 28 day old male Wistar and pooled in six well tissue culture dishes. Following devitalisation by freeze-thawing, pulps were decontaminated by a wash step in 70% ethanol for two h and exposure to UV irradiation for 10 min. Pulpal matrices were then cultured as previously described in protocol 1.

3.2.1.2 Identification of bacterial species isolated from pulpal matrix post-culture.

Aspirated bacterial suspension from cultured pulpal matrix was plated out on FAA plates which were incubated anaerobically or aerobically. Resulting bacterial growth was initially identified as SAG isolates if colonies appeared smooth, pale yellow in colour and around 0.5 mm in diameter, as seen in the colony morphology of the isolates used to inoculate the broth. The colonies also produce a characteristic caramel-like odour. The identity of all isolates, including those which did not appear to be SAG bacteria, was confirmed using 16S rDNA sequencing, as previously described (**2.2.2.2**).

3.2.1.3 Enumeration of bacterial cells attached to devitalised matrix

Following culture for 4, 8 or 24 h as previously described (**3.2.1.1**), pulpal matrix was removed from the culture dish and macerated using a sterile scalpel blade. The remaining matrix and associated bacterial cells were re-suspended in 1ml of PBS and vortexed for one min. This suspension was serial diluted to 1 x 10^{-6} and a 50 µl sample of each serial dilution spiral plated on FAA. Plates were incubated anaerobically for 72 h prior to colony counting. Counts were taken from three dilutions for each condition and the average obtained to give the count after each incubation period.

3.2.2 Co-culture of SAG bacteria and vital tooth slices

Upper and lower incisors were dissected from 28 day old male Wistar rats and cut into 2 mm thick transverse sections, as previously described (**2.2.5**). Tooth slices were cultured in DMEM supplemented with 10% heat inactivated foetal calf serum and 0.15 mg/ml vitamin C for a minimum of 5 days before further culture in DMEM+10%BHI containing SAG clinical isolates.

An overnight culture of *S. constellatus* 45386 or *S. anginosus* 39/2/14A was prepared in DMEM+10%BHI broth. One ml of this culture was transferred to 9 ml of fresh broth and further incubated for 4 h at 37°C, 5% CO_2. The suspension was then diluted

to 10^2 cfu/ml before being incubated under agitation with the tooth slices for 4, 8 or 24 h at 37°C, 5% CO_2, 60 rpm. Control tooth slices were cultured in sterile DMEM+10% BHI for the co-culture period. In all conditions tooth slices were cultured in a volume of 2 ml of media or bacterial suspension.

Following culture, tooth slices were fixed in 10% neutral buffered formalin overnight demineralised in formic acid for 72 h under constant agitation and manually processed or stained with acridine orange and ethidium bromide, as previously described (**2.2.6**). Tooth slices which were manually processed were stained with H&E and histological sections were examined by light microscopy. Nuclear counts were performed over 50 μm^2 areas of the pulp and odontoblast layer, as previously described (**2.2.7**).

3.2.3 Fluoroscein Diacetate (FDA) staining of SAG bacteria

Bacteria were prepared for inoculation of the tooth slices system in DMEM+10% BHI as previously described (**3.2.2**). One gram of FDA (Sigma F7378) was dissolved in 100 ml of acetone to give a 1% solution (w/v). Following dilution of the bacteria to 10^2 cfu/ml, 20 µl of 1% FDA in acetone was added for every millilitre of bacterial suspension. The bacteria were then incubated at room temperature in the presence of the FDA stain for 30 min before being filtered using a 0.2 µm cellulose acetate disposable filter (VWR, Leicestershire, UK). Bacteria captured in the filter were then re-suspended in sterile DMEM+10% BHI and used to inoculate the tooth slice system. In the control condition sterile media was used in place of bacterial suspension. Tooth slices were incubated for 4, 8 and 24 h in inoculated or sterile media before they were transferred to formalin for 24 h for fixation. Tooth slices were fixed, demineralised and manually processed as previously described (2.2.6). Sections were cut and viewed under UV microscopy.

3.2.4 Expression of inflammatory markers in healthy and infected pulpal tissue

3.2.4.1 RNA Extraction

Tooth slices were cultured as previously described in supplemented DMEM for 5 days before being transferred to sterile DMEM+10% BHI or a suspension of *S.*

constellatus 45386 in DMEM+10% BHI at a concentration of 10^2 cfu/ml. The tooth slices were further cultured for 24 h before pulps were extirpated and pooled in 350 µl RLT lysis buffer from the RNeasy™ mini kit (Qiagen Ltd, Crawley, UK) containing 10% β-mercaptoethanol (Sigma M3148). Tissue was homogenized using a rotot-stator homogeniser followed by passing through a QIAshredder spin-column (Qiagen Ltd, UK) by centrifugation at 13,600g for 2 min. Isolation of total RNA was then carried out using a Qiagen RNeasy mini kit (Qiagen Ltd, UK) which consisted of RW1 and RPE wash buffers and spin columns to bind the mRNA. An equal volume of ethanol was added to the lysate collected from the QIAshredder spin-column and this was then added to an RNeasy spin column for centrifugation at 12,000 g. The mRNA attached to the membranes within the spin column whilst other cell components passed through the membranes and were discarded. 350 µl of RW1 wash buffer was added to the column prior to another centrifugation step at 12,000 g for 30 s. Ten microlitres of DNase enzyme (Qiagen) were then added to the column to digest any traces of DNA that may have been contaminating the sample. The sample was then incubated at room temperature for 15 min before repeating the RW1 wash step. This was followed with two washes with 500 µl of RPE buffer which were carried out by centrifugation at 12,000 g for 30 s each. Finally, mRNA was eluted by adding 40 µl of sterile water and centrifuging at 12,000 g for 1 min. All reagents were stored on ice throughout the experiment and steps were performed rapidly at room temperature to minimise RNA degradation.

3.2.4.2 Reverse transcription

Extracted RNA was converted to cDNA prior to PCR reaction. This was carried out in a sterile environment at room temperature with all reagents stored on ice.

250 ng of extracted RNA was added to a sterile 0.25 ml PCR tube with 1 µl of random primer and made up to 15 µl sterile water. This was then incubated at 70°C for 5 min in a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd, Essex, UK). Following cool down this was then stored on ice whilst a reverse transcription master mix was prepared. The master mix consisted of 5 µl of 5x MMLV reaction buffer (Promega), 1.25 µl dNTPs, 0.6 µl RNasin (Promega), 1 µl

MMLV enzyme (Promega) and 2.15 µl sterile water for each reaction. 10 µl of master mix was added to the RNA/primer mix and incubated at 37°C for 1 h in the thermal cycler to convert the RNA to cDNA. cDNA was then stored at -20°C until required for PCR reactions.

3.2.4.3 Polymerase Chain Reaction (PCR)

PCR was performed on cDNA from both healthy and infected tooth slices to amplify the genes for tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6. These cytokines were chosen as they are widely documented as playing an important role in inflammation. The rat housekeeping gene β-actin was used as a positive control.

1 µl of cDNA products and RT negative control were added to a PCR mix containing primers for amplification of one of the aforementioned gene sequences. Primer sequences are listed in table 3.1. PCR mix was prepared on ice and consisted of 10 µl GoTaq flexi buffer (Promega), 3 µl 1.5 mM magnesium chloride, 1 µl 0.2 mM dNTPs, 0.5 µl GoTaq DNA polymerase (Promega) and 2.5 µl of forward and reverse primers at a concentration of 0.04 µg/µl. The volume of the reaction mixture was made up to 50 µl with nuclease free water. Reactions were run on a G-storm™ GS1 thermal cycler (Genetic Research Instrumentation Ltd) with an initial denaturing step of 95°C for 5min, followed by 30 cycles of a 1 min 95°C denaturing step, a 1 min 56°C annealing step and a 1 min 72°C extension step. A final extension step at 72°C for 5 min was run, ending the reaction. All PCR products were stored at 4°C until visualisation.

PCR products were visualised on TBE agarose gels containing ethidium bromide. 5x TBE buffer containing 0.5 M tris-base (Sigma Aldrich), 0.5 M boric acid (Sigma Aldrich), 0.03 M EDTA (Sigma Aldrich) with a pH of 8.1 was used to make agarose gels and was also used as a running buffer. The TBE buffer was diluted 10 times to a concentration of 0.5x prior to use. To prepare the gel, 1g of agarose (Sigma) was added to 100 ml of the diluted TBE buffer and heated in a microwave for approximately 45 s prior to the addition of 5 µl of 10 mg/ml ethidium bromide

(Promega). The agarose solution was immediately poured into a casting tray and left to cool.

10 µl of each PCR product were loaded into wells on the agarose gel. A molecular marker was run in the first well, which consisted of 7 µl of a 100bp DNA ladder (Promega). The loaded gel was run in 0.5x TBE buffer at 120 mA for 45 min. Gels were removed from the casting tray and placed on a Gel Doc™ scanner (Bio-Rad, Hemel Hempstead, UK) for visualization by UV light. Images were captured using Quantity One image analysis software (Bio Rad) and band intensity was measured using Image Pro-Plus version 6.0.0.260 image analysis software (Media Cybernetics Inc. Bethesda, MD).

Primer	**Sequences 5'-3'**	**Source**
TNF-α	F:CCAGCGTGCCAACGCCCTCCTGGCCAAT R:GGGGTCAGAGTCGGGGACAGGGGCTGGG	SigmaGenosys primer design
IL-6	F:CCGCAAGAGACTTCCAGCCAGTTGCCTT R:TGGATGGTCTTGGTCCTTAGCCACTCCT	SigmaGenosys primer design
IL-1β	F:GCCCGTGGAGCTTCCAGGATGAGGACCC R:CTCCAGCTGCAGGGTGGGTGTGCCGTCT	SigmaGenosys primer design
β-actin	F:TGAAGATCAAGATCATTGCTCCTCC R:CTAGAAGCATTTGCGGTGGACGATG	Gatto *et al.* 2008

Table 3.1 Primer sequences for PCR

3.2.5 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 (**2.2.7**). One-way analysis of variance (ANOVA) was performed to determine the relative significance of the difference between experimental cell counts and the controls. Tukey-Kramer's test was used in conjunction with ANOVA to compare the significant difference between all possible pairs of means. This allowed comparison of the mean cell counts between tooth slices exposed to different bacterial species, in addition to comparison with the control.

3.3 Results

3.3.1 Co-culture of SAG bacteria and devitalised pulpal matrix

3.3.1.1 SAG growth on devitalised pulpal matrix

Protocol 1 – Co-culture of SAG bacteria and unsterilised pulpal matrix

SAG bacteria were found to attach to the surface of devitalised pulpal matrix after 24 h incubation at 37°C, 5% CO_2 (Fig. 3.1). Baclight LIVE/DEAD stain was used primarily for the staining of bacteria but also stains the remnants of the pulpal cells in the matrix which were seen in the control tissues as oval shaped cells fluorescing red, indicating that they were no longer vital due to the freeze-thaw cycles (Fig 3.1C). Bacterial and mammalian cells could be distinguished on the basis of size as mammalian fibroblasts are typically around five times larger than *Streptococcus spp.* cells. At x20 magnification the bacteria appeared as green fluorescence extending across the pulpal matrix (Fig 3.1A), with a red fluorescence from the pulpal cells beneath showing through in areas where bacteria have not grown across the matrix. These areas were more pronounced at higher magnifications (Fig 3.1B), where the dead pulpal cells can be clearly seen through the bacterial lawn, indicating intermittent areas of sparse and dense bacterial attachment and growth.

The bacterial lawns seen on the pulpal matrix using this method appeared to consist of different bacterial types. In Fig. 3.1A the black circle indicates cocci shaped bacteria which are typical of SAGs, whilst the white circles indicate the presence of bacilli, which appear as groups of rod-shaped green fluorescence. This suggests that there is contamination from other bacterial species.

Protocol 2 – Co-culture of SAG bacteria and sterilised pulpal matrix

SAG bacteria formed a more dense bacterial lawn when grown on previously sterilised pulpal matrix, with bacteria appearing more closely associated with each other and the majority of the pulpal matrix being covered by bacterial growth. This was seen as bright green fluorescence across the pulpal matrix with minimal red fluorescence from dead pulpal cells showing through (Fig. 3.2). When the matrix was viewed at low magnifications (x20), to observe the attachment of the bacteria across

the whole sample, only small areas of the pulp were visible between the areas of bacterial growth (Fig. 3.2a). In contrast, when the bacteria were incubated with pulpal matrix which had not been sterilised there were extensive breaks between the areas of bacterial growth (Fig. 3.1a). Even at higher magnifications (Fig 3.2B), where breaks in the bacterial lawn were more apparent, the enhanced growth of the SAG bacteria on sterilised pulp was evident, with red fluorescence from pulpal cells almost completely absent. This indicates that the presence of pulpal contaminants in untreated pulp may have an adverse effect on the growth of SAG bacteria introduced into the system. Pulpal contaminants compete with the SAG bacteria for nutrients and space for attachment so when they are eradicated this provides a more beneficial growth environment for the SAG bacteria.

3.3.1.2 Identification of bacterial species isolated from pulpal matrix post-culture.

The FAA plates inoculated with the post-culture media from protocol 1 showed extensive growth of bacteria that appeared to be a mixture of SAG bacteria and a number of unidentified isolates. This was seen on the plates as colonies which matched the morphology of SAG isolates as previously described (**3.2.1.2**) growing amongst other colonies of mixed morphologies distinct from those of the SAG bacteria. All plates inoculated with post-culture media from protocol 2 showed only pure growth of SAG bacteria. 16S rDNA sequencing was performed to identify the isolates (Table 3.2). This confirmed that the colonies appearing to be SAG bacteria were formed from the strain with which the system had been inoculated and the sequence data matched that which had been previously recorded (**2.2.2.2.3**). The contaminants that grew on the matrix were found to consist of species that would be expected to be found as commensals on rats, e.g. *Escherichia coli, Streptococcus thoraltensis* and *Enterococcus faecalis*, found in the environment the rats have been caged in, e.g. *Bacillus subtilis,* or to have come from the dissection environment e.g. *Staphylococcus aureus.*

Species	Accession number of type strain	% homology to type strain
Escherichia coli K12	U00096	100
Streptococcus thoraltensis S69	109007	99.3
Staphylococcus sciuri DSM	20345	99.7
Bacillus subtilis 168	Z99104	100
Staphylococcus aureus MSSA476	BX571857	100
Enterococcus faecalis V583	AEO16830	100
S. anginosus 39/2/14A	AF104678	99.9

Table 3.2 Bacterial species isolated from unsterilised pulp following culture with SAG bacteria.

3.3.1.3 Enumeration of bacterial cells attached to devitalised matrix

The number of bacteria recovered from the pulp increased with increased incubation time (Fig 3.3). The bacterial numbers rose from 2.1 x 10^6 cfu/ml at 8 h to 1.9 x 10^7 cfu/ml at 24 h, which ANOVA statistical analysis indicated was significant ($P<0.05$). A significant rise in bacterial numbers recovered from the pulp suggests that in addition to bacterial attachment to the pulp there was also bacterial reproduction occurring, as the pulp was only initially inoculated with 10^2 cfu/ml.

3.3.2 Co-culture of SAG bacteria and vital tooth slices

3.3.2.1 Histology and viability staining

Following co-culture with *S. anginosus* 39/2/14A and *S. constellatus* 45386 for 4 h, tooth slices showed few signs of tissue damage with the majority of cells still appearing histologically normal and comparable to control sections (Fig 3.4 and 3.5). Odontoblasts were columnar with basal nuclei and remained in densely packed layers in contact with the dentine layer whilst pulp cells were darkly stained with round nuclei and staining visible in the surrounding matrix. However, histological sections

of tooth slices inoculated with *S. anginosus* 39/2/14A showed evidence of cocci shaped organisms attached to the pulp in small clumps that were stained pink by the H&E (Fig 3.5A).

After 8 h of incubation a greater number of bacteria appeared to be attached to the pulp as the foci of bacterial attachment increased in size (Fig 3.6 and 3.7) and an associated breakdown of the surrounding collagen matrix was apparent. This matrix degradation was seen in the photomicrographs as clear areas between cells where there had previously been staining and is particularly pronounced around areas of bacterial attachment (Fig 3.6b and 3.7b). This indicates that the bacteria may be producing enzymes which digest the matrix, enabling their growth and reproduction.

After 24 h of incubation *S. constellatus* 45386 showed extensive attachment to the pulp in localised areas, with a noticeable decrease in viable pulpal cells (Fig 3.8). However, there were still a large number of viable odontoblasts surrounding the pulp and cell death within the pulp appeared much less extensive than that which occurred with *S. anginosus* 39/2/14A (Fig 3.9). With this isolate there is much less attachment seen but the pulpal matrix is completely disintegrated with almost no remaining viable cells (Fig 3.9b). Cell death can also be seen to have extended into the odontoblast layer (Fig 3.9a).

Viable cell counts were performed using Image Pro-Plus software to count the average number of viable cells in a 50 μm^2 area at each time point. Counts were performed on cells in the pulp and odontoblast layer. The results from the counts in the pulp showed a trend of decreasing cell number with increasing incubation time with SAG bacteria when compared to the controls (Fig. 3.10), which supports the observations made from studying the histological sections. Also, the observation that *S. anginosus* 39/2/14A appears to cause cell damage to the tooth slice more rapidly than *S. constellatus* 45386 was supported by the statistical analysis of the cell counts. These showed that after 4 h incubation with *S. constellatus* 45386, whilst there was a decrease in cell number compared to the control, it was not statistically significant. However, after 4 h incubation with *S. anginosus* 39/2/14A there was a significant decrease in cell number ($P=0.0057$), indicating that this strain is more efficient at rapid colonisation and infection of the dental tissues.

Cell counts in the odontoblast layer confirmed that, as seen in the histological examination of the tooth slices, these cells remained unaffected by the presence of SAG bacteria for up to 24 h of incubation (Fig. 3.11). Incubation with *S. constellatus* 453856 showed no significant decrease in odontoblast number after the maximum incubation time of 24 h, despite the significant decrease in pulpal cells at this time point. Similarly, there was no significant cell death in the odontoblast layer after 8 h incubation with *S. anginosus* 39/2/14A and the decrease in cell number at 24 h has a higher *P* value than that observed in the pulpal layer, indicating that there is less significance to this decrease than that seen in the pulpal region.

ANOVA analysis showed that after 8 and 24 h of incubation both strains caused a significant amount of cell death in the pulp ($P<0.0001$). Tukey-Kramer's test was used to compare the means of all possible pairs and indicated there were no significant differences in cell counts between tooth slices incubated with different bacterial species.

Viability was related to cell counts and histology by staining co-cultured tooth slices with acridine orange and ethidium bromide, as seen in Fig. 3.12. After 4 h (Fig. 3.12a) there was very little red staining visible, indicating that after 4 h of infection with SAG bacteria there were minimal dead cells. There was an increased amount of red staining seen when the tooth slices were incubated with bacteria for 8 h, relating to an increased number of dead cells being present on the surface of the tooth slice (Fig. 3.12b). There was almost no green fluorescence visible on the surface of the tooth after 24 h incubation with the bacteria (Fig. 3.12c), indicating almost total cell death. These experimental findings confirm that the viability decreases with increasing exposure to the bacteria and reflect the results seen in histological sections.

3.3.2.2 FDA staining

FDA staining of bacteria was used to confirm that the bacteria were attaching to the tooth slices as observed in the histological examination of the sections. Sections

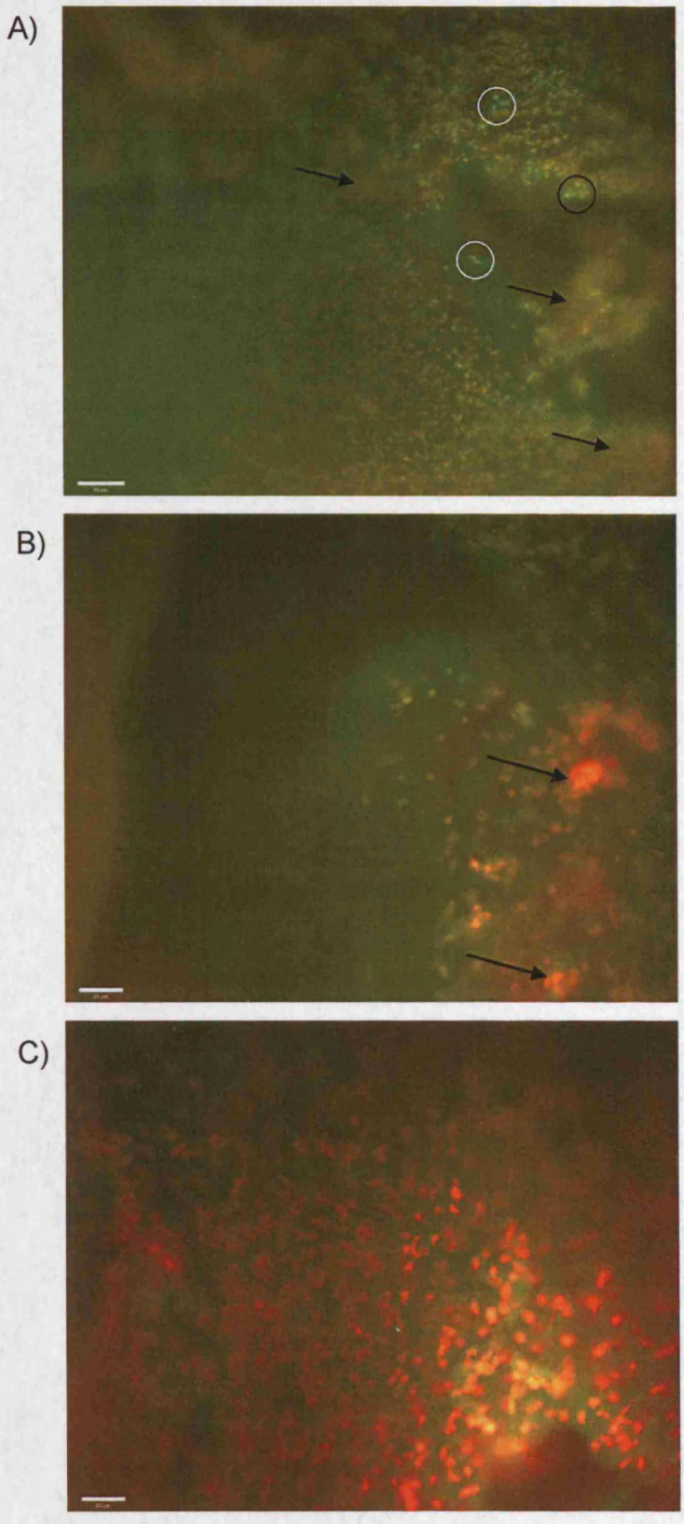

Fig. 3.1 UV microscopy of *S. anginosus* 39/2/14A on unsterilised devitalised pulpal matrix. Baclight LIVE/DEAD stain results in live cells fluorescing green and dead cells fluorescing red. A) x20 mag. White circles indicate rod-shaped bacteria and black circles indicate groups of cocci. Arrows point to areas of red fluorescence from pulpal cells of the matrix where no bacterial lawn has grown. B) x40 mag. Arrows point to areas of red staining which is caused by dead pulpal cells. These are around ten times larger than bacterial cells C) x40 mag. of control pulp with no bacteria.

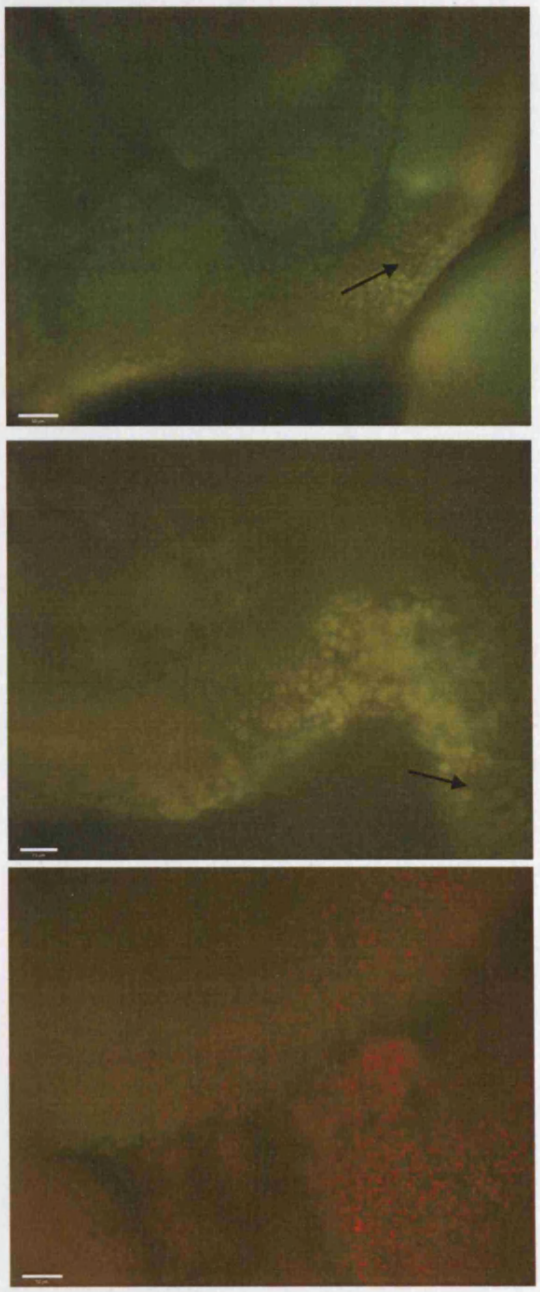

Fig. 3.2 UV microscopy of *S. anginosus* 39/2/14A on sterilised devitalised pulpal matrix. Baclight LIVE/DEAD stain results in live cells fluorescing green and dead cells fluorescing red. A) x20 mag. Arrow indicates one small area where bacterial lawn is absent and red fluorescence from pulpal cells can be seen. B) x40 mag. Arrow indicates red fluorescence from pulpal cells. C) x20 mag. of control pulp with no bacteria.

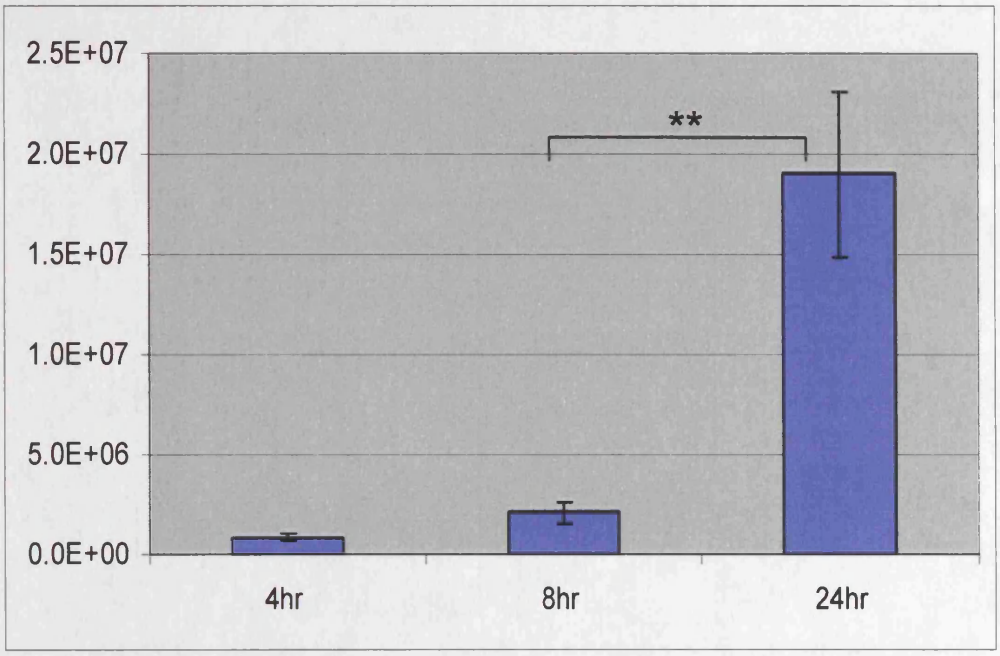

Fig. 3.3 Bacterial numbers recovered from devitalised pulpal matrix after 4, 8 and 24 hrs incubation with SAG bacteria in BHI. Bacterial load is significantly increased at 24 hours, indicating bacterial growth has occurred on the matrix. Error bars represent standard error. ANOVA statistical analysis was used to determine significance. **=significant, $P<0.01$.

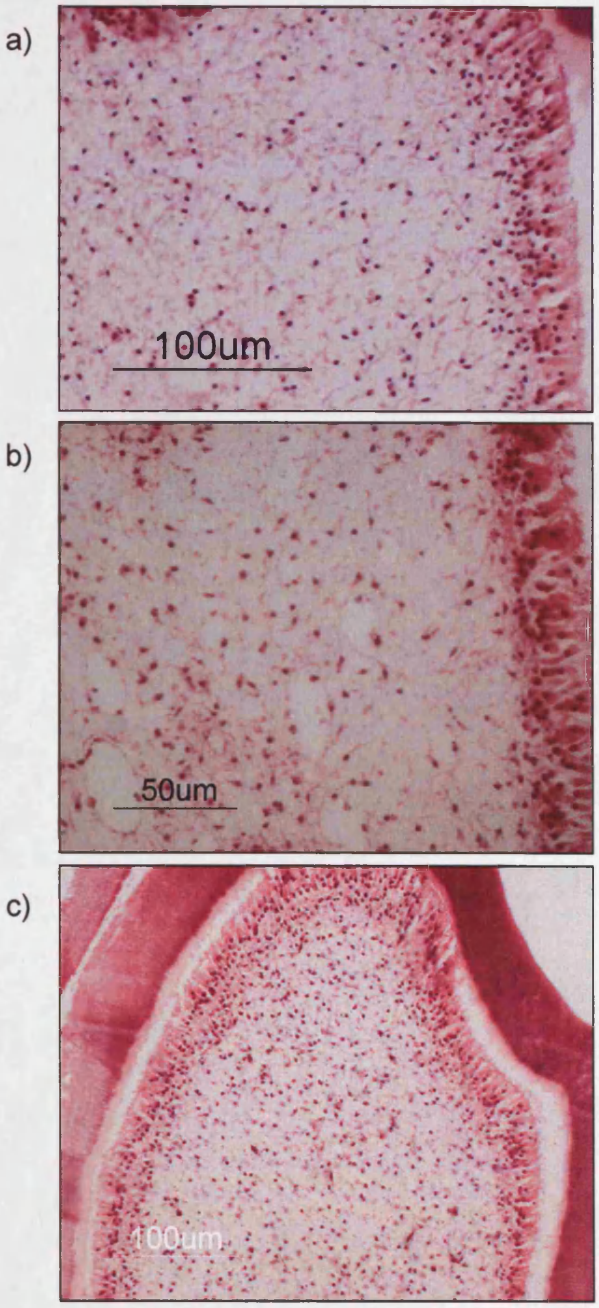

Fig. 3.4 4 hr co-culture of *S. constellatus* 45386 and tooth slices. Cells appear darkly stained and tissue architecture remains unaltered compared to control. a) Co-culture x20 mag. b) Co-culture x40 mag. c) Control x20 mag.

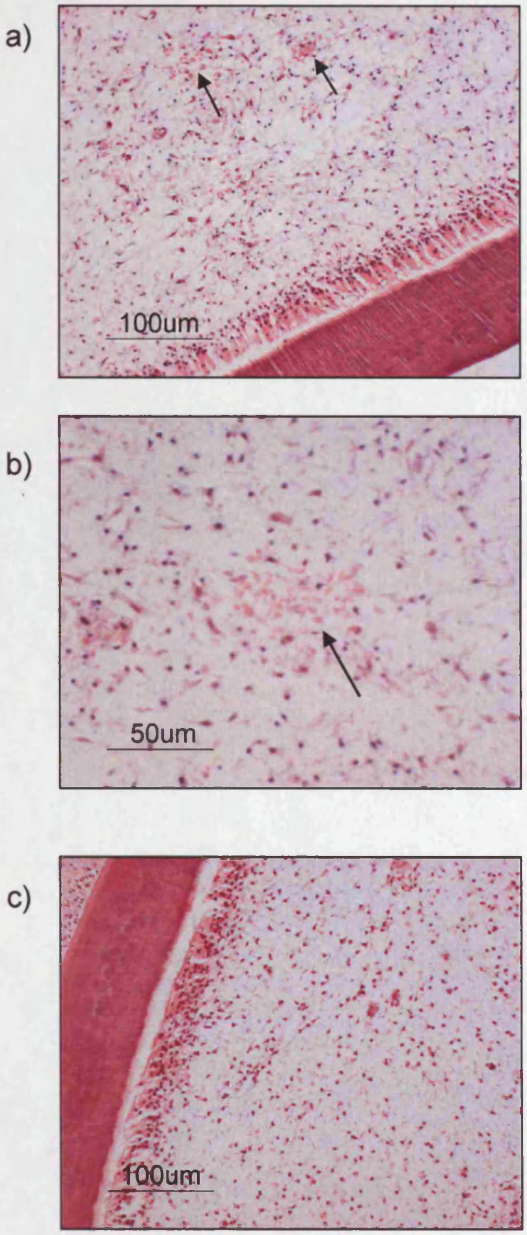

Fig. 3.5 4 hr co-culture of *S. anginosus* 39/2/14A and tooth slices. After 4 hrs some bacterial attachment is evident but cells in the infected tooth slices appear darkly stained and tissue architecture remains unaltered compared to control. a) Co-culture x20 mag. Arrows indicate attached bacteria in the centre of the pulp. Mammalian cells appear healthy despite bacterial attachment. b) Co-culture x40 mag. Arrow indicates bacterial attachment. c) Control x20 mag.

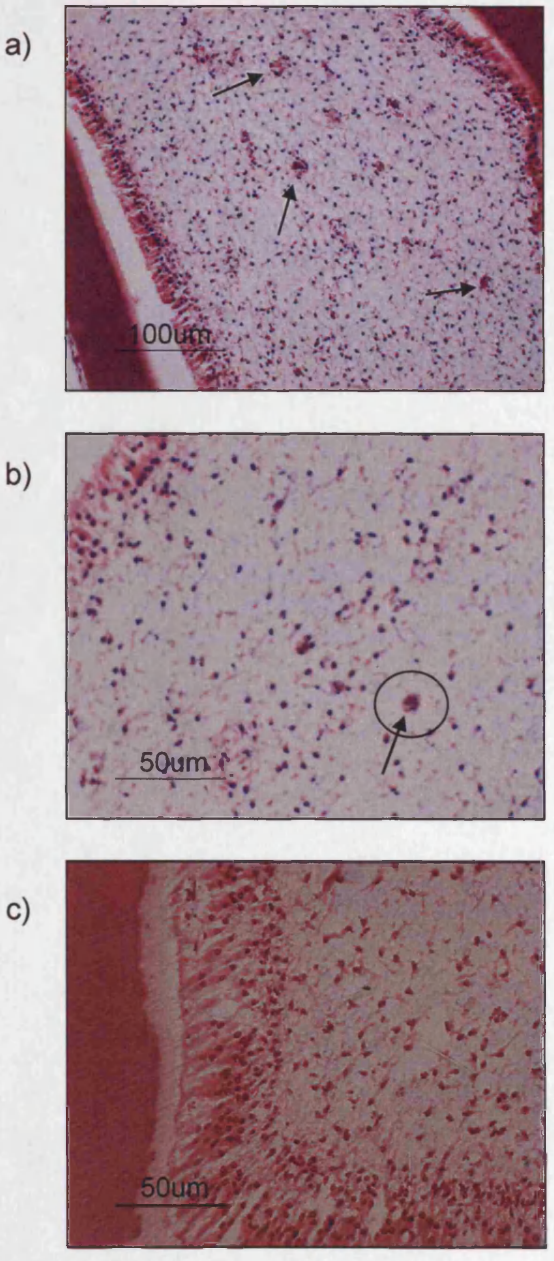

Fig. 3.6 8 hr co-culture of *S. constellatus* 45386 and tooth slices. Bacterial attachment is visible as areas of dense staining. At higher magnifications the breakdown of the matrix is apparent as a clear area between cells which is stained in controls. a) Co-culture x20 mag. Arrows indicate bacterial attachment. b) Co-culture x40 mag. Arrow indicates bacterial attachment surrounded by an area free from staining, indicated by the circle, which suggest a breakdown of the matrix. c) Control x40 mag.

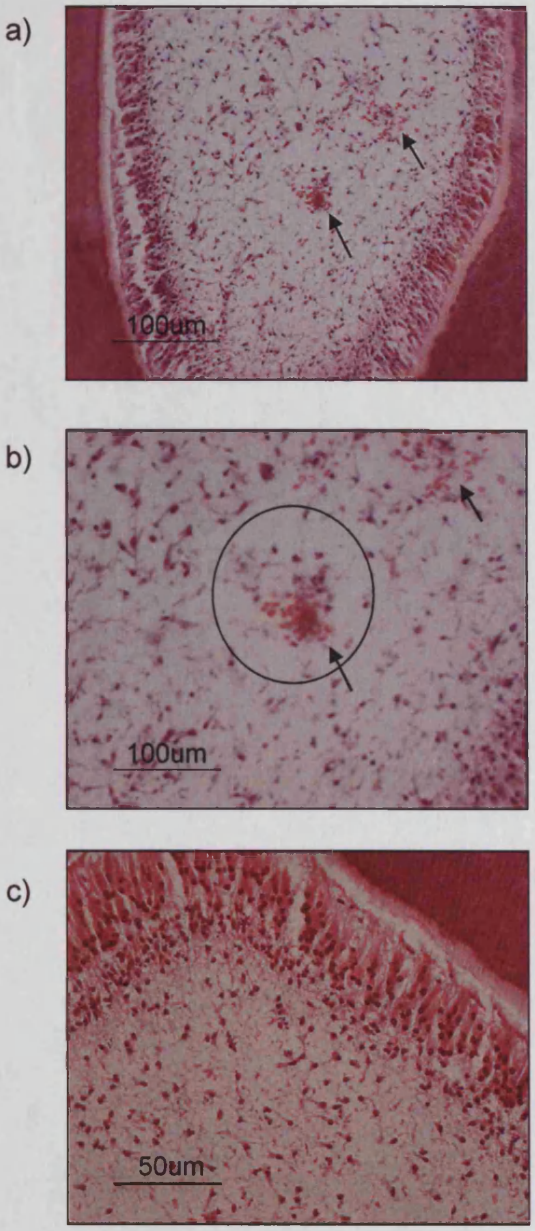

Fig. 3.7 8 hr co-culture of *S. anginosus* 39/2/14A and tooth slices. Cells appear more sparse but many are still viable, particularly in the odontoblast layer. Clear areas between fibroblasts are present indicating matrix breakdown. a) Co-culture x20 mag. Arrows represent bacterial attachment in the centre of the pulp. b) Co-culture x40 mag. Arrows represent bacterial attachment. Black circle highlights matrix breakdown in the surrounding area of the bacteria. c) Control x40 mag.

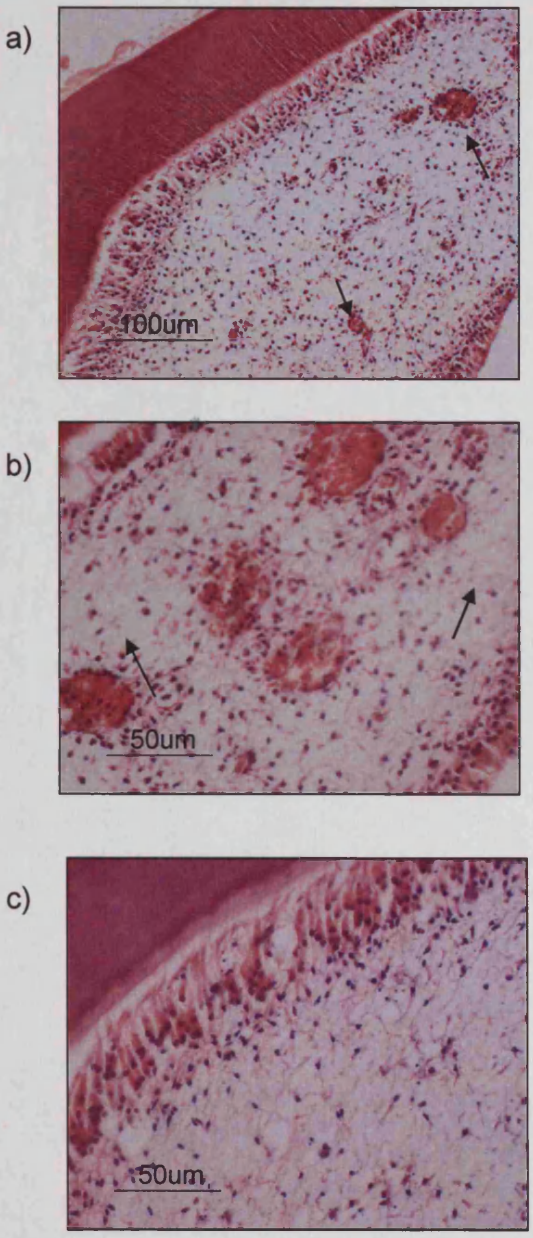

Fig. 3.8 24 hr co-culture of *S. constellatus* 45386 and tooth slices.. a) Co-culture x20 mag. Arrows indicate bacterial attachment. b) Co-culture x40 mag. Large areas of bacterial attachment can be seen as pink stained areas in the centre of the pulp. Arrows point to breakdown of matrix. c) Control x40 mag.

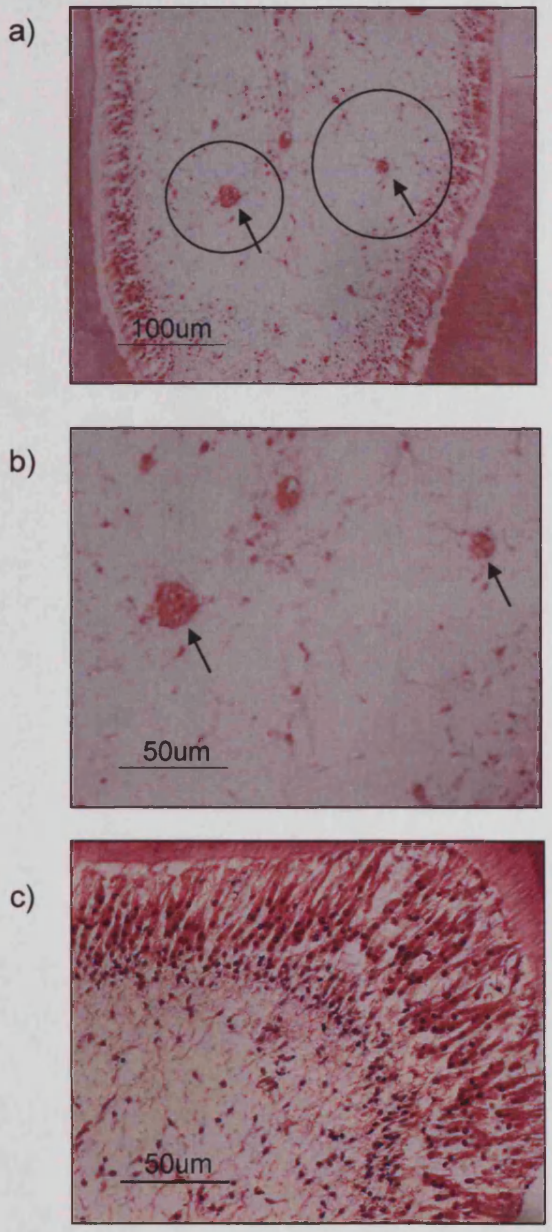

Fig. 3.9 24 hr co-culture of *S. anginosis* 39/2/14A and tooth slices. a) Co-culture x20 mag. Arrows indicate bacterial attachment surrounded by extensive cell death which is marked by black circles. Very few cells within the pulp remain viable though a large number of odontoblasts remain viable. b) Co-culture x40 mag. Arrows indicate bacterial attachment. A complete breakdown of the matrix can be seen with only very sparse staining between few remaining cells. c) Control x40 mag.

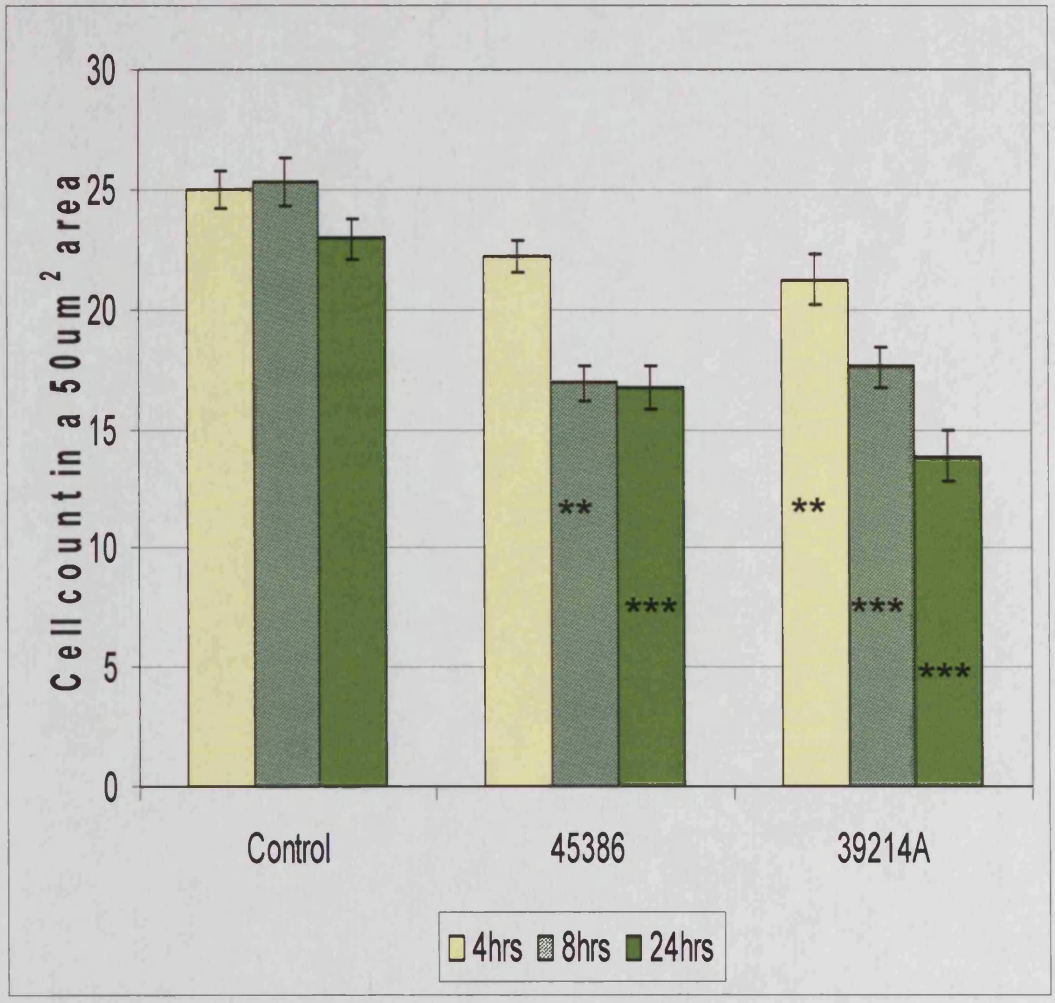

Fig. 3.10 Pulpal cell counts for tooth slices cultured in DMEM+10% BHI with SAG bacteria. Nuclear counts were performed to provide an average cell number in a 50µm2 area of the pulp. Counts indicated that culture in the presence of *S. constellatus* 45386 there was a significant decrease in cell number after 8 hours and that cell number continues to significantly decrease up to 24 hours in culture. Culture in the presence of *S. anginosus* 39/2/14A resulted in a significant decrease in cell number after 4 hours incubation with increasing cell loss at 8 and 24 hours. Error bars represent standard error.

** = P<0.01 *** = P<0.001

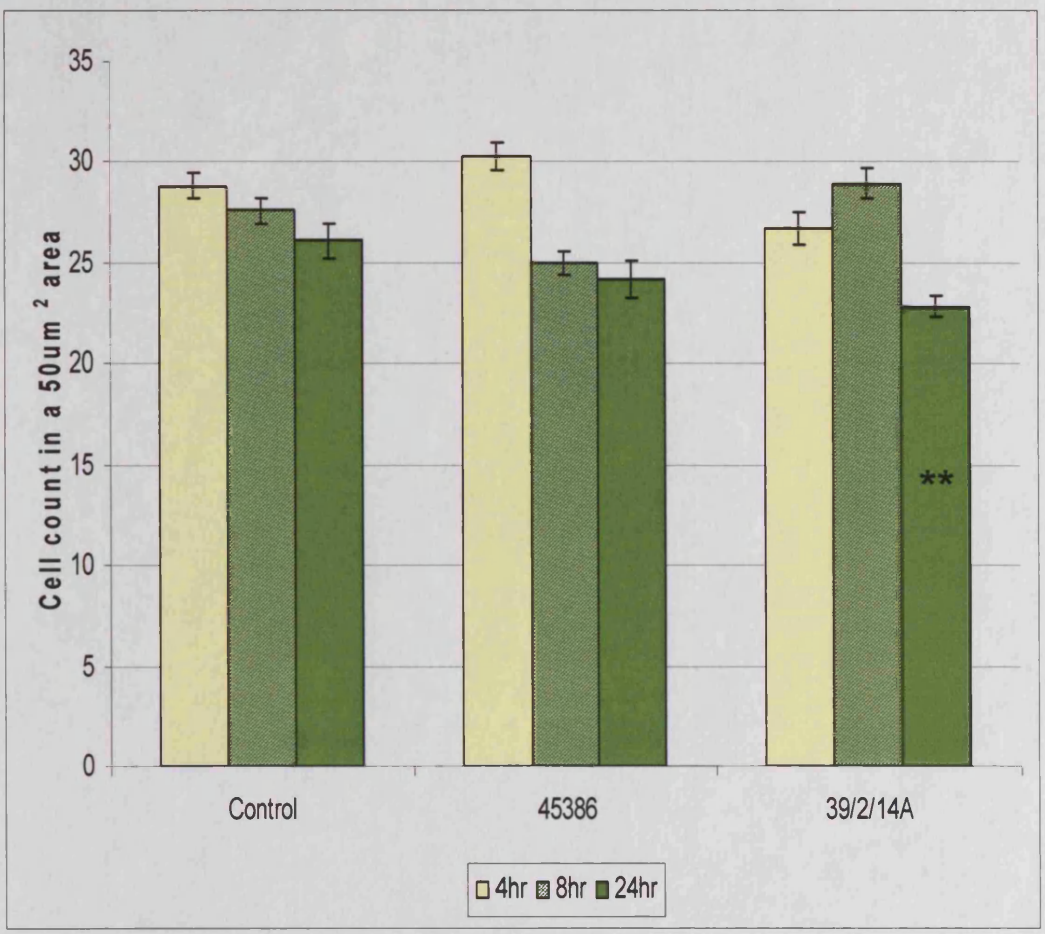

Fig. 3.11 Odontoblast counts for tooth slices cultured in DMEM+10% BHI with SAG bacteria. Nuclear counts were performed to give an average cell number for a 50μm^2 area of the odontoblast layer. There was no significant decrease in cell number in those slices incubated with *S. constellatus* 45386. A significant decrease was seen in tooth slices incubated with *S. anginosus* 39/2/14A for 24 hrs. There was no significant difference between the results seen for either SAG species.
** = $P < 0.01$

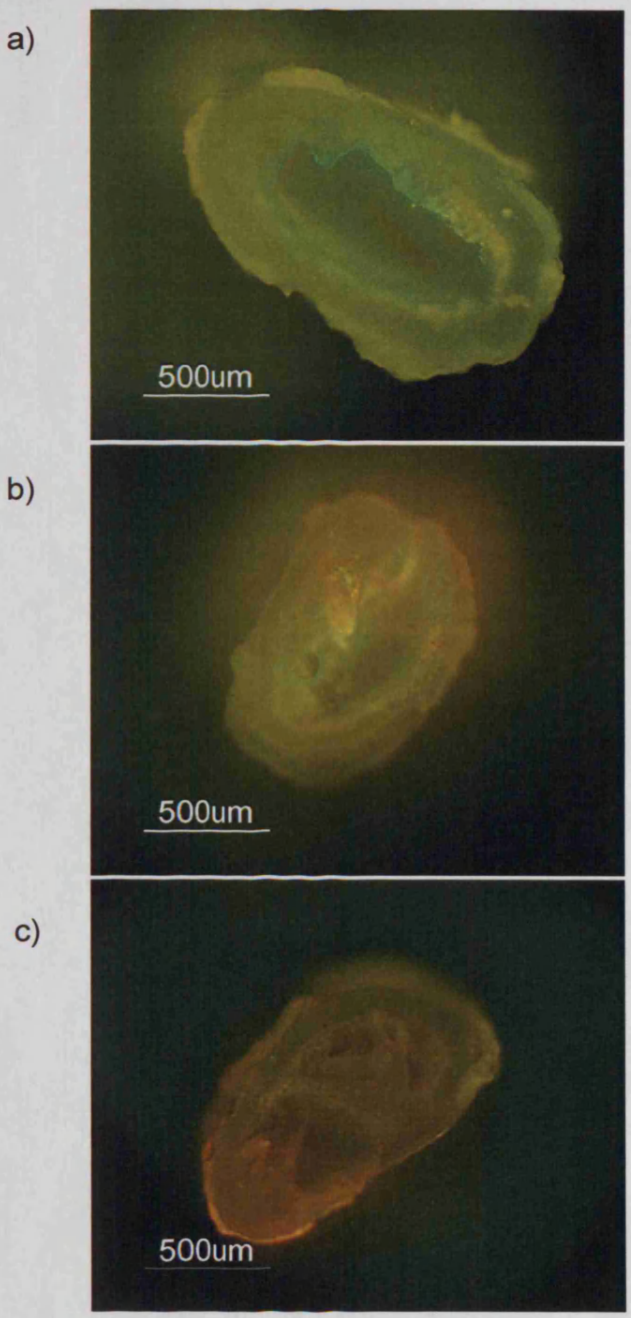

Fig. 3.12 Staining of tooth slices with acridine orange and ethidium bromide following incubation with SAG bacteria for 4, 8 and 24 hrs. a) 4 hrs, x 4 mag. Predominantly green fluorescence indicates minimal cell death. b) 8 hrs, x 4mag. Increased red fluorescence indicates an increase in cell death. c) 24 hrs, x 4mag. Extensive cell death is indicated by predominantly red fluorescence.

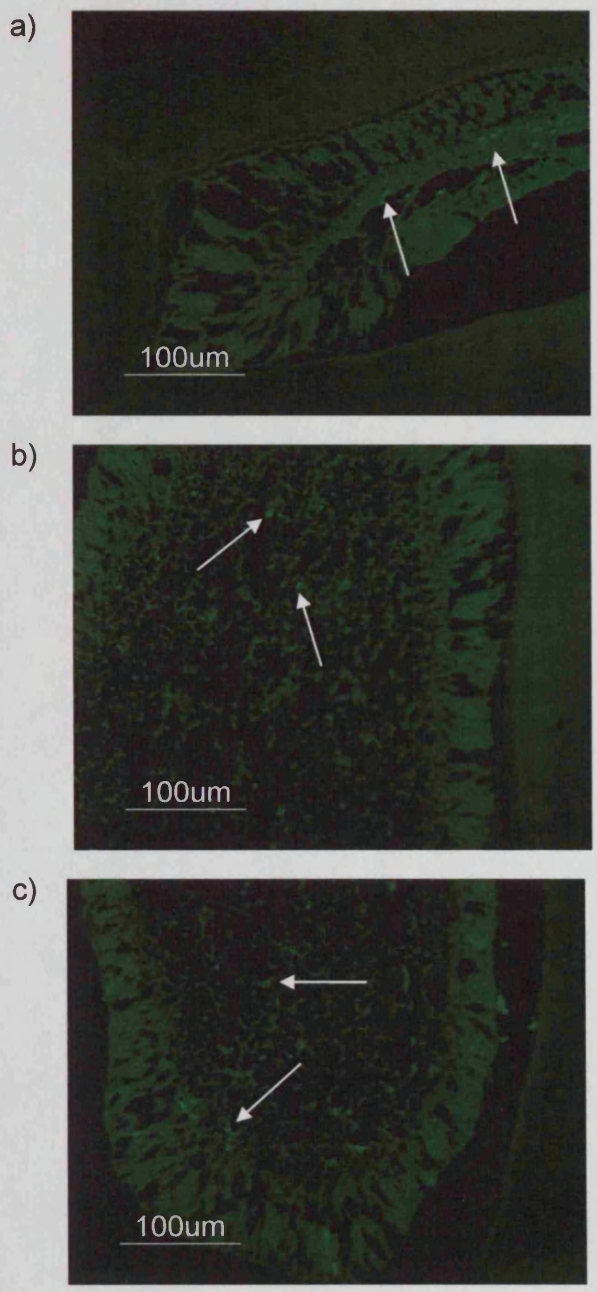

Fig. 3.13 FDA staining of bacteria on tooth slices following incubation for 4, 8 and 24 hrs. a) 4 hrs, x20mag. b) 8 hrs, x20mag. c) 24 hrs, x 20mag. Arrows indicate areas of bacterial attachment.

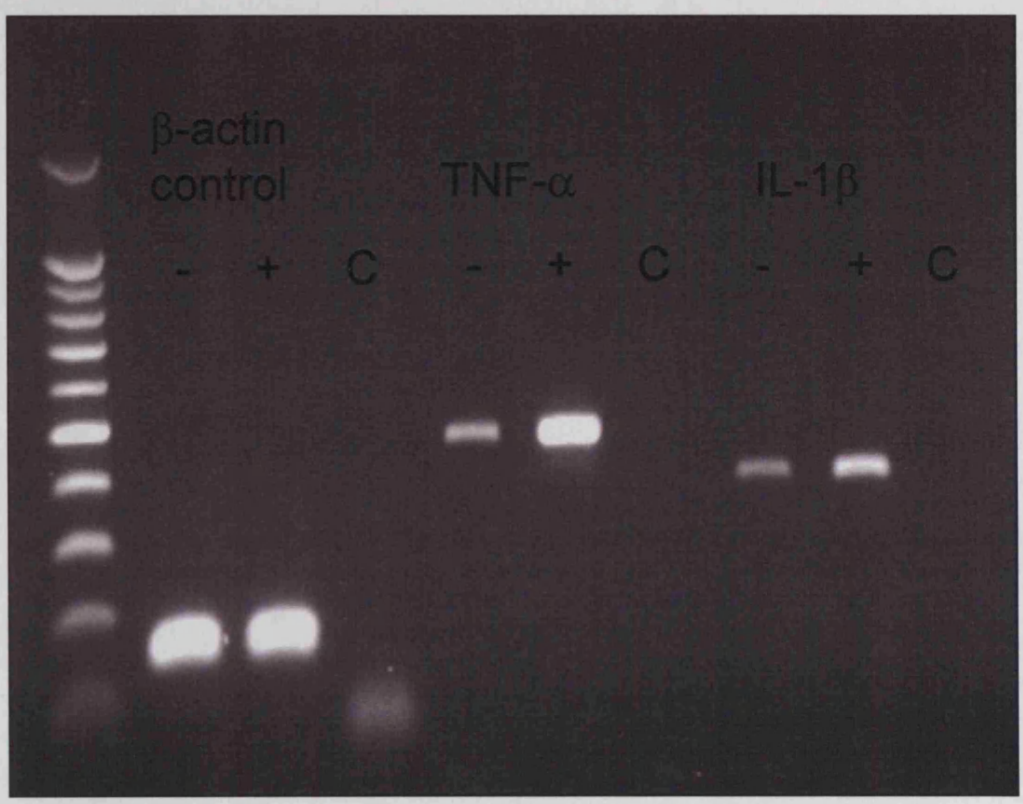

Fig. 3.14 PCR of inflammatory markers in sterile (-) and infected (+) pulp. Bands for TNF-α (438bp) and IL-1β (359bp) expression are noticeably stronger in pulp extracted from tooth slices infected with SAG bacteria compared to those which were cultured in a sterile environment. The rat house-keeping gene β-actin (155bp) was used as a control and expression remained the same in both sterile and infected cultures. Water was used as a negative control (C).

3.4 Discussion

During the initial stages of the development of the model used in this study the SAG bacteria were exposed to devitalised pulpal tissues in BHI, the standard SAG growth medium. This allowed observation of the bacteria interacting with the pulpal matrix without altering the growth conditions. This was an important factor to consider as the pulp is a unique substance with very different characteristics from the normal solid phase media that bacteria are grown on and it was not previously known if SAG bacteria would be able to attach to this matrix. In addition, the presence of host defence proteins and cytokines which may be found in the pulp could also inhibit bacterial growth. The successful growth and attachment of the bacteria on the pulp confirmed that this tissue is a suitable growth substrate for the bacteria and that there are no components within this to prevent infection of the viable tissue in the tooth slice model.

Bacteria were initially grown directly on pulp that had been devitalised by freeze-thaw cycles but had no other pre-treatment prior to inoculation. When samples of the post-culture media were plated out on FAA plates it was found to be infected by a number of organisms which were not part of the SAG bacteria. These were identified using 16S rRNA sequencing and were found to be commensals from the rat or the dissection environment. *Escherichia coli, Streptococcus thoraltensis* and *Enterococcus faecalis* are all commonly found in the normal flora of the gut of mammals (Facklam 2002; Madigan 2005) and are likely to have been present on the skin of the animal from contamination from faeces in the cage. Similarly, *Bacillus subtilis* is commonly found in soil or hay (Madigan 2005) and is likely to have come from the rats bedding. *Staphylococcus sciuri* is a commensal organism which can be isolated from a variety of animals, particularly rats (Hauschild et al. 2010), whilst *Staphylococcus aureus* is frequently isolated from humans (Madigan 2005) and as such was likely to be present in the dissection environment.

The effect that pulpal contaminants were having on the growth of SAG bacteria was unknown and it is possible for the presence of other bacteria to enhance or decrease growth of SAG bacteria. In some cases of co-infection, particularly in abscesses, a synergistic relationship between certain species has been observed (Kuriyama et al.

2000; Nagashima et al. 1999). Other studies have shown that the growth of one species may impair the growth of those bacteria also in the environment due to the production of antimicrobials or by causing a change in pH (Hibbing et al. 2010; Horiuchi et al. 2009). It was therefore important to eliminate infection of the model by these contaminants to assess the ability of the SAG to attach and grow on the pulp without interference from other species. This was achieved by the addition of a disinfection step to the protocol prior to inoculation with SAG. Alcohol washes and UV irradiation eradicated the contaminants and appeared to provide a more favourable environment for the growth of the SAG bacteria, as their growth on the pre-treated pulp appeared denser and to spread across the entire surface with less breaks between areas of aggregated bacteria. In the contaminated sample the bacteria showed less dense growth, which is likely to be caused by competition between the different species for nutrients and resources, in addition to the previously outlined techniques that bacteria employ to compete with other species. With the contaminating bacteria eliminated from the pulp, SAG bacteria were able to grow across the entire surface, forming a bacterial lawn. As the bacteria are in close association with each other they may be able to begin the formation of a biofilm. This is an organised community of bacterial cells which are embedded in a matrix which has been secreted by the bacteria (Hall-Stoodley and Stoodley 2009). Biofilms have been shown to provide protection to the bacteria against environmental factors such as heat and irradiation and increase their chances of survival against antimicrobials (Cos et al. 2010). This would be of particular importance when considering the uses of this model, as bacterial senstitivity is often tested *in vitro* in suspension where they are much more susceptible to antimicrobials (Aslam 2008) so the use of a model which allows biofilm formation and antimicrobial testing would be particularly advantageous. Therefore, in addition to identifying the ability of SAG bacteria to attach to the pulpal tissue, these experiments also emphasise the importance of the system to be free from any environmental contaminants as they may effect the growth of the bacteria being studied.

Further imaging and investigation of the infected pulp would be required to confirm bacterial numbers and if there is biofilm formation, as exact quantitative measurement of bacterial numbers using the LIVE/DEAD stain is not possible. In this study bacterial numbers have been estimated by macerating the infected pulp, washing in

PBS and plating out the resultant bacterial suspension. However, if a biofilm does begin to form this may not give an entirely accurate representation of bacterial numbers, as this may not be sufficient to remove all bacteria from the tissue. Also, although this was effective in estimating total bacterial numbers, it would not be sufficient for accurately recording numbers of different species. An alternative to this may be to use scanning electron microscopy, although this also has limitations as the processing required to view the tissues often causes the bacteria to detach completely from the matrix, giving an inaccurate measurement of numbers when the tissue is examined.

Following culture with the devitalised matrix, SAG bacteria were co-cultured with the mammalian tooth slices under the modified culture conditions that had been previously defined (**3.2.2**). Areas of pink staining by H&E were confirmed as bacteria attaching to the pulp using FDA fluorescence staining. Bacteria appeared to attach to the pulp in focal points which increased in size with increasing incubation time, indicating that bacteria in the media may be attaching to those bacteria already fixed onto the pulpal matrix. Progressive attachment of bacteria to the enamel during the formation of plaque and caries has been extensively reported (Love and Jenkinson 2002), with streptococci being identified as the primary colonisers of the oral cavity whose attachment to the salivary pellicle on the enamel is essential to the colonisation of the tooth. The streptococci have been shown to express multiple adhesins that allow them to attach to these salivary components, as well as enabling them to attach to other microbial cells and host cells (Hasty et al. 1992). Of particular interest is the antigen I/II adhesin, the production of which has been shown to be up-regulated by a collagen type I signal characteristically produced by the collagen fibrils found in dentinal tubules (Heddle et al. 2003). However, there is little research on the attachment of bacteria to pulpal tissues. The data from this study suggests that the chain-forming ability of streptococcal species may be important in pulpal invasion, as in caries formation, and that the attachment of streptococcal species which has been seen to occur in the dentinal tubules may also happen in the pulp. This also supports suggestions that SAG are involved in the primary colonisation of the pulp (Aderhold et al. 1981), and that they may enable further invasion by other bacterial species by providing an anchor for attachment of other species which can not directly adhere to the pulpal matrix but are able to attach to the adhesins on the surface of the SAG

bacteria, similar to the processes involved in plaque formation (Love and Jenkinson 2002). In addition to this, the action of SAG bacteria changes the gaseous environment and available nutrients as they advance further into the pulpal tissues, providing a environment more favourable to anaerobic species than that found at the site of initial infection (Fisher and Russell 1993). For example, at the dentine surface during initial pulpal exposure the cavity is still in contact with the oral cavity and so bacteria such as SAG, which are able to metabolise carbohydrates, are at an advantage. However, as the cavity advances further into the tooth direct contact with the oral cavity is lost, which affects the availability of nutrients and the gaseous environment. In such inflamed environments asaccharolytic bacteria may thrive as they are able to utilise the amino acids and peptides released from tissues decomposed during inflammation (Djais et al. 2006). Without the primary colonisers of the pulp such as SAG, which change the nutrient availability and gaseous conditions of the infection environment, these anaerobic bacteria with different growth requirements would be unable to infect the root canal. Therefore, by eliminating early infection this may help prevent inflammation progressing and causing extensive pulpal damage.

The formation of the bacterial clumps seen in the histological examination of the SAG infected tooth slices appeared to be associated with a breakdown of the collagen matrix, which can be seen as an absence of staining between fibroblasts. It is particularly pronounced around areas of bacterial attachment, indicating that the attached bacteria are having a direct localised effect on the surrounding tissues. It is possible that the bacteria are secreting enzymes which breakdown the matrix, such as hyaluronidase and chondroitin sulphatase. These enzymes are known to be produced by SAG bacteria (Jacobs and Stobberingh 1995; Whiley et al. 1990) and dental pulp is rich in their substrates (Bartold et al. 1995), suggesting that their breakdown, along with that of collagen, is important in the advancement of pulpal infection.

Hyaluronidase has been thought to be of particular importance in the invasion of SAG bacteria into connective tissues (Unsworth 1989), though many researchers argue that this enzyme is not widely produced in all SAG strains, particularly *S. anginosus* strains (Whiley et al. 1990). Also, it has been suggested that the production of hyaluronidase in SAG bacteria is not as important to the infection process as the production of chondroitin sulphatase (Jacobs and Stobberingh 1995). Chondroitin

sulphatase has been shown to play an important role in facilitating bacterial spread throughout a tissue and also liberating nutrients from the surrounding area (Shain et al. 1996). Further investigation of the SAG infection process using this model should consider in more detail the bacterial production of such hydrolytic enzymes and the role they play in pulpal infection and necrosis.

In addition to the tissue damage caused by SAG infection, there was also a direct effect on viable cell numbers within the pulp and odontoblast layer. Cell numbers were assessed using Image Pro Plus software which has been previously described (Section 2.2.7). Cells within the pulp and odontoblast layer were counted in a 50 μm^2 area after 4, 8 and 24 h of infection with SAG bacteria. ANOVA and Tukey-Kramer's statistical testing showed that there was a significant decrease in viable cell numbers after 8 and 24 h of infection with both *S. anginosus* 39/2/14A and *S. constellatus* 45386 ($P<0.0001$). This confirms that incubation of the tooth slices with these strains for 8 h or more results in a significant amount of cell death which is caused by the presence of the bacteria and that no other changes to the culture conditions are resulting in this cell death. Odontoblast counts did not show the same pattern of cell death, with cells resisting significant levels of necrosis for up to 24 h incubation with *S. constellatus* 45386. Incubation with *S. anginosus* 39/2/14A did not result in a significant decrease in odontoblast number until 24 h incubation ($P<0.01$). In all tooth slices incubated with SAG bacteria, attachment of the bacteria appeared to be focussed in the central areas of the pulp and was absent from the odontoblast layer. This resistance to cell necrosis in the odontoblast layer indicates that when SAG bacteria are incubated with tooth slices in this model, the bacteria preferentially attach to and destroy cells of the pulp. The preferential attachment to the pulp may be due to the large amounts of collagen found in the matrix surrounding the pulpal fibroblasts, as streptococcal species express adhesins to allow attachment to collagen (Heddle et al. 2003). Also, the densely packed nature of the odontoblast layer may prevent the bacteria from accessing appropriate points of attachment and may limit the space for bacterial growth. In the pulp there are large areas between fibroblasts which consist mostly of matrix components, and these areas may provide a more favourable environment for attachment and growth of SAG bacteria. These attachment patterns are likely to play a role in the pattern of cell necrosis that is seen in the tooth slices, as the increased cell death is seen in the regions surrounding the areas of bacterial

attachment, whilst less cell necrosis is seen in areas without bacterial attachment, such as the odontoblast layer. This suggests that the bacteria may be having a mechanical effect on the matrix and surrounding cells, with their growth causing a physical disruption to the adjacent mammalian cells. However it is also possible that this localised cell death is caused by the enzymes and toxins which are being secreted by the attached bacteria as they diffuse into the surrounding tissues. This would account for the increased necrosis in the pulp and the limited effect on the odontoblast layer, as it would require longer for the bacterial products to reach the odontoblast layer and may be limited to later incubation times when the bacterial number has increased to a sufficient level to produce enough supernatant to diffuse out into the odontoblasts.

Although some bacterial attachment and matrix breakdown was observed in H&E staining after 4 h of infection with *S. anginosus* 39/2/14A, there was no significant loss in cell numbers. Similarly, infection of the tooth slices with *S. constellatus* 45386 did not result in significant cell death after 4 h and H&E staining for this isolate did not reveal any bacterial attachment. This suggests that it may take at least 8 h for the bacteria to establish an infection of the pulp that leads to production of sufficient levels of enzymes to cause significant cell damage. This suggests that early intervention in pulpal infection may help to arrest the infection and further study on how this may impact on treatment regimes should be considered. This data also confirms previous observations that there are differences in the growth and infection rates between the different isolates. Growth curves showed that *S. anginosus* 39/2/14A had a higher total bacterial yield than *S. constellatus* 45386 and its growth rate was higher, particularly in early stages of incubation (**2.3.3**). This may account for its enhanced ability to attach and cause damage to the tooth slices at an earlier time point.

After 24 h of incubation with *S. anginosus* 39/2/14A there was extensive cell death in both the pulp and the odontoblast layer, whilst cell death in the tissues incubated with *S. constellatus* 45386 did not chang significantly from that which occurred after 8 h of incubation. Cell death also seemed to be confined mainly to the pulpal area rather than the odontoblast layer, suggesting that this particular strain of *S. constellatus* may be less adept at penetrating the mammalian tissues, as the production of hydrolytic enzymes that promote tissue invasion has been found to be variable between strains

(Grinwis et al. 2010; Jacobs and Stobberingh 1995). The more extensive cell death seen in those tooth slices incubated with *S. anginosus* 392/14A may also be due in part to the ability of *S. anginosus* to reach a higher bacterial yield than *S. constellatus* over the same time period when grown in the modified medium at 37°C and 5% CO_2. Such extensive cell death as seen with *S. anginosus* would have a severe impact on the ability of the pulp to repair itself. However as it does not seem to reach irreversible levels until at least 8 h of infection, early treatment may prevent pulpal necrosis and allow the tooth to be saved. In addition to this, the cells within the tooth and the host immune response may produce cytokines which may help to protect the cells of the tooth.

The production of cytokines as part of the immune response within the tooth slice is limited due to the lack of blood supply. However there is an increased expression of TNF-α and IL-1β in infected tooth slices, which indicates that the model responds to the introduction of SAG bacteria into the system. These cytokines have been found to be expressed early on in the response to infection (Silva et al. 2007; Stashenko et al. 1998), as seen in this study, and so this *ex-vivo* model accurately represents events that occur *in vivo*. This is important when considering a model for use for testing antimicrobials and other anti-inflammatory treatments. These cytokines are often produced *in vivo* by cells such as macrophages and T-lymphocytes but, due to the lack of circulatory system in this model, they are most likely to be produced by the fibroblasts themselves, in addition to any immune cells residing within the pulp, as has also previously been reported in other studies (Silva et al. 2005). This non-specific immune response seen in the model is representative of the early *in vivo* situation, where non-specific inflammation occurs prior to a specific response led by immune cells in the blood stream which enter the pulp through the blood vessels which extend through the tissue (Hahn and Liewehr 2007a, b).

Although IL-6 expression was not detected in sterile or infected tooth slices this may be detectable if the infection period was extended, as TNF-α and IL-1β are thought to be able to induce its expression (Silva et al. 2007), so it may become detectable later in the cytokine cascade.

The ability to quantify the damage caused to cells in the tooth slice as a result of incubation with SAG bacteria enabled this model to be used to study pulpal infection in a controlled system which reduced the use of animal materials. Histological examination of tooth slices has shown that whilst cell necrosis and tissue breakdown was associated with bacterial attachment, there appeared to be production of enzymes or toxins which were able to spread throughout the tissue and cause cell death at sites distant from the bacteria. The role of these bacterial products is investigated in Chapter 4. In addition to evaluating the effects of bacterial infection on the mammalian tissues this model has also been used to study the host responses which may occur during a pulpal infection. A model such as this, which will allow elucidation of these inflammatory processes, is essential for further research into possible antimicrobials for use in endodontic treatments.

Chapter 4: Characterisation of SAG bacterial supernatants and their introduction into the tooth slice culture system

4.1 Introduction

Bacterial attachment to the pulpal tissues of teeth has been shown in the previous chapter to cause significant and quantifiable damage to the tissues. The development of this model provides a template for investigation into endodontic infection of pulpal tissues using novel methods which allow examination of the direct interaction of bacteria with dental tissues cultured in an organotypic system. However, in the early stage of infection, when caries has not advanced through the dentine, pulpal damage may still occur due to the bacterial production of enzymes and toxins which can diffuse from the dentinal tubules into the pulpal cavity and illicit an immune response (Bergenholtz 1981; Love and Jenkinson 2002). These products include enzymes that are capable of degrading the matrix, such as hyaluronidase and chondrotin sulphatase, toxins and components of the bacterial cell wall such as LPS, lipotechoic acid, flagella, peptidoglycans and lipopeptides, as well as the nuclear material of bacteria lysed by the host immune system (Akira et al. 2006).

Invasion of the dentinal tubules is important to pulpal inflammation as, in addition to the diffusion of bacterial products into the pulp, it also causes a reduction in outward flow of fluid from the tubules, causing an increase in pressure in the pulpal chamber. This increase in pressure may contribute to pulpal necrosis which hinders the natural reparative ability of the tooth (Heyeraas and Berggreen 1999). The generation of a model system which is representative of this clinical situation may provide a method of testing anti-inflammatory capping agents that could be used in future treatments.

In the clinical situation, the bacterial products diffuse through the dentinal fluid that surrounds the odontoblast process in the dentinal tubules. When bacteria are cultured *in vitro* these products are excreted into the culture medium. The bacterial products can then be collected and applied to tissues to assess their enzymatic and toxic effects.

Previously, the cause of pulpal inflammation prior to pulpal colonisation has been attributed to caries bacteria and their products diffusing through the dentine into the

pulp (Hahn and Liewehr 2007a, b). Recently, there has been an increased interest in the role of different bacterial species, such as SAG bacteria and other anaerobes, which reside in the dentinal tubules and later advance into the pulpal cavity and colonise the pulpal tissues. Studies on the effect of supernatants from black pigmented *Bacteroides* have revealed that such bacteria are capable of producing cytokines and enzymes which result in pulpal necrosis and matrix degradation (Chang et al. 2002; Yang et al. 2003c). The effects of supernatants from SAG bacteria on pulpal fibroblasts have not been previously studied, and the experiments with other endodontic bacteria have been carried out using pulpal fibroblasts which are cultured in an *in vitro* environment and are separated from the odontoblasts (Killough et al. 2010, Dabija-Wolter et al. 2009). As such the model which has been developed in this study would be beneficial for investigating the effect of SAG bacteria supernatants on the pulpal matrix, fibroblasts and odontoblasts when cultured *in situ* in an organotypic model.

Characterisation of the SAG bacteria supernatants is needed to give an indication of what components are present and which of these are having an effect on the mammalian tissues. The characterisation of the supernatants produced by the different SAG species will also highlight differences in protein expression that exist between the species. It has previously been established that certain strains of SAG are more likely to be associated with infection than others, due to their increased pathogenicity and expression of virulence factors (Jacobs and Stobberingh 1995; Unsworth 1989).

The colonisation of the dentinal tubules and diffusion of the bacterial products into the pulpal chamber is an important phase in the development of a pulpal infection. It is important to understand these events as they can lead to the formation of tertiary dentine and the stimulation of repair mechanisms within the tooth. It would be advantageous to be able to use the model to represent the events that occur during this time. To do this the diffusible products produced by the bacteria must be collected and applied to the tooth slice in a controlled system at a known concentration. A bicinchoninic acid (BCA) assay was used to calculate the total protein concentration in bacterial supernatants. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine differences in supernatants between the species.

These supernatants were then applied to the established model and histological examination was used to identify their effect on the pulpal tissue.

4.2 Methods

4.2.1 Supernatant collection

S. constellatus 45386 and *S. anginosus* 39/2/14A were inoculated into BHI and incubated overnight at 37°C, 5% CO_2. Resultant ONCs containing bacteria in stationary phase were centrifuged at 4000 rpm for 10 min to pellet the bacterial cells which were then discarded. The remaining supernatant was filtered through a 0.2 μM Millipore filter and stored at -20°C in 1ml aliquots until required. A control supernatant was prepared by treating sterile BHI with the same process.

4.2.2 Supernatant analysis

4.2.2.1 SDS-PAGE

Pre-cast NuPAGE Bis-Tris mini gels (Invitrogen, Paisley, UK) with a 4-12% gradient were used to analyse the protein composition of the bacterial supernatants, in order to identify differences in protein expression between the strains. SDS-PAGE was performed according to manufacturer's instructions. 2.5 μl of NuPage LDS sample buffer and 1.5 μl of reducing agent was added to10 μl of each supernatant. Samples were then heated at 70°C for 10 min in a heating block. The Bis-Tris mini gel was loaded into the XCell SureLock mini-cell (Invitrogen) and the anode and cathode chambers filled with running buffer consisting of 50 ml 20x NuPage MES SDS running buffer made up to 1 L with deionised water. 200 ml of running buffer was added to the inner chamber and 500 μl of NuPage antioxidant was added. The remaining running buffer was used to fill the outer chamber of the cell. 10 μl samples were then loaded into the gel and run at 200 volts for 35 min. Supernatants were run on the gels, either undiluted or diluted 1:10. Each run was performed in triplicate.

4.2.2.2 BCA Assay

A BCA protein assay kit (Pierce, Northumberland, UK) was used to determine the total protein concentration in the bacterial supernatants and the BHI broth control. The BCA assay works by adding the BCA reagent which contains bicinchoninic acid and cupric sulphate to the protein solution. The peptide bonds in the protein reduce

the copper ions in the cupric sulphate which are then chelated by the bicinchoninic acid which causes the formation of a purple product which can be measured by absorbance. As the amount of copper ions reduced is proportional to the amount of the protein in the sample, the colour change and associated change in absorbance is relative to the protein concentration.

For each assay, a standard curve was generated by adding 25 µl of known BSA standards in a range of concentrations from 20 µg/ml to 2,000 µg/ml to wells of a 96 well plate. On the same plate, 25 µl of bacterial supernatant and sterile BHI was also placed into triplicate wells. 200 µl of BCA reagent was then added to each of the standards and samples. This BCA reagent was prepared combining a sodium bicinchoninate solution with a cupric sulphate solution in a 50:1 ratio. Absorbance at 570nm was read using on a Microplate™ reader (BioTek Instruments Limited, Bedfordshire, UK). A standard curve was plotted by using the absorbance values from the BSA standards and a best-fit line was generated. The equation of the best-fit line allowed the concentrations of the bacterial supernatants to be calculated.

4.2.3 Culture of tooth slices in the presence of bacterial supernatants

Two protocols were devised to examine the effects of the bacterial supernatants on the cultured tooth slices. The initial method described in protocol 1 below appeared to inhibit bacterial growth and it was therefore necessary to alter this method.

Protocol 1

Upper and lower incisors from 28 day old Male Wistar rats were dissected and sliced into 2mm transverse sections as previously described (**2.2.5**). Tooth slices were then cultured for 5 days in DMEM which contained no BHI. Tooth slices were then transferred to sterile DMEM+10% BHI within a section of dialysis tubing. The enclosed tooth slices were then transferred to a well containing DMEM+10% BHI inoculated with SAG bacteria and cultured for 4, 8 and 24 h. The dialysis tubing was then removed from the well, washed in PBS and cut open to remove the tooth slice. Tooth slices were then fixed in formalin for histological processing (**2.2.6**). If visible

turbidity was not observed in the inoculated medium a sample was plated onto fastidious anaerobe agar and incubated overnight to confirm bacterial growth.

Protocol 2

Upper and lower incisors from 28 day old Male Wistar rats were dissected and sliced into 2mm transverse sections as previously described (**2.2.5**). Tooth slices were then cultured in DMEM which contained no BHI for 5 days. Following this a culture medium was prepared which consisted of supplemented DMEM+10% BHI and 0.6ug/ml of bacterial supernatant or BHI supernatant control. This medium was then applied to the tooth slices which were cultured in its presence for 4, 8 and 24 h before being fixed for histological examination (**2.2.6**). Cell counts were performed on 5 tooth slices which were representative of each experimental group. 5 sections were taken from each tooth with 5 counts performed within 5 random fields of view for each section.

4.2.4 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 (**2.2.7**). One-way analysis of variance (ANOVA) was performed to determine the relative significance of the difference between experimental cell counts and the controls. Tukey-Kramer's test was used in conjunction with ANOVA to compare the significant difference between all possible pairs of means. This allowed comparison of the mean cell counts between tooth slices exposed to supernatants from different bacterial species, in addition to comparison with the control.

4.3 Results

4.3.1 Supernatant analysis

4.3.1.1 SDS-PAGE

The proteins in the supernatants produced by *S. constellatus* 45386 and *S. anginosus* 39/2/14A were separated using SDS-PAGE and stained using silver staining to allow a comparison of the protein content of the supernatants between the two species (Fig 4.1). To obtain adequate staining on the gels, supernatants were run undiluted. Preliminary runs used supernatants diluted 1:10 in PBS which did not give sufficient staining, bands became faint and it was difficult to distinguish between different bands. Undiluted supernatant samples were then run in triplicate to allow comparison of the different samples and ensure similar banding was seen in all replicates. It was found that the supernatant produced by *S. constellatus* 45386 had a far greater number of distinct proteins than that produced by *S. anginosus* 39/2/14A and that it appeared as easily distinguishable bands without smearing. A band was visible at approximately 130kDa in the supernatant produced by *S. anginosus* 39/2/14A which shows streaking. There were also visible bands at around 100 and 200kDa in the supernatants of both bacteria. Whilst these were visible in both protein profiles they appeared more densely stained in the profile produced by *S. constellatus* 45386. In addition, this supernatant also produced a distinct and densely stained band at approximately 45kDa, which was absent from the supernatant of *S. anginosus* 39/2/14A.

4.3.1.2 BCA assay

A standard curve was plotted (Fig. 4.2) that generated an equation that relates absorbance (y) to protein concentration (x). From this, the protein concentration of bacterial supernatants was calculated. The total protein concentration in the supernatants was approximately 6μg/ml for each of the different SAG strains. This was repeated in triplicate, with average concentrations and standard deviation displayed in Table 4.1.

Supernatant sample	Protein concentration (µg/ml)
S. anginosus 39/2/14A	5.66 (S.D = 0.36)
S. constellatus 45386	5.98 (S.D = 0.43)

Table 4.1 Protein concentration in supernatant samples from overnight culture at 37°C, 5% CO_2 in BHI. S.D = standard deviation.

4.3.2 Culture of tooth slices in the presence of bacterial supernatants

Protocol 1

The turbidity of the inoculated media in which the dialysis tubing containing the sterile tooth slice was incubated remained unchanged after 24 h of incubation, suggesting that bacterial growth was inhibited, as a noticeable change in turbidity is normally seen when SAG strains are incubated in DMEM+10% BHI for 24 h. A sample of the inoculated media was plated onto fastidious anaerobe agar and incubated overnight at 37°C in an anaerobic cabinet. No bacterial growth was evident on the plate, confirming that the bacteria which had been inoculated into the media had died and there had been no growth in the media. Subsequently, there was no supernatant formation and the tooth slice inside the dialysis tubing was unaffected. This protocol was then abandoned in favour of applying collected supernatants directly to the tooth slice by adding them to the culture media, eliminating the need for concurrent bacterial growth.

Protocol 2

After 4 h incubation in the presence of supernatants collected from *S. anginosus* 39/2/14A and *S. constellatus* 45386 tooth slices appeared similar to the control slices when viewed at lower magnifications, indicating the cells were healthy and viable throughout the sections (Fig 4.3A and 4.4A). However, when viewed at higher magnifications, some degree of tissue breakdown was observed (Fig 4.3B and 4.4B). This suggested that at this time point there was only a minimal effect on the cells and that the majority of damage is to the surrounding connective tissue. The control sections which were incubated with a sterile sample of the bacterial growth media which was filtered in the same manner as the collected supernatants had no effect on the tooth slices, with them appearing healthy and with extensive staining of the

connective tissues between cells at both low and high magnifications (Fig 4.3 C and 4.4 C). This confirmed that the effects seen in the sections incubated with the supernatants were due to bacterial products and not components of the media.

Sections which were incubated for 8 h with the bacterial supernatants appeared to have a notable decrease in cells in the pulpal area (Fig 4.5A and 4.6 A). Histological examination indicated that there was a decrease in the structural integrity of the tissues as there was less staining seen surrounding the cells (Fig 4.5 B and 4.6 B). This may indicate matrix breakdown by bacterial enzymes secreted into the supernatant. However, the majority of damage appears to be confined to the pulpal area with the cell-rich zone clearly distinguishable and the odontoblast layer remaining relatively unaffected, with densely packed cells that appear darkly stained and healthy.

Incubation of the tooth slices with bacterial supernatants for 24 h resulted in an increase in cell death and further effects on the structural integrity of the matrix throughout the pulp, with effects seen to extend into the odontoblast layer. In these sections the cell-rich zone became indistinguishable in some areas (Fig. 4.7b and 4.8a) and widespread matrix breakdown was observed. The most notable difference between the tooth slices incubated for 24 h and those incubated for shorter time periods was the effect on the odontoblast layer. At longer incubation times the odontoblasts appeared to be less densely packed with fewer stained nuclei visible (Fig. 4.7b and 4.8b).

The observed changes in cell number which were seen in the histological sections were confirmed using average cell counts over a 50 μm^2 area for each time point. These counts demonstrated that for both *S. constellatus* 45386 and *S. anginosus* 369/2/14A there was a significant decrease in pulpal cell number after 4 h of incubation with the supernatants and that this decrease continued with increasing incubation time (Fig. 4.9). The counts also confirmed that the odontoblasts remained relatively unaffected by the supernatants until 24 h of incubation, when a significant decrease was seen to occur (Fig. 4.10). Prior to this, the average cell count for a 50 μm^2 area was approximately 25, which was not significantly different from the control counts.

The significance of the cell count results was tested using ANOVA and Tukey Kramer's test which also indicated that in addition to a significant decrease in cell number compared to the control, there was also a significant difference in cell number over the same area for the different incubation times.

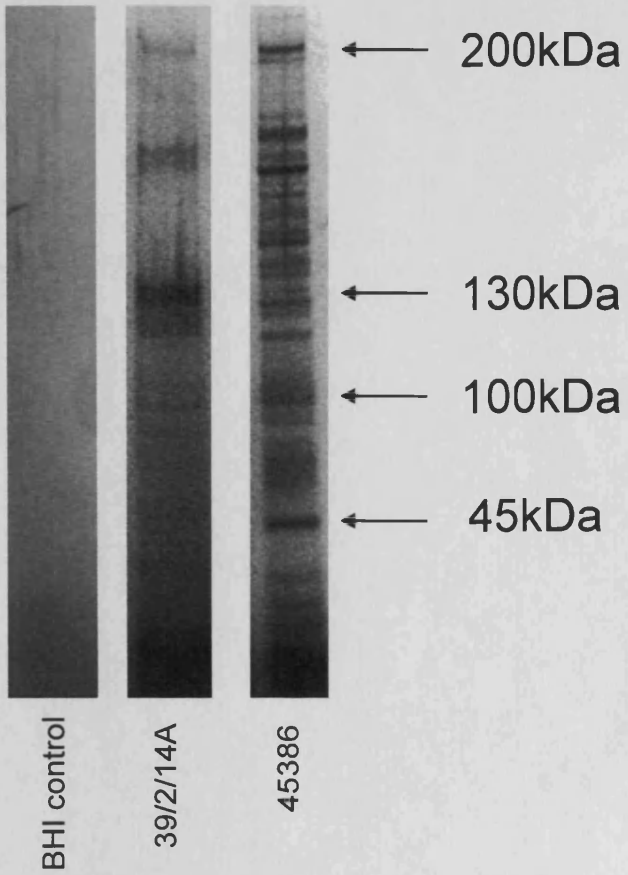

Fig. 4.1 SDS-PAGE analysis of supernatants collected from overnight growth of *S. constellatus* 45386 and *S. anginosus* 39/2/14A in BHI. Protein bands are seen in both supernatants at 200 and 100kDa. The densely stained area at 130kDa in the supernatant from *S. anginosus* 45386 may represent unseparated proteins. A number of proteins appear in the supernatant from *S. constellatus* 45386 which are not seen in *S. anginosus* 39/2/14A. BHI control shows no protein bands.

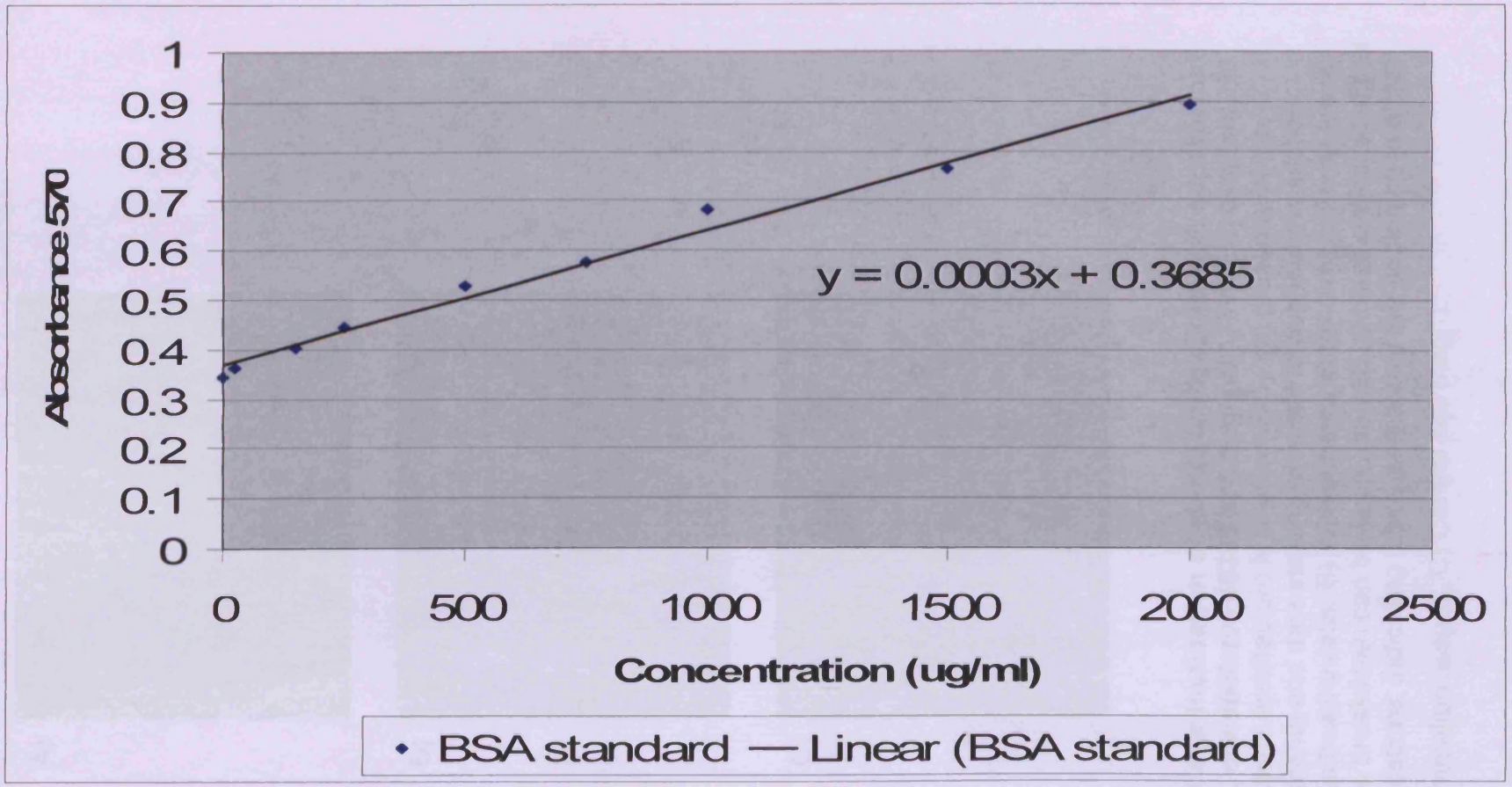

Fig. 4.2 Standard curve generated from BCA assay relating protein concentration to absorbance. The equation of the line y=0.0003x + 0.3685 allowed calculation of protein concentration in the supernatants of a known absorbance.

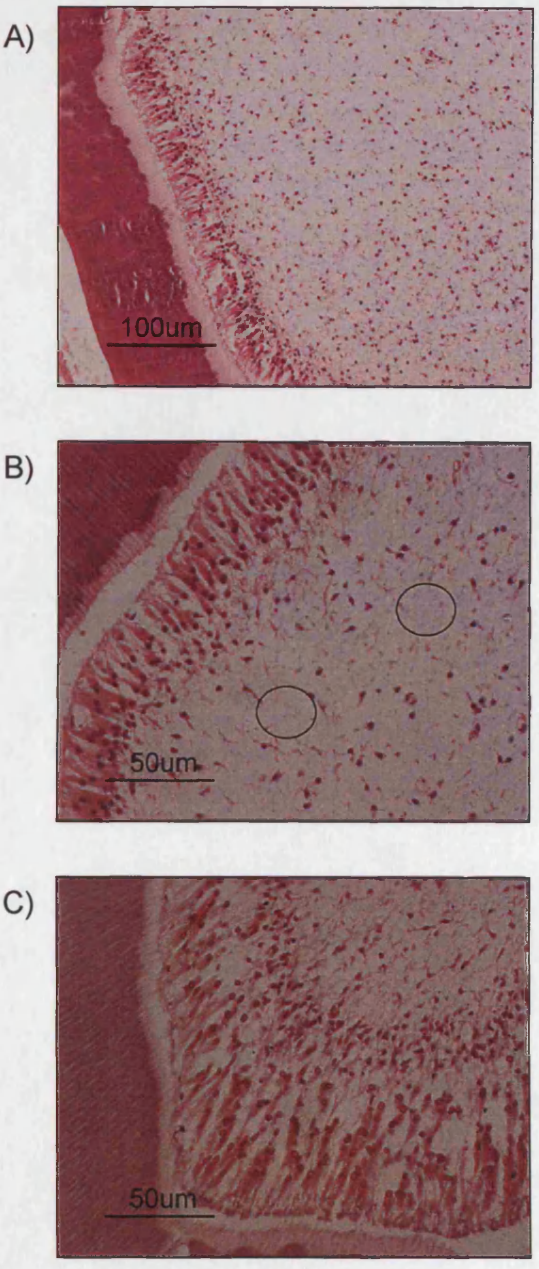

Fig. 4.3 4 hr incubation of sterile tooth slices with supernatant from *S. constellatus* 45386 at 0.6µg/ml. A) With supernatant x20 mag. Tissue architecture is unaffected by the presence of the supernatants and dark staining of nuclei throughout the section indicates healthy cells. B) With supernatant x40 mag. Some areas of tissue breakdown can be seen (marked by black circles) at higher magnifications, indicating that supernatants are beginning to effect the mammalian tissues. C) Control x40 mag.

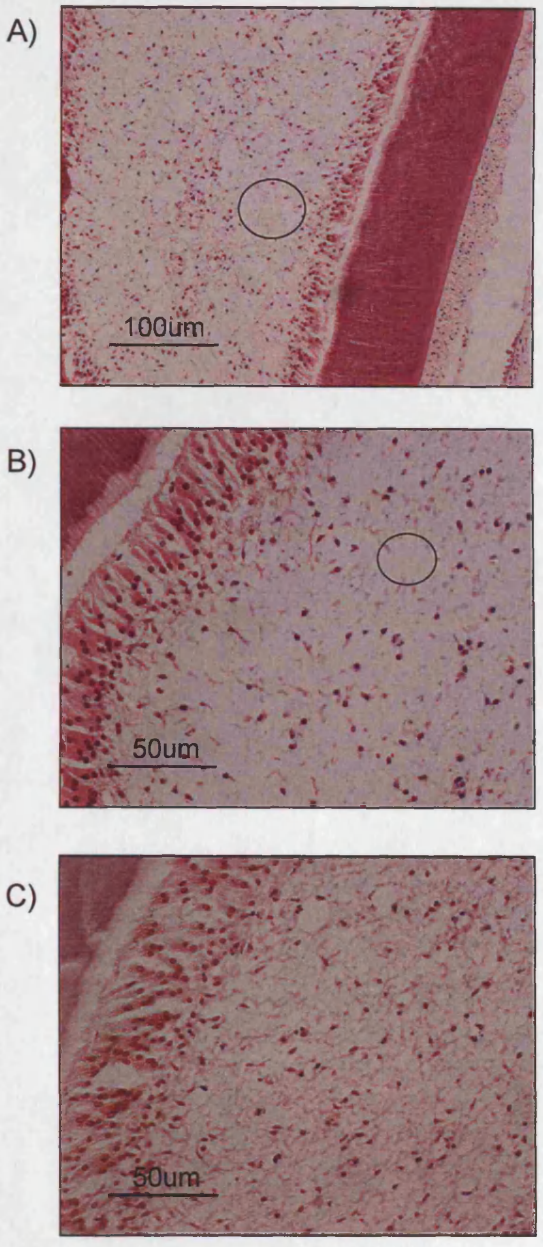

Fig. 4.4 4 hr incubation of sterile tooth slices with supernatant from *S. anginosus* 39/2/14A at 0.6µg/ml. A) With supernatant x20 mag. Areas where cells are absent can be seen (indicated by circle). B) With supernatant x40 mag. Some areas of tissue breakdown can be seen (marked by black circle) at higher magnifications, indicating that supernatants have begun to degrade the mammalian tissues. C) Control x40 mag. Staining of the matrix between cells confirmed tissue breakdown seen in test slices is an effect of supernatants and not a processing artefact.

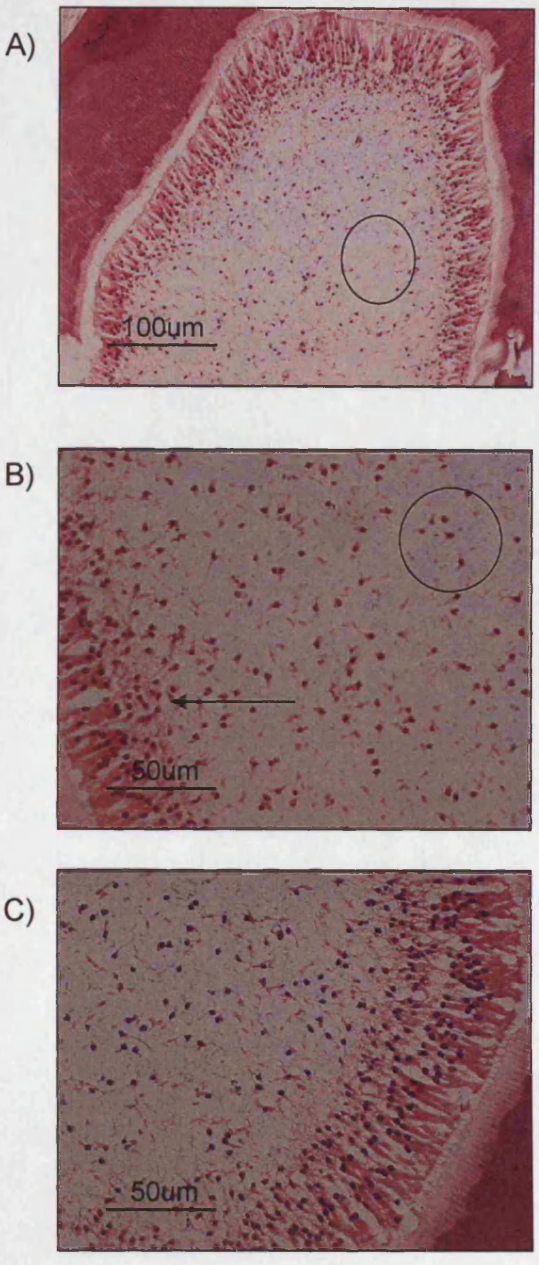

Fig. 4.5 8 hr incubation of sterile tooth slices with supernatant from *S. constellatus* 45386 at 0.6µg/ml. A) Co-culture x20 mag. Cell numbers appear to be decreasing with large areas with no cells present being apparent (marked by circle) B) Co-culture x40 mag. Tissue breakdown around the cells is apparent and can be seen as a lack of dense staining in the surrounding area (indicated by circle). Majority of damage appeared to be confined to the pulpal area, cell-rich zone and odontoblasts appear relatively unaffected (Arrow). C) Control x40 mag.

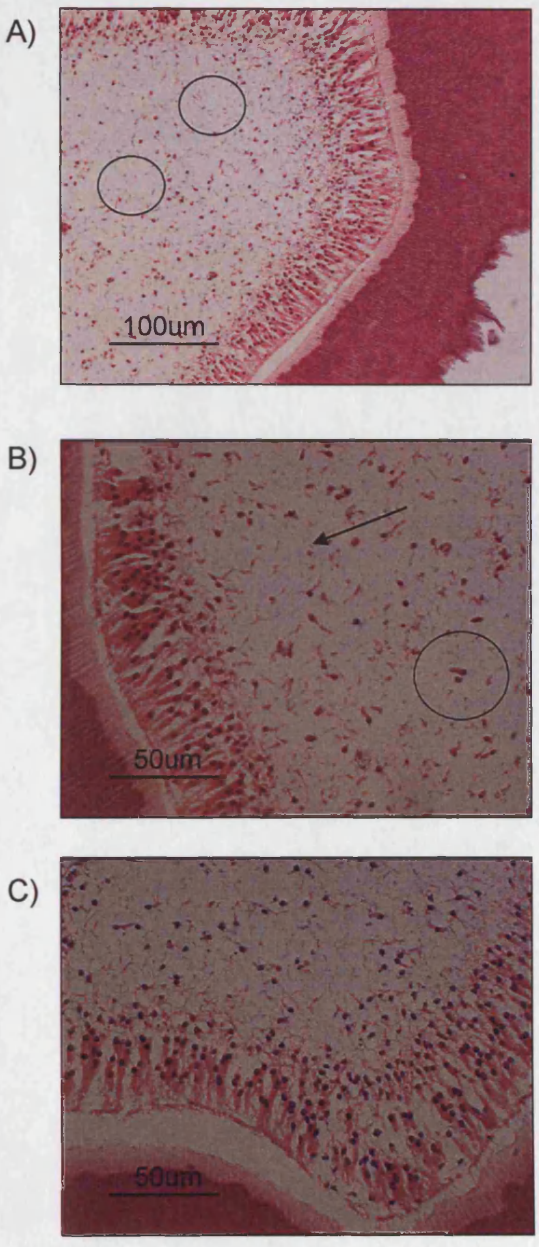

Fig. 4.6 8 hr incubation of sterile tooth slices with supernatant from *S. anginosus* 39/2/14A at 0.6 µg/ml. A) With supernatant x20 mag. Acellular areas can be seen throughout the pulp (indicated by circles). B) With supernatant x40 mag. Acelllular areas are more apparent (indicated by arrow) and breakdown of tissues surrounding the cells can be seen as a lack of matrix staining (indicated by circle). C) Control x40 mag.

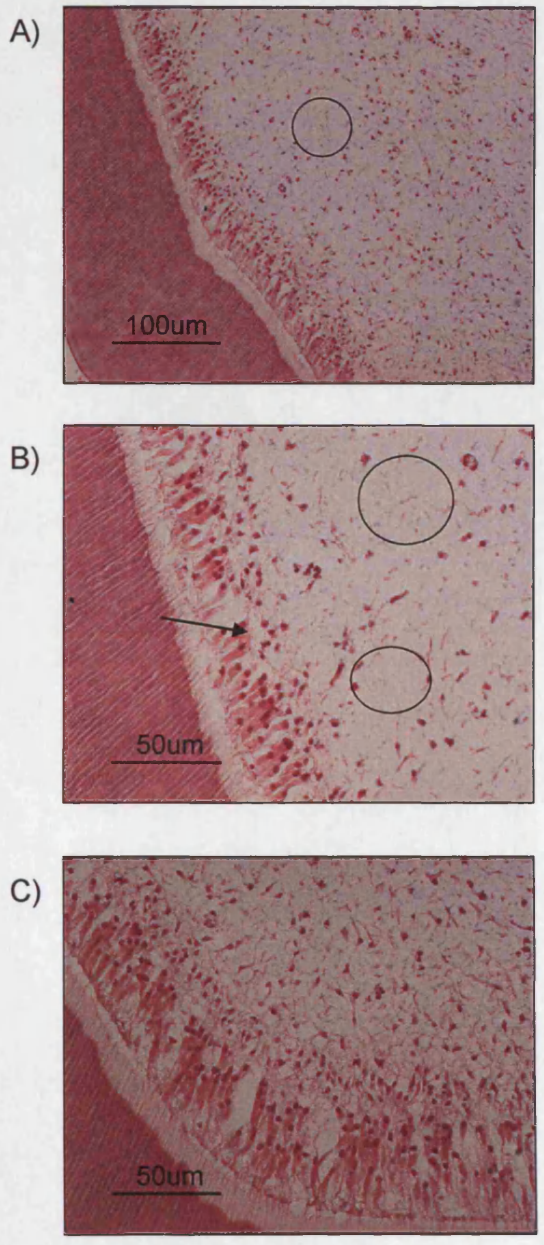

Fig. 4.7 24 hr incubation of sterile tooth slices with supernatant from *S. constellatus* 45386 at 0.6mg/ml. A) Co-culture x20 mag. Large areas of tissue breakdown and an absence of viable cells (indicated by circle). Odontoblast layer appears less densely packed. B) Co-culture x40 mag. Cell rich zone appears markedly depleted of cells (indicated by arrow). Cell death in the pulpal region is seen as areas without staining (indicated by circles). C) Control x40 mag.

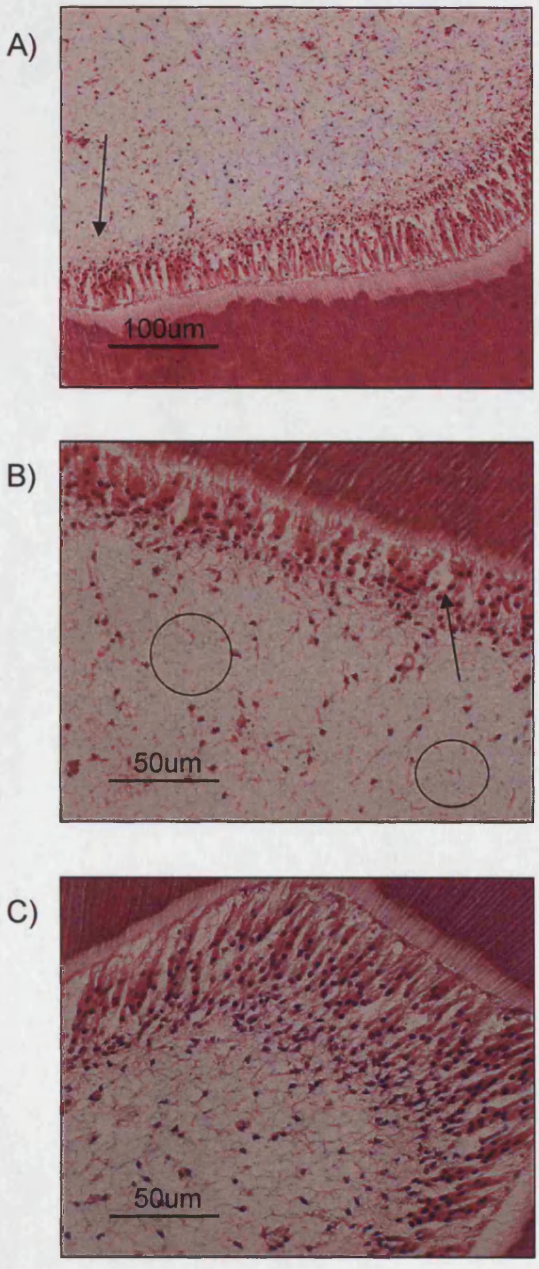

Fig. 4.8 24 hr incubation of sterile tooth slices with supernatant from *S. anginosus* 39/2/14A at 0.6mg/ml. A) Co-culture x20 mag. Cell numbers appear reduced, particularly in the cell-rich zone which has become indistinguishable from the odontoblasts in some areas (indicated by arrow). B) Co-culture x40 mag. Large areas of tissue breakdown and absence of viable cells are visible (indicated by circles). Odontoblast layer appears less densely packed with increasing gaps between cells (indicated by arrow). C) Control x40 mag.

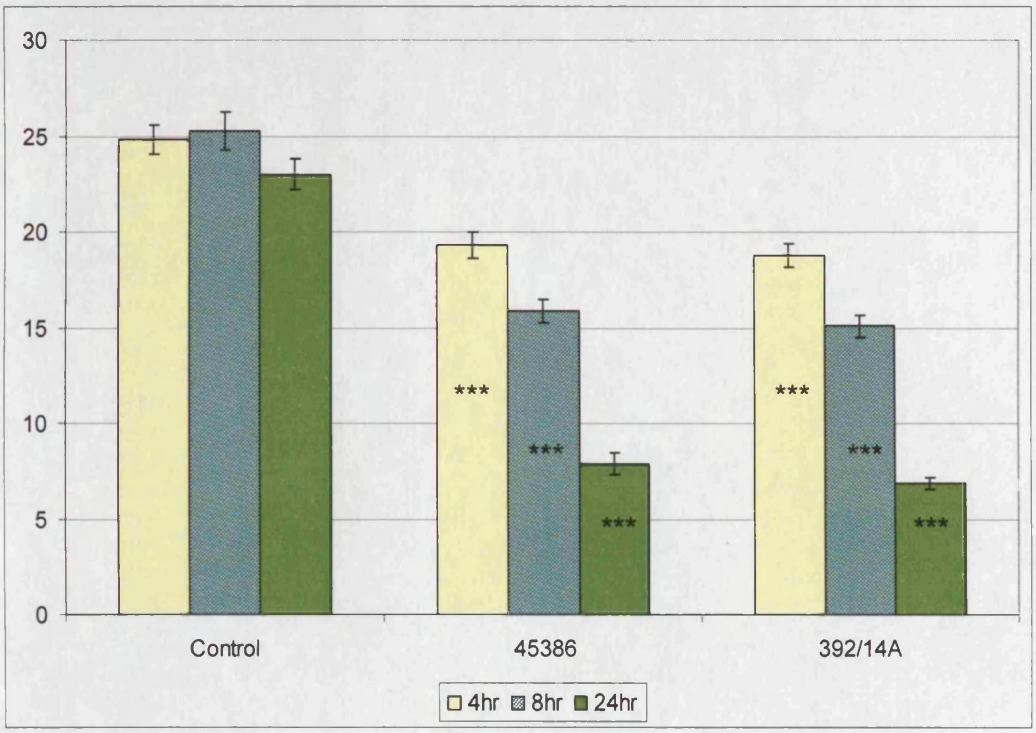

Fig. 4.9 Pulpal cell counts for tooth slices cultured in DMEM+10% BHI with an addition of approximately 0.6 µg/ml supernatant protein. Nuclear counts were performed to provide an average cell number in a $50\mu m^2$ area of the pulp. Counts indicated that culture in the presence of supernatants from *S. anginosus* 39/2/14A and *S. constellatus* 45386 causes a significant decrease in cell number after 4 hours and that cell number continues to significantly decrease up to 24 hours in culture. Error bars represent standard error.

*** = P<0.001

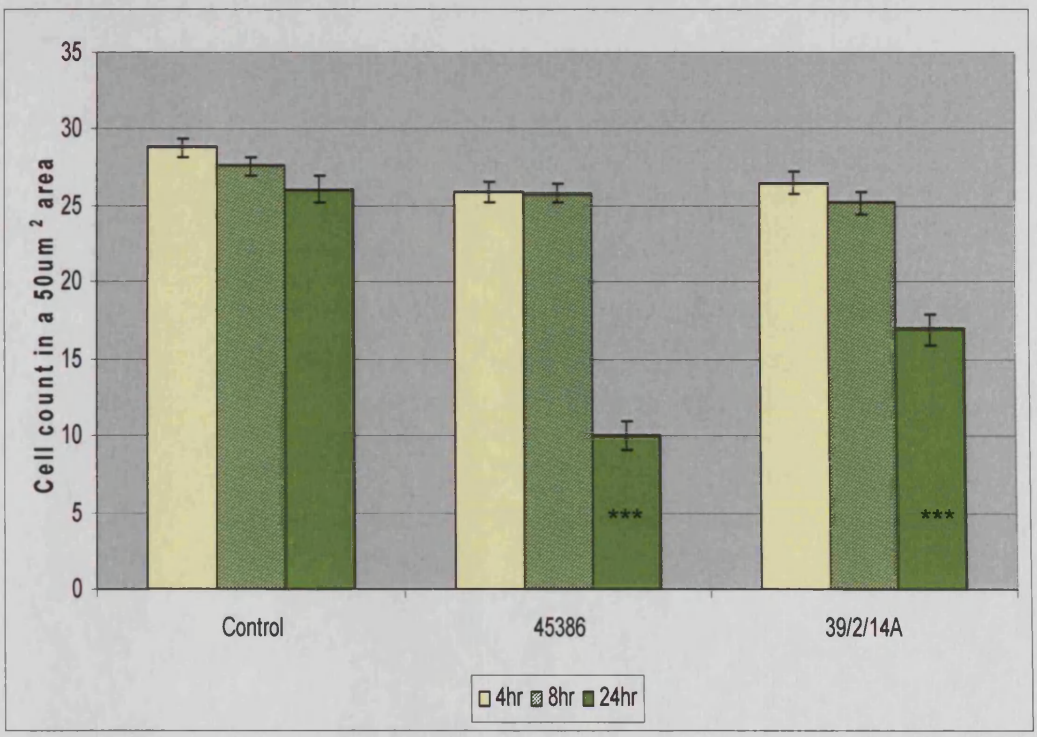

Fig. 4.10 Odontoblast counts for tooth slices cultured in DMEM+10% BHI with an addition of approximately 0.6 µg/ml supernatant protein. Nuclear counts were performed to give an average cell number for a 50µm^2 area of the odontoblast layer. There was no significant difference between the results seen for either SAG species. Cell counts at 4 and 8 hours were similar and showed no significant difference from the control counts. After 24 hours incubation there was a significant decrease in odontoblast number.

*** = P<0.001

4.4 Discussion

The introduction of supernatants into the tooth slice culture system resulted in a significant decrease in cell number within the pulpal tissues of the tooth. Similar results were observed for both *S. constellatus* 45386 and *S. anginosus* 39/2/14A, suggesting that the proteins which are present in both supernatants play a major role in the matrix breakdown and cell death seen in tooth slices.

Analysis of the supernatants produced by the different SAG species was important to establish any differences or similarities between the supernatants. SDS-PAGE was used to separate proteins in the supernatants according to their molecular weight. Silver staining was then used to visualise the proteins. SDS-PAGE of the BHI control showed no bands, indicating that the control was free of protein. A comparison of the protein profiles produced by supernatants from *S. anginosus* 39/2/14A, *S. constellatus* 45386 and the BHI control revealed that there were a far larger number of distinct proteins in the supernatant of *S. constellatus* 45386. Using the protein molecular weight ladder as a reference, the approximate molecular weight of these proteins was calculated, as the ladder consists of protein extracts of known weight which separate on the gel according to their weight. Comparing the position on the gel of the bands from the supernatants with those in the DNA ladder, their weight could be estimated. There were 2 distinct bands that were apparent in both supernatants and these proteins were of molecular weights of approximately 200 and 100kDa. Previous studies have identified a variety of proteins in the supernatants of SAG bacteria, including neuraminidase, α-galactosidase and N-acetyl-β-D-glucosaminidase (Willcox et al. 1995). The enzymatic activities of these proteins may have a role in infection as they are able degrade host tissues and facilitate the spread of bacteria. They have also been found to assist in the adhesion of other bacteria to the infected tissue by modifying surrounding host proteins (Beighton and Whiley 1990).

Previous studies have identified an enzymatically active form of hyaluronidase that has a molecular weight of 107kDa and is expressed by streptococcal species (Berry et al. 1994). This suggests that the densely stained band seen in *S. constellatus* 45386 supernatant at approximately 100kDa may be produced by a hyaluronidase enzyme. The band at the same molecular weight in the supernatant produced by *S. anginosus*

39/2/14A is fainter and may indicate that there is less of this protein being produced or that this is a different protein of a similar weight, as strains of *S. constellatus* have been shown to have an increased incidence of expression of hyaluronidase (Homer et al. 1993; Takao 2003) compared to other SAG species.

The band seen at 200kDa is in the molecular weight range of a number of bacterially produced proteases which degrade host proteins including albumin, collagen and salivary proteins (Juarez and Stinson 1999). Although further analysis is required to confirm the identity of this protein, the tissue degradation seen in histological examination of tooth slices cultured with the SAG supernatants could be explained by the presence of proteases, in particular a collagenase as there are large amounts of collagen in the pulpal matrix (Linde 1985).

A band can be seen in the protein profile produced from the *S. constellatus* 45386 supernatant at approximately 45kDa that is absent in the supernatant from *S. anginosus* 39/2/14A. This band may indicate the expression of chondroitin sulfatase as this enzyme has been shown to have a molecular weight of around 45kDa (Yoshida et al. 2002) and has also been shown to be produced by *S. constellatus* species. In contrast, a number of *S. anginosus* isolates which have been tested have been found not to express chondoritin sulfatase (Homer et al. 1993; Jacobs and Stobberingh 1995).

The densely stained band at approximately 130kDa that is present in the supernatant of *S. anginosus* 39/2/14A does not appear to have a corresponding band in the supernatant of *S. contellatus* 45386. Although it was not possible to ascertain a potential identity of this protein from the literature it is unlikely to be a result of unseparated proteins as replicates of the experiment showed similar results, with a larger number of bands in the supernatant of *S. constellatus* 45386.

The difference in protein profiles of the supernatants is not unexpected as a number of previous studies have identified different protein expression between the SAG species (Grinwis et al. 2010; Jacobs and Stobberingh 1995; Willcox et al. 1995). Also, despite there being an increased number of distinct proteins present in the supernatant of *S. constellatus* 45386 compared to that of *S. anginosus* 39/2/14A, the total protein

concentration of each supernatant was calculated to be approximately 0.6 μg/ml and appeared to have a similar effect on tooth slices when introduced into the culture system.

As in the tooth slices incubated directly with bacteria, the cell death seen in the tooth slices incubated with supernatants is likely to be the result of necrosis, rather than apoptosis. This is characterised by the loss of nuclei that were present in control sections and the presence of cells with a cloudy appearance in the histological sections. These factors result in a decreased cell count compared to control sections, as the cells which do not fall within set parameters were not counted.

Following incubation of tooth slices with collected supernatants, histological examination showed a significant decrease in cell number over a 50 μm^2 area after 4 h of incubation, from an average of 25 cells in the control to 19 cells in those incubated with bacteria. Cell death continued to increase with increased periods of incubation, with extensive necrosis seen after 24 h. This was seen as a decrease in cell number to an average of 7 cells in a 50 μm^2 area, with fewer cells stained throughout the pulp, gaps which appeared between the previously densely packed cells of the odontoblast layer and the degradation of the cell-rich zone. The pattern of cell necrosis and matrix degradation appeared to be similar to that which was observed when tooth slices were incubated directly with bacterial suspension (**3.3.2**), as pulpal cells were affected first, with cell death and matrix breakdown increasing with longer incubation times. However, the effect seen when the tooth slices were incubated with the supernatants did appear to be more rapid and less localised. When tooth slices were incubated directly with the bacteria, a significant decrease in pulpal cell numbers was not seen in those tooth slices incubated with *S. constellatus* 45386 until 8 h of incubation, whilst a significant amount of cell death had already occurred after 4 h incubation with *S. constellatus* 45386 supernatant. Effects seen after 8 h of incubation were similar for both supernatants and bacteria, whilst after 24 h of incubation there were fewer cells in those tooth slices incubated with supernatants, an average of 7 cells per 50 μm^2 area, compared to those tooth slices incubated directly with bacteria, which still had an average of 15 cells per 50 μm^2 area.

In addition to an increase in pulpal necrosis, the histological examination of tooth slices incubated with SAG supernatants also confirmed that there was a significant decrease in cell numbers in the odontoblast layer after 24 h incubation with the supernatants. Prior to this, the decrease in cell number was limited to the pulpal region, as seen in the tooth slices incubated directly with SAG bacteria. Whilst the tooth slices incubated with the bacteria appeared to have a pattern of cell death which was focussed in the centre of the pulpal chamber, those slices incubated with supernatants showed cell death throughout the pulp. Tooth slices incubated directly with bacteria and those incubated with supernatants showed a similar pattern of matrix degradation which was seen as a decrease in the staining around the cells and, in tooth slices incubated directly with bacteria, was associated with microcolonies of SAG that attached to the pulp. However, there was no significant decrease in cell number of the odontoblast layer after 24 h of incubation, this being observed only in those tooth slices incubated with SAG supernatants. This suggests that the supernatant production by bacteria in dentinal tubules may be of particular importance in the clinical situation as the degradative and cytotoxic components appear to be able to spread freely throughout the pulp, rather than being limited to areas of bacterial attachment. This highlights the significance of dentinal tubule infection and its role in pulpal inflammation and necrosis. These findings suggest that in addition to antimicrobials that are targeted towards eradicating the presence of infecting organisms, substances which protect the pulp from their products should also be investigated. Studies have shown that albumin is able to inhibit the cytotoxicity of zinc-oxide eugenol *in vivo* (Schmalz et al. 2000). Whilst this compound is found as an ingredient in filling materials rather than being a bacterial product, it indicates that there is potential for development of other such substances which may be of use in pulp protection against bacterial supernatants which enter the pulp from the dentinal tubules.

The increased incidence of pulpal necrosis seen in tooth slices incubated with sterile supernatants can be accounted for because of the difference in bacterial protein levels at the initial point of culture. When tooth slices were incubated with bacterial suspension, the bacterial numbers were at approximately 10^2 cfu/ml when they were introduced into the co-culture system and were in the log phase of growth. The supernatant which was collected for introduction into the tooth slice system was

collected from an overnight culture of bacteria which contained an excess of 10^8 cfu/ml of bacteria which were in the stationary phase of growth. Whilst the bacteria which were introduced into the system would require some time to begin producing sufficient level of enzymes and toxins to have a visible effect on the tooth slices, the collected supernatants already contain proteins produced from all bacterial growth phases. The protein concentration is also likely to be higher than that initially produced by the viable bacteria due to the higher bacterial load from which the supernatant was collected.

Cell counts which were performed over a $50\mu m^2$ area on tooth slices which had been exposed to the supernatants from the different species for the same length of time showed no significant different in cell number, indicating that the supernatants from the two species had a similar effect on the tooth slices, despite their different protein profiles. This further supports the hypothesis that the proteins which are present in both profiles play a role in tissue breakdown. Further investigation into the identity of these proteins and their role in infection may be beneficial to understanding the processes involved in pulpal infection.

The role of the supernatants of SAG bacteria in pulpal inflammation and necrosis has not been reported in the literature, although there are some studies which focus on the effect of other bacterial supernatants and by-products, particularly those produced by the black-pigmented *Bacteroides.* Pulpal fibroblasts which have been exposed to supernatants from these bacteria have been shown to have an inflammatory response, producing cytokines such as IL-6 and IL-8 (Yang et al. 2003a; Yang et al. 2003b) and stimulating production of matrix metalloproteinases (MMPs) (Chang et al. 2002). Secretion of such products by pulpal fibroblasts has been shown to result in matrix degradation and stimulation of inflammatory cells. Tissue breakdown that is associated with the presence of SAG supernatants may therefore indicate that they are stimulating a similar response from the pulpal fibroblasts.

The results presented in this chapter have demonstrated that the bacterial products which diffuse into the surrounding growth media play an important role in the cell necrosis and matrix breakdown that is seen in tooth slices incubated with SAG bacteria. Investigation of the effects of the supernatants in the absence of bacterial

attachment suggested that bacterial proteins may be the principal cause of the tissue damage observed, rather than mechanical action of bacterial attachment, as cell counts revealed that there was an increased incidence of cell death in tooth slices incubated with supernatants. Although this cell death may have been more pronounced due to the increased protein concentration in these experiments, it suggests that bacterial products are important in the infection process, as the tissue breakdown that was observed is likely to release nutrients for further bacterial growth. SDS-PAGE analysis, in conjunction with calculation of protein concentration of the supernatants, suggested that whilst different SAG isolates may produce a variety of different proteins, their overall effect on mammalian tissues is similar, resulting in no significant difference in cell death between different isolates. In summary, these results have highlighted the importance of SAG supernatants in pulpal inflammation and cell death and have demonstrated a further use of the model presented in this study.

Chapter 5: Antimicrobial activity of chlorhexidine and triclosan against SAG bacteria *in vitro.*

5.1 Introduction

The use of antimicrobials in endodontic infection has been an important development in eradicating bacteria from the root canal. This is important as re-infection is one of the major reasons for treatment failure (Dahlen 2002; Siqueira and Rocas 2008) and improved methods of root canal disinfection have been shown to increase the chances of treatment success and therefore decrease the chances of subsequent tooth loss (Abou-Rass and Bogen 1998).

Calcium hydroxide is the most commonly used antimicrobial in fillings but, as previously discussed (**1.5**), there is no specific action of this antimicrobial on the bacteria, its activity being based upon the pH change that it incurs (El Karim et al. 2007; Desai and Chandler 2009), and this may not be effective against all bacterial species present in the root canal (Desai and Chandler 2009). To further improve the disinfection of the tooth before filling, novel antimicrobials need to be developed which have a specific bacterial activity, particularly against those organisms which have been identified as playing a role in endodontic infection. As inflammation plays an important role in pulpal necrosis and impacts upon the resolution of infection in the root canal, there has been particular interest in antimicrobials that also have anti-inflammatory properties (Farges 2009), such as triclosan (Mustafa et al. 1998), or utilisation of naturally occurring anti-inflammatory antimicrobial peptides such as defensins (Dhople et al. 2006).

The standard protocol for testing the efficacy of antimicrobials against specific bacterial species involves calculation of the minimum inhibitory concentration (MIC). When bacteria grow in liquid media under standard conditions, the media will become turbid or 'cloudy.' The inhibition of this visible growth is used to measure the efficacy of an antimicrobial, with the MIC being defined as the lowest concentration of the antimicrobial which inhibits visible growth after overnight incubation. Whilst the MIC of an antimicrobial relates to the level required to prevent the bacterial growth, it does not always cause bacterial death and decrease of cell number. The

lowest concentration of an antimicrobial that causes bacterial cell death is known as the minimum bactericidal concentration (MBC), though this is not as widely used as a test of antimicrobial efficacy as the MIC test.

In this study, the effect of triclosan on SAG growth was studied. Triclosan was chosen as it has already been incorporated into a number of dentifrices and its anti-inflammatory properties, in addition to its action as an antimicrobial, make it a potentially useful substance in controlling pulpal disease (Mustafa et al. 1998). Whilst the effect of triclosan has been studied on many organisms such as *Pseudomonas aeruginosa* (Escalada et al. 2005), *Escherichia coli* (Yu et al. 2010) and *Staphylococcus aureus*, including methicillin-resistant strains (Zafar et al. 1995), its efficacy against SAG bacteria has not been previously studied. The efficacy of triclosan against SAG bacteria was compared to the efficacy of chlorhexidine gluconate (CHX) in its commercially available forms. CHX is effective against both Gram positive and negative bacteria through disruption of the bacterial membrane. Triclosan is also thought to act by disruption of the membrane, as previously described (**1.7**). CHX has previously been shown to be effective against *S. anginosus* biofilms *in vitro* (Chavez de Paz et al. 2010), thus providing a suitable antimicrobial for comparison with triclosan.

The efficacy of triclosan and CHX was tested using 2 different methods – one which counts viable cells following exposure to the biocides and one which employs the Bioscreen C system. This is an automated incubator which is able to measure turbidity using a technique that is more sensitive than a spectrophotometer. The Bioscreen C system allows analysis of the bacterial growth kinetics following biocide exposure.

The development of the co-culture model in this study may be useful in the testing of novel antimicrobials, and the suitability of existing antimicrobials such as triclosan and chlorhexidine, for use as endodontic disinfectants. As the model allows for the quantification of tissue damage by the bacteria (**3.3.2**), it may be further developed to assess the efficacy of antimicrobials in reducing this bacterial tissue damage. In addition, it provides a model which can be used in preliminary testing of antimicrobial toxicity, which may have benefits over existing models. As previously outlined (**1.6**), *in vitro* models using single mammalian cell populations do not consider the

importance of spatial arrangement of the dental cells and the effect this may has on dental repair and bacterial attachment. Similarly, there are drawbacks of the available *in vivo* models due to ethical implications, costs and the complications of systemic influences and commensal bacteria. The model proposed here may overcome some of these problems with current model systems with the aim of improving the accuracy of antimicrobial testing.

5.2 Materials and Methods

5.2.1 Minimum inhibitory concentration of triclosan

Triclosan, under the brand name Irgasan, was dissolved in 5% (w/v) β-cyclodextrin in DMEM+10% BHI to give a triclosan concentration of 512 μg/ml. A doubling dilution series was produced to give a series of triclosan concentrations ranging from 512 – 0.5 μg/ml. 180 μl of each dilution was transferred to the wells of a 96-well plate to produce a dilution series of triclosan in the plate. A control well containing only 5% β-cyclodextrin in DMEM+10% BHI with no triclosan was set up for each dilution series. This was performed in triplicate for each strain being tested.

Overnight cultures of *S. anginosus* 39/2/14A and *S. constellatus* 45386, grown in DMEM+10% BHI, were diluted to an absorbance of Ab550nm = 0.5 to give a bacterial count of 10^8 cfu/ml. 20 μl of diluted suspension was then added to each of the wells containing broth. The plate was then incubated overnight at 37°C, 5% CO_2. The MIC was recorded as the lowest concentration of triclosan which inhibited visible bacterial growth following overnight incubation.

5.2.2 Biocide contact time

To identify an appropriate time period needed for chosen biocides to be effective on SAG bacteria, 5 proposed contact times were tested. Overnight cultures of *S. constellatus* 45386 and *S. anginosus* 39/2/14A grown in BHI broth were centrifuged at 4000 rpm for 10 min. Supernatant was discarded and the resultant pellet was re-suspended in sterile PBS to an absorbance of 0.5 at 550 nm, giving a bacterial suspension of 10^8 cfu/ml. 1 ml of diluted bacterial suspension was added to 1 ml of biocide or control. The biocides used were chlorhexidine gluconate at 4, 0.5 and 0.2%, and triclosan dissolved in 5% (w/v) β-cyclodextrin at 64 and 125 ug/ml. PBS was used as a control. The suspension and biocide mix were incubated at room temperature with a 100 μl sample taken at 10, 20, 30, 45 and 60 min. The sample was transferred to 900 μl of neutraliser to immediately neutralise the effect of the biocide and prevent further cell death. The resulting bacterial sample was then serially diluted and spiral plated onto FAA to allow cell counting and calculation of bacterial numbers in the original suspension.

5.2.3 Bioscreen C antimicrobial testing

Control protocol

Overnight cultures of *S. anginosus* 45386 and *S. constellatus* 39/2/14A grown in DMEM+10% BHI broth were centrifuged at 4000 rpm for 10 min. Supernatant was discarded and the resultant pellet was re-suspended in sterile PBS to an absorbance of 0.5 at 550 nm, giving a bacterial suspension of 10^8 cfu/ml. This suspension was then diluted ten-fold to give a dilution series from 10^8 cfu/ml to 10^1 cfu/ml. 350 µl of sterile DMEM+10% BHI broth was added to 90 wells of a 100 well Bioscreen plate (Oy Growth Curves AB Ltd, Helsinki, Finland). To each of the test wells 50 µl of the diluted suspensions were added to each of the test wells to give 10 wells of each dilution. Into the final 10 wells 50 µl of sterile PBS was added. The plate was then incubated in the Bioscreen for 20 h with an absorbance reading taken using a wide band filter every 15 min. Viability counts were performed from dilutions from 10^4 cfu/ml to 10^1 cfu/ml to allow calculation of the number of viable bacteria in the original inoculum. 10 µl drops were plated in triplicate on FAA and incubated overnight at 37°C, 5% CO_2.

Biocide test protocol

Overnight cultures of *S. anginosus* 45386 and *S. constellatus* 39/2/14A were cultured and diluted to 10^8 cfu/ml as previously described. The biocides and controls used are listed in Table 5.1. As for the control plate, 350 µl of DMEM+10% BHI was added to 80 wells, allowing 10 wells for testing of each of the biocides and their controls and 10 wells for the PBS growth control. 1 ml of bacterial suspension was then added to 1ml of biocide or control and incubated for 10 min. 1 ml of each biocide and suspension mixture was then transferred to 9 ml of neutraliser and mixed. 50 µl of each mix was then added to 10 wells of the plate. In the final 10 wells 50 µl of PBS was added to the broth. The plate was then incubated in the Bioscreen for 20 h with an absorbance reading taken using a wide band filter every 15 min.

	Biocide
1	4% chlorhexidine
2	0.5% chlorhexidine
3	0.2% chlorhexidine
4	125μg/ml triclosan in 5% β-cyclodextrin
5	64μg/ml triclosan in 5% β-cyclodextrin
6	PBS control

Table 5.1 List of biocides and controls used in the Bioscreen C test

5.3 Results

5.3.1 Minimum inhibitory concentration of triclosan

Following overnight incubation at 37C, 5% CO2 in the presence of various triclosan dilutions dissolved in 5% β-cyclodextrin solution, both *S. anginosus* 39/2/14A and *S. constellatus* 45386 were found to be inhibited by 125 μg/ml triclosan. Inhibition of growth was determined by visual examination of the plate and the MIC was defined as the lowest triclosan concentration that inhibited visible turbidity, with media remaining the same absorbance as that in the control wells. Control wells containing 5% β-cyclodextrin with no triclosan showed no inhibition of bacterial growth.

5.3.2 Biocide contact time

Bacteria were initially cultured at a concentration of 10^8 cfu/ml. Following dilution in the biocide and neutralisation, bacterial concentration was 10^3 cfu/ml. The minimum contact time that the bacteria were exposed to biocides was 10 min. Following the 10 min incubation period there was no growth on the FAA plates inoculated with bacteria that had been exposed to the chlorhexidine solutions. The maximum contact time tested was 60 min. After this time point, strains which were exposed to triclosan continued to grow. Bacterial counts were taken for each strain following each incubation period (Fig 5.1 and 5.2). Bacterial isolates incubated with triclosan at a concentration of 125 μg/ml showed a greater reduction in number at shorter incubation times. For *S. anginosus* 39/2/14A, a 10 minute incubation with 125 μg/ml triclosan resulted in a reduction of bacterial numbers by 2.5 x 10^3 cfu/ml whilst incubation with 64 μg/ml triclosan over the same time period resulted in a reduction of 1.9 x 10^3 cfu/ml (Fig. 5.1). However, at 60 min, both concentrations showed a reduction of approximately 2.3 x 10^3 cfu/ml, indicating that higher concentrations of triclosan only have an increased biocidal effect at shorter incubation times. Bacteria incubated with controls had a bacterial count of 4.1 x 10^3 cfu/ml. Results for *S. constellatus* 45386 were similar, with bacterial numbers being reduced by approximately 2.2 x 10^3 cfu/ml after 60 min incubation with both 64 and 125 μg/ml triclosan (Fig. 5.2). This indicates that at these concentrations triclosan has a similar effect on the different SAG strains.

5.3.3 Bioscreen testing of antimicrobial efficacy

Following the results of the biocide contact time experiment, bacteria were incubated with biocides for 10 min prior to growth in the bioscreen system. The control for each strain consisted of growth curves generated by bacterial suspension, which had not been exposed to the biocide, with different bacterial concentrations at the start point. Each diluted suspension of *S. anginosus* 39/2/14A showed standard growth curve characteristics with a defined lag, log and stationary phase (Fig 5.3). Diluted suspensions of *S. constellatus* 45386 showed a lag and log phase, although those suspensions which had an initial bacterial load of 10^6 cfu/ml or below did not reach stationery phase after 20 h of incubation (Fig 5.4). However, as this strain was shown to have slower growth when grown in DMEM+10% BHI (Fig 2.3), this represents normal growth under these conditions and it unlikely to be a result of exposure to triclosan. From these curves, an absorbance value was taken which related to a point at which all suspensions had reached the log phase of growth. For these bacteria the absorbance value was 0.2. This was then plotted to give an equation that related the time taken to reach an absorbance of 0.2 with bacterial number (Figs 5.5 and 5.6). Growth curves were then plotted for bacterial suspensions which were exposed to biocides prior to growth in the bioscreen system. The equation relating absorbance to time to reach an absorbance of 0.2 was used to calculate the bacterial numbers following biocide exposure.

As seen in the previous experiments where bacteria were plated out immediately after exposure to biocides, all chlorhexidine solutions demonstrated a complete eradication of both *S. anginosus* 39/214A and *S. constellatus* 45386. *S. anginosus* 39/2/14A suspensions which were exposed to triclosan and the control solutions of 5% β-cyclodextrin and PBS showed similar growth characteristics following neutralisation which mirrored the growth seen in the control (Fig 5.7). However, the bacteria which had been exposed to the biocides showed an increased lag period, indicating that the bacteria had been adversely affected by the treatment with biocides. *S. constellatus* 45386 was also eradicated by the chlorhexidine solutions used. More variation was seen in the growth curves of bacteria incubated with triclosan and control solutions than was observed with *S. anginosus* 39/2/14A (Fig 5.8). In addition to this, *S. constellatus* 45386 did not enter stationary phase as seen with *S. anginosus* 39/2/14A.

Cells appeared to start dying after the log phase, with the maximum absorbance reached at approximately 800 min after incubation.

Using the information from the bioscreen analysis of the SAG bacterial growth following exposure to the biocides, the efficacy of the biocides was assessed by calculating the bacterial reduction (Fig 5.9).

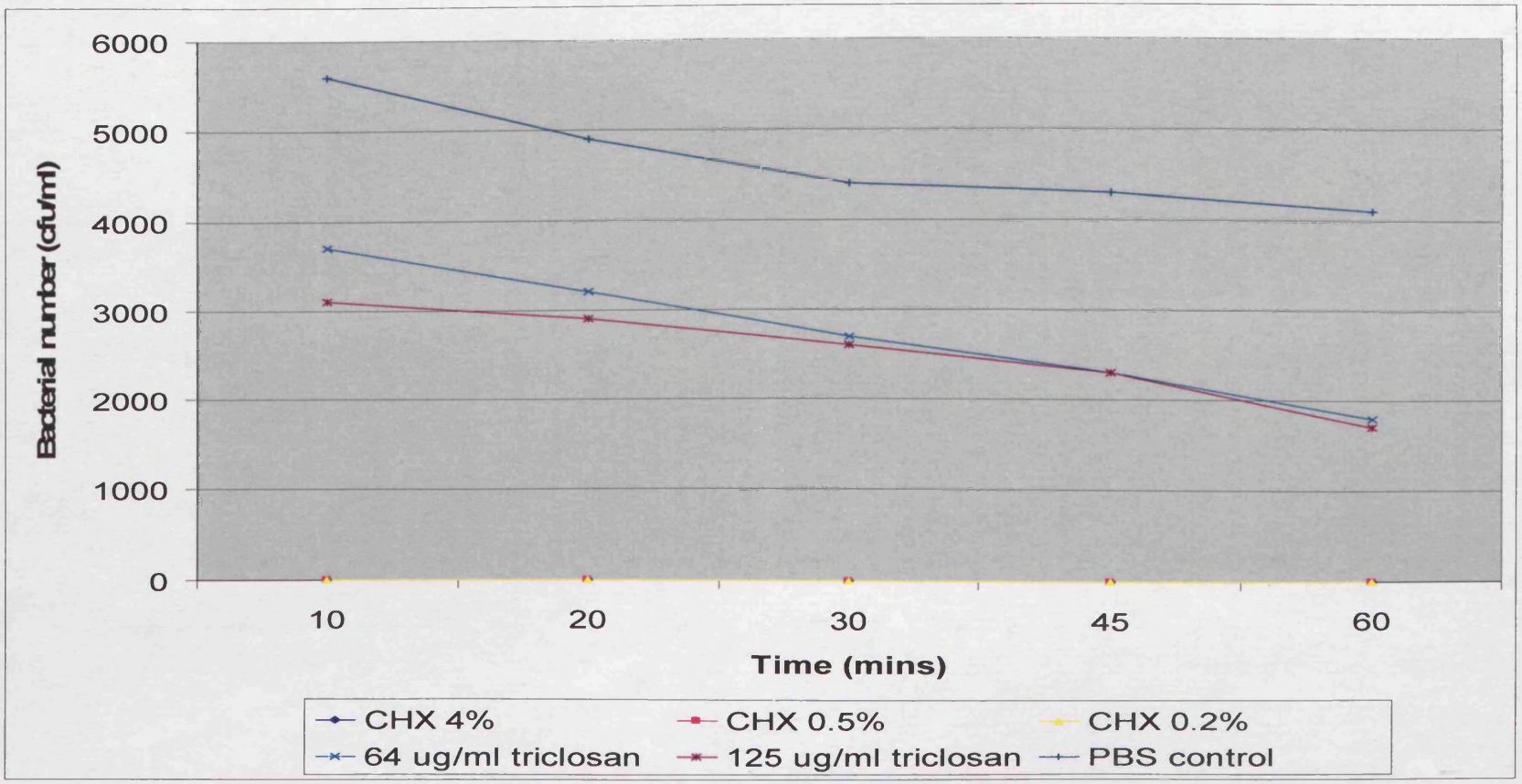

Fig. 5.1 Reduction in numbers of *S. anginosus* 39/2/14A following incubation with biocides for up to 60 min. Incubation with all chlorhexidine solutions results in eradication of bacteria after 10 min incubation. Triclosan solutions cause a reduction in viable bacterial numbers but do not fully eradicate growth after maximum incubation time. Higher concentrations show an increased reduction in bacterial numbers at shorter incubation times.

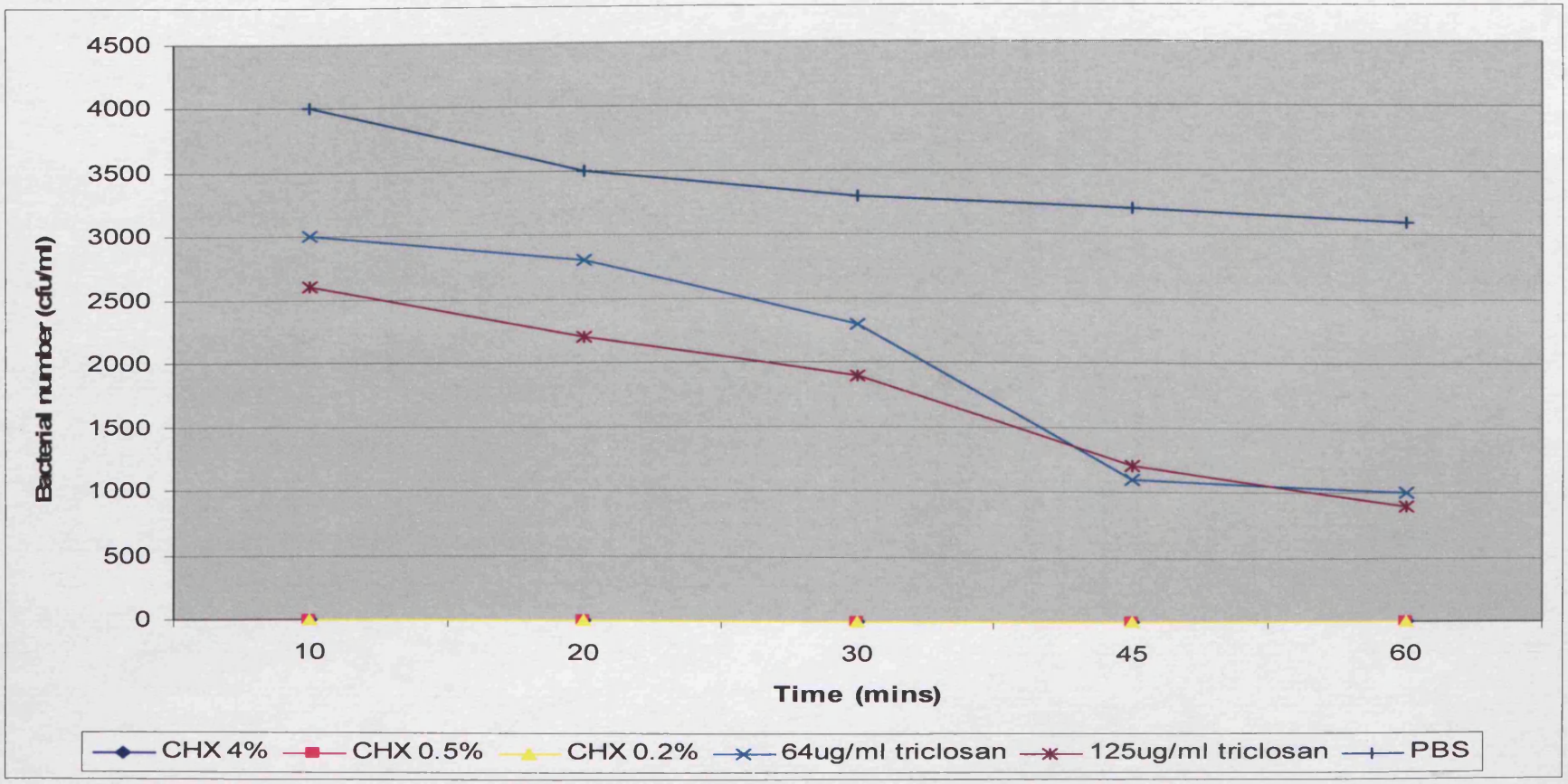

Fig. 5.2 Reduction in numbers of *S. constellatus* 45386 following incubation with biocides for up to 60 min. Incubation with all chlorhexidine solutions results in eradication of bacteria after 10 min incubation. Triclosan solutions cause a reduction in viable bacterial numbers but do not fully eradicate growth after maximum incubation time. Higher concentrations show an increased reduction in bacterial numbers at shorter incubation times.

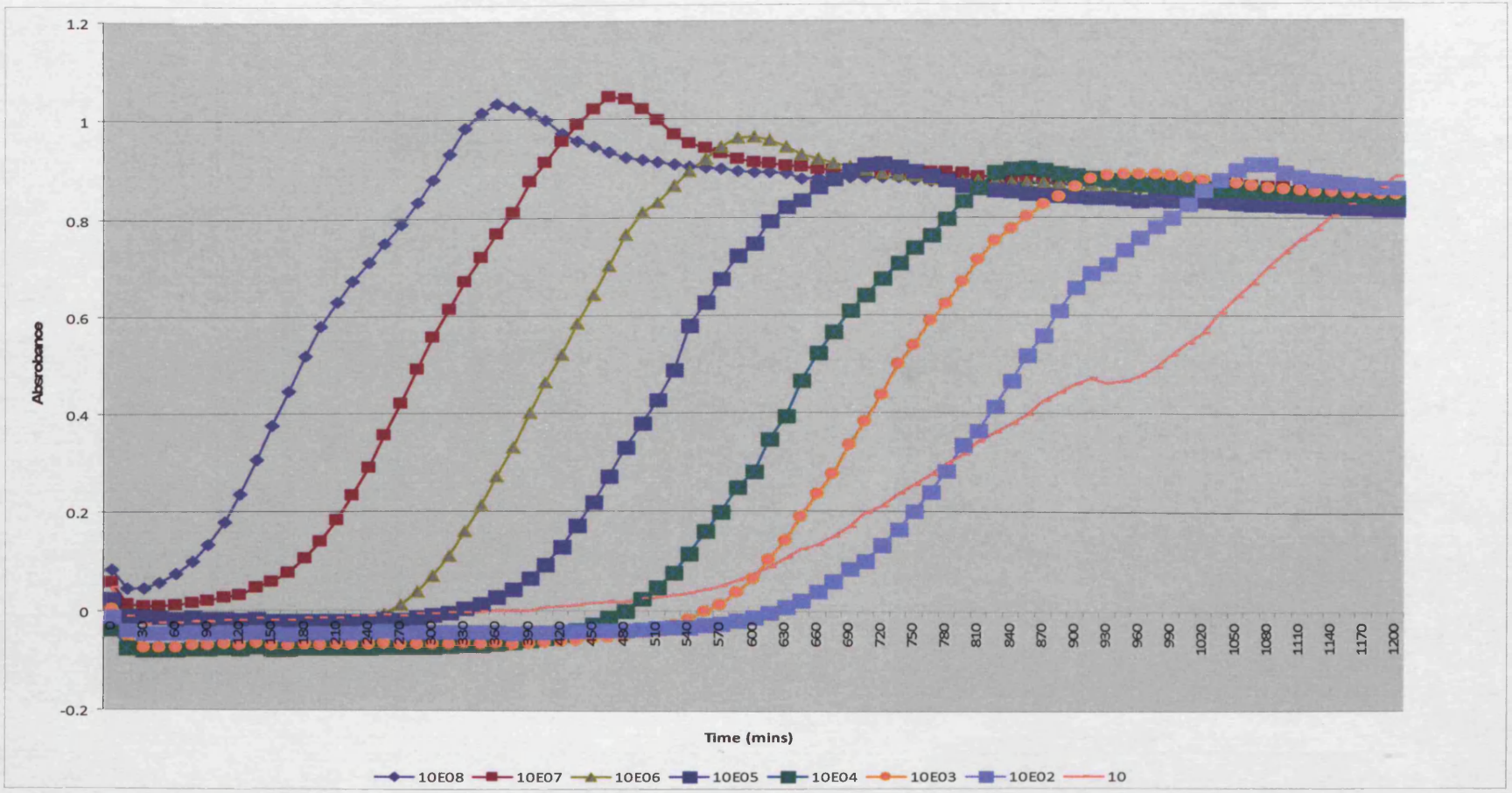

Fig 5.3 Growth curves generated by cultures of *S. anginosus* 39/2/14A incubated for 20 hr in the Bioscreen system. Suspensions ranging from 10^8 cfu/ml to 10^1 cfu/ml were cultured and absorbance measured every 15 min. Each dilution shows a standard bacterial curve with defined lag, log and stationary phases.

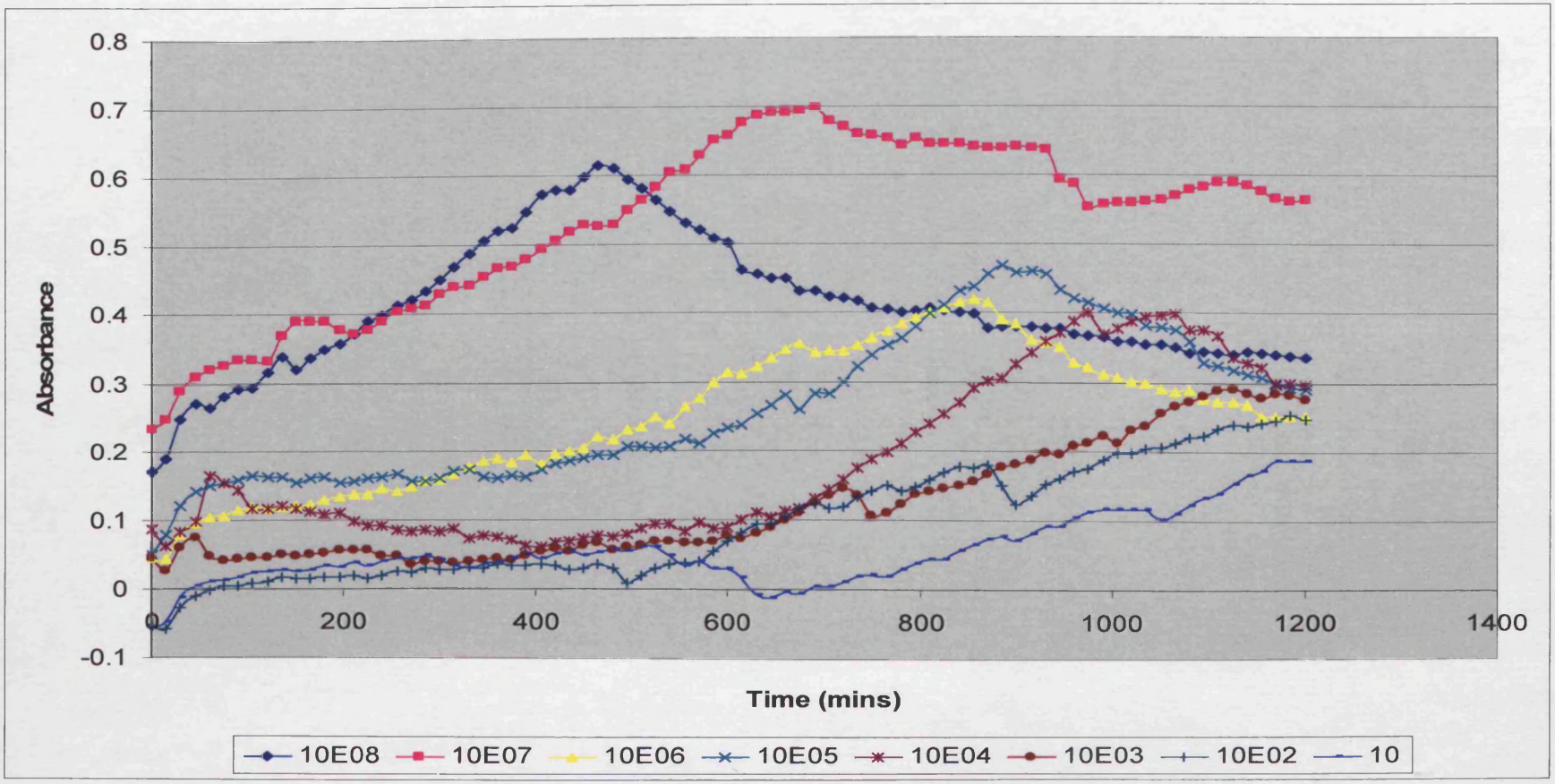

Fig 5.4 Growth curves generated by cultures of *S. constellatus* 45386 incubated for 20 hr in the Bioscreen system. Suspensions ranging from 108 cfu/ml to 101 cfu/ml were cultured and absorbance measured every 15 min. Diluted cultures show a lag and log phase of growth. However suspensions which had an initial bacterial number of 10^6 cfu/ml or below do not appear to reach stationery phase after 20hr of incubation.

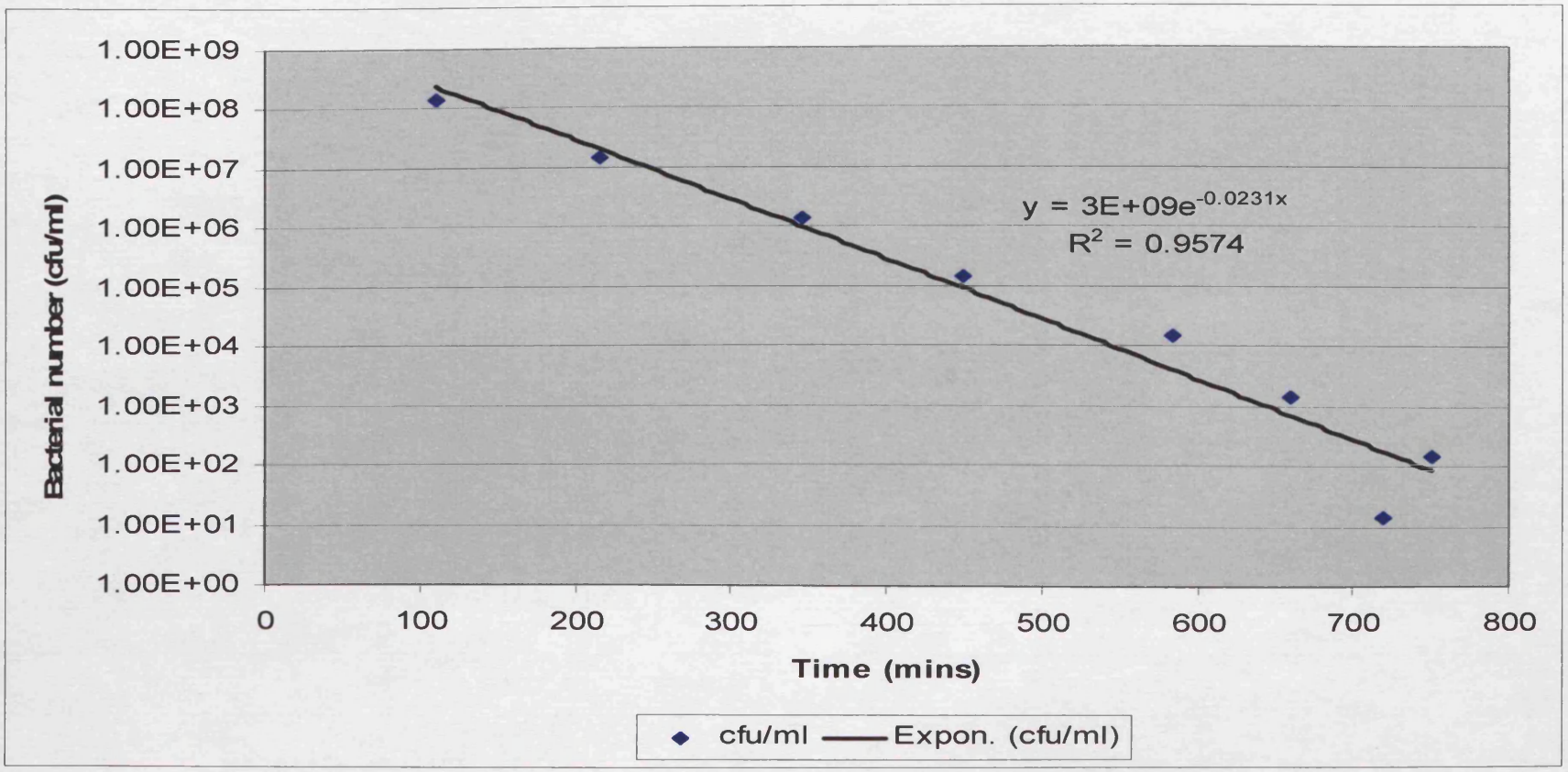

Fig. 5.5 Time taken for diluted cultures of *S. anginosus* 39/2/14A to reach an absorbance of 0.2 when grown in a bioscreen system. The gradient of the line allows calculation of bacterial number of an initial solution by using the time taken to reach an absorbance of 0.2 in a bioscreen system.

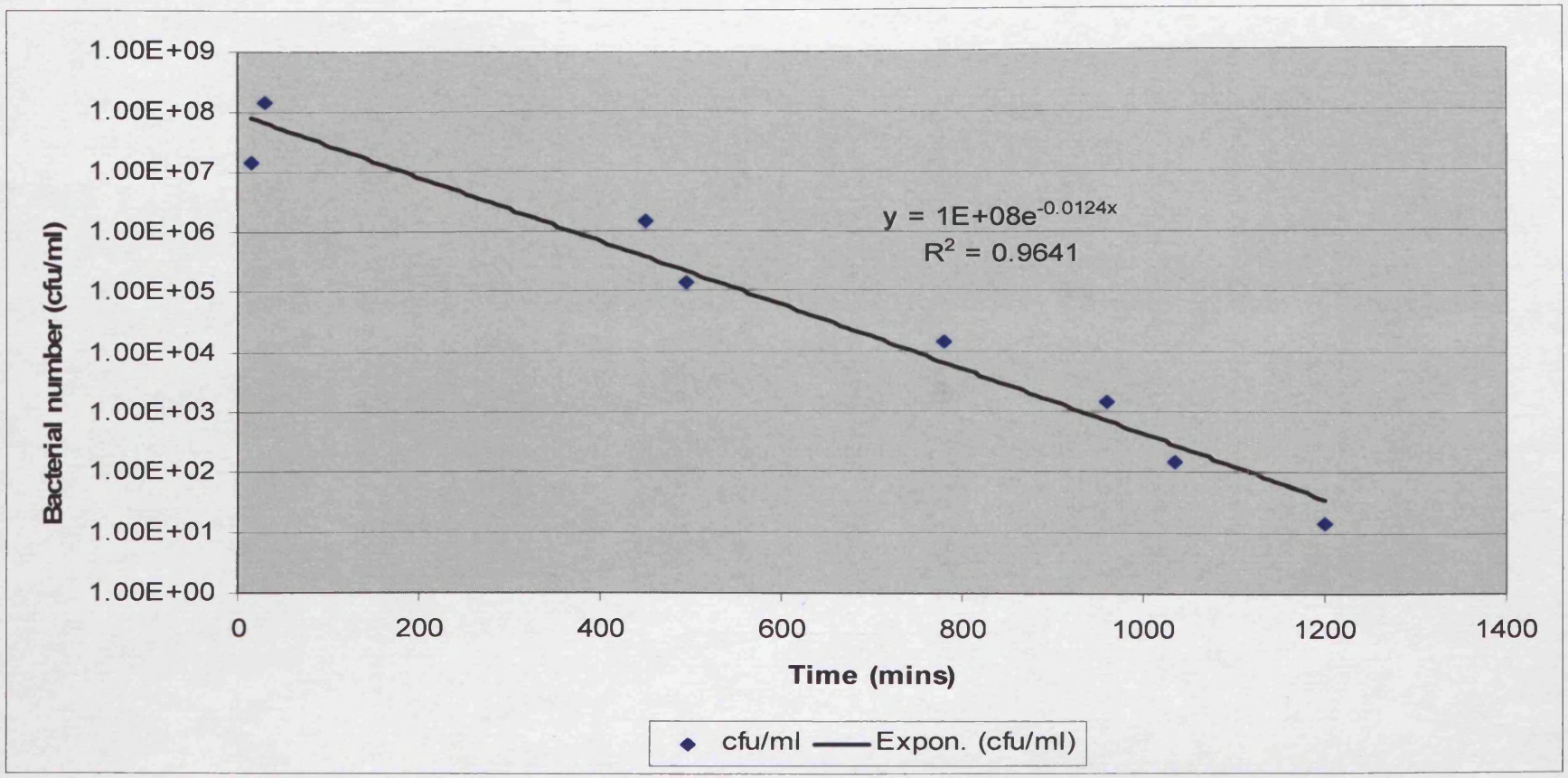

Fig. 5.6 Time taken for diluted cultures of *S. constellatus* 45386 to reach an absorbance of 0.2 when grown in a bioscreen system. The gradient of the line allows calculation of bacterial number of an initial solution by using the time taken to reach an absorbance of 0.2 in a bioscreen system.

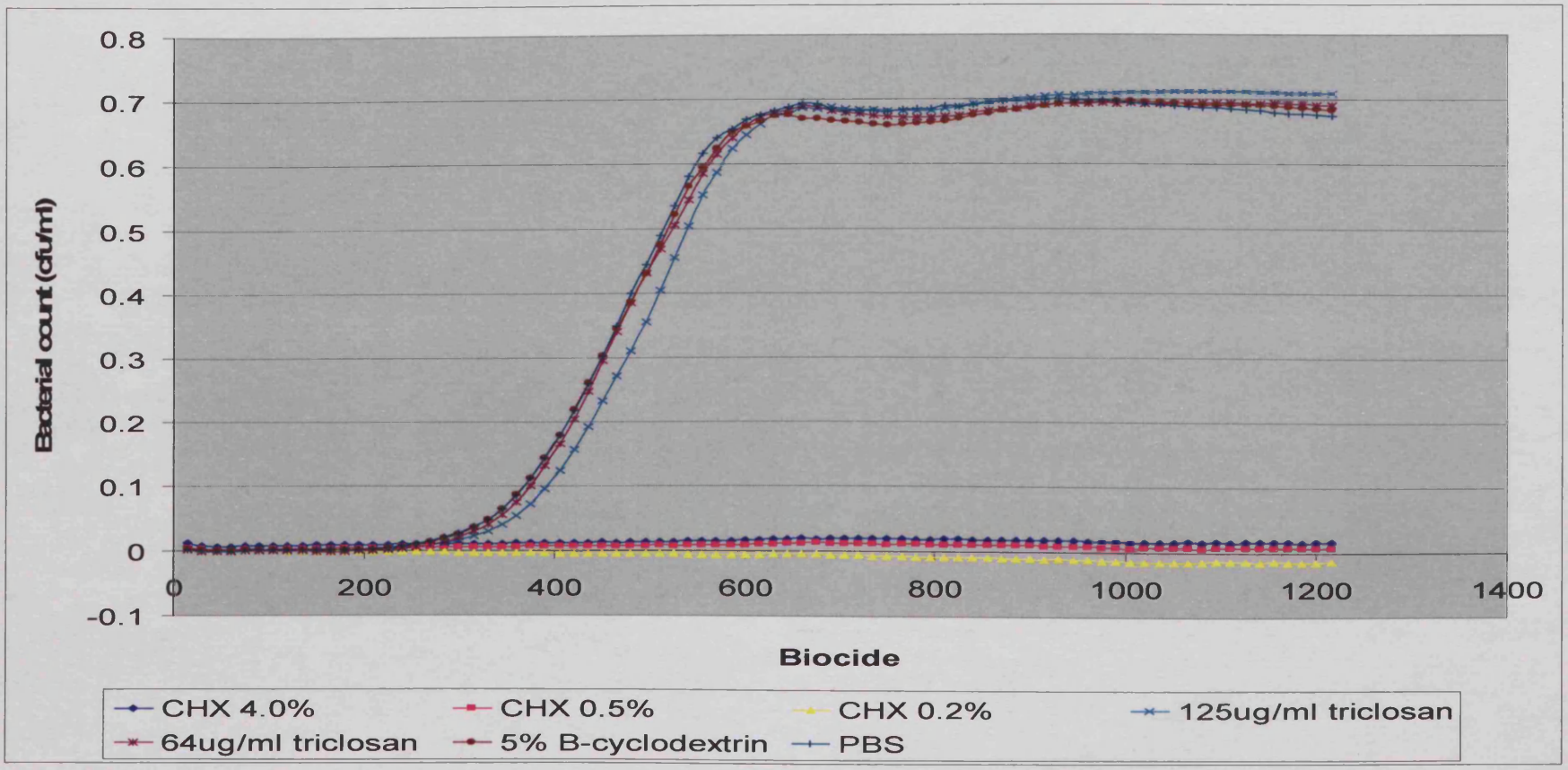

Fig. 5.7 *S. anginosus* 39/2/14A growth curves following 10 minute incubation with biocides. Suspensions incubated with chlorhexidine show no growth. All other suspensions show a standard bacterial growth curve with defined lag and log phase and all reach stationary phase at approximately 700 min.

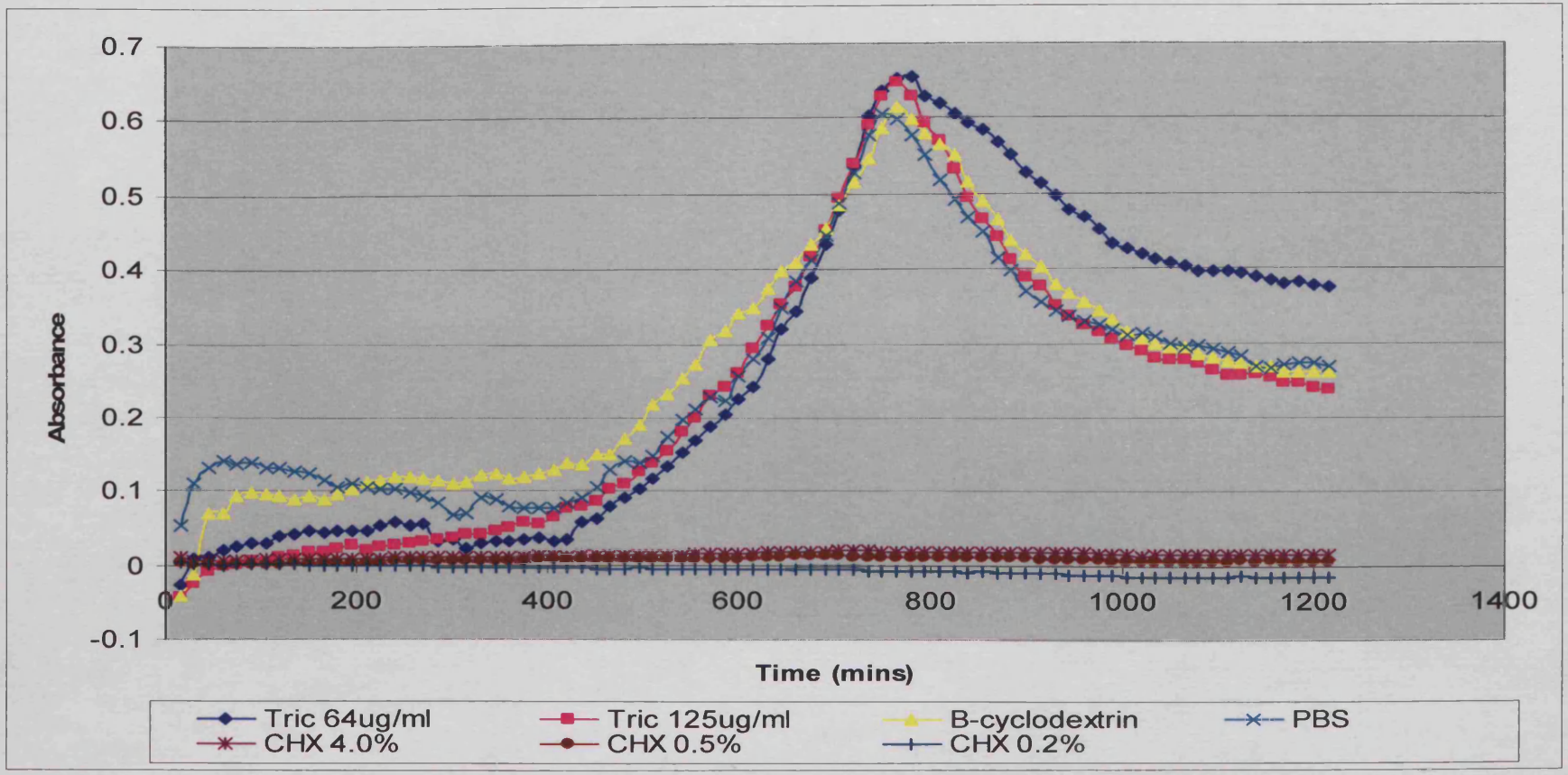

Fig. 5.8 *S. constellatus* 45386 growth curves following 10 minute incubation with biocides. Suspensions incubated with chlorhexidine show no growth. All other suspensions show a standard bacterial growth curve with defined lag and log phase prior to reaching an absorbance of around 0.6, at which the bacterial numbers begin to decrease. This point was reached at approximately 800 min after incubation.

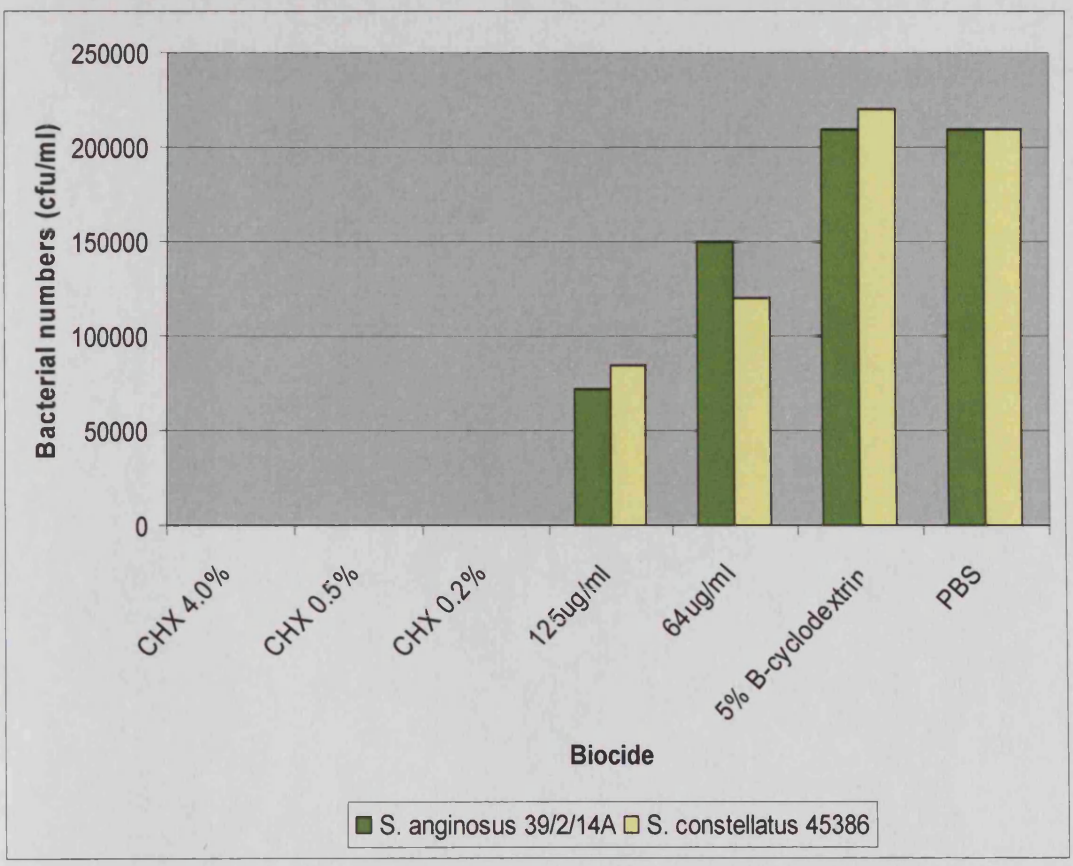

Fig. 5.9 Bacterial numbers remaining following 60 minute incubation of SAG bacteria with biocides. Chlorhexidine exhibits complete biocidal activity against the bacteria, whilst triclosan does not completely inhibit growth after 60 minutes. For *S. anginosus* 39/2/14A, 125ug/ml triclosan results in a 3-fold reduction of bacterial number. 64ug/ml triclosan results in only a 1.5-fold reduction. For *S. constellatus* 45386, 125ug/ml triclosan results in a 2.5-fold reduction of bacterial number. 64ug/ml triclosan results in a 1.8-fold reduction.

5.4 Discussion

Prior to this study, there had been very limited investigation into the efficacy of chlorhexidine and triclosan against SAG bacteria. This is possibly due to the relatively limited research into the role of SAG bacteria as endodontic pathogens that ha been conducted in the past. However, as recent research in this area has advanced and revealed the significance of these organisms in pulpal infection and abscess formation, it has become apparent that investigation into antimicrobial control of these bacteria is necessary. The results from the study in this chapter have demonstrated that at concentrations from 0.2% and above, chlorhexidine is able to completely eradicate SAG bacteria in suspension after a 10 minute exposure period. In contrast, whilst triclosan has been shown to cause a reduction in bacterial number, it did not demonstrate an ability to completely eradicate bacterial infection.

The different methods used in this chapter highlight the importance of thorough testing of potential antimicrobials. As previously discussed, bacteria behave differently under different growth conditions (**2.1**) and have been shown to have an increased resistance to antimicrobials when growing in a biofilm, as they become embedded in an extracellular matrix which infers protection against antimicrobials to the bacterial cells within (Aslam 2008; Fux et al. 2005). Plating out bacterial suspensions that had been exposed to biocides and counting the colonies allowed calculation of the number of surviving bacteria. This gave an indication of bactericidal efficacy and revealed that chlorhexidine was more effective than triclosan against SAG bacteria. However, it did not reveal any information about the effect the biocides had on the surviving bacteria. This is important as it has been shown that the use of biocides can select for a bacterial sub-population which may have different growth characteristics (Maillard 2007) and which may then have a different effect on the host tissues. Bacteria that survive exposure to sub-MIC levels of biocides may develop a resistant to the biocide, and in some cases this can lead to further resistance to other biocides or antibiotics which have a similar mechanism of action (Cottell et al. 2009). This may be of particular significance with SAG bacteria due to their role in abscess formation, as antibiotics are commonly used in treatment of abscesses. It is therefore important to identify the risks of resistance to a biocide that is being

proposed for use in endodontics and to assess the likelihood that cross-resistance to antibiotics could occur.

Another important factor that should be considered when testing a novel antimicrobial for use in endodontics is the effect it has on the bacteria that remain viable after its use. If bacteria that survive are able to function and grow as normal, such a biocide may not be suitable for use in endodontic treatment as the survival of bacteria following endodontics is one of the major reasons for treatment failure (Abou-Rass and Bogen 1998; Sundqvist et al. 2003). In this study, Bioscreen C has been used to assess the growth of bacteria following exposure to biocides. This showed that the exposure to triclosan did not have an effect on the growth curves of the surviving bacteria. The bacteria showed normal growth when compared to their growth prior to biocide exposure and reached a similar maximum absorbance level. This indicates that triclosan does not affect all of the bacteria within a population at the concentrations that were used in this study. The mode of action of triclosan has been shown to involve incorporation of the biocide into the bacterial membrane, disturbing its function and preventing replication of the bacteria (Villalain et al. 2001). As not all bacteria were affected in this study, it may indicate that the concentration of triclosan was too low to act on all bacterial cells present, and that whilst it may have been incorporated into the membranes of some of the cells, others were left unaffected. Therefore a higher concentration or a longer incubation time may assist in reducing bacterial numbers further. This mode of action may be adequate for use of triclosan in applications where a reduction in bacterial numbers is sufficient, such as handwashing and cleaning of surfaces. However, in endodontics this is not appropriate as the bacteria which would remain in the pulpal chamber would be able to grow as normal on the removal of triclosan. Failure to fully disinfect a pulpal chamber following endodontic treatment can lead to re-infection of the surrounding periodontal tissue, which is a leading cause of treatment failure and tooth loss. As triclosan does not appear to completely eradicate all SAG bacteria it may not be an appropriate antimicrobial agent for use in endodontics.

Whilst chlorhexidine has been widely used in antibacterial mouthwashes for several decades (Beighton et al. 1991; Daneshmand 1978; Okada 1980), its use in endodontics may be considered as a more recently investigated application. Despite

this, it has already been shown that chlorhexidine has activity against a wide range of Gram positive and negative bacteria, as well as some fungi, including *Candida albicans* (Karami et al. 2009; Valera et al. 2009). In addition to its broad spectrum of activity, chlorhexidine is an attractive antimicrobial for use in endodontics due its substantivity, as the control of bacterial numbers following treatment is particularly important for a successful outcome. As this study has demonstrated it is also effective against SAG bacteria, chlorhexidine may be an antimicrobial of interest to study using the co-culture model that has been developed. Chlorhexidine has previously been shown to be effective against *S. anginosus* biofilms *in vitro* (Chavez de Paz et al. 2010), so to progress from this its efficacy needs to be tested on bacteria that are attached to the tissue structure of the dentine-pulp complex. The model presented here provides a method in which to study this in a controlled system.

The use of triclosan as an antibacterial agent has been widely documented, and it has been incorporated into a number of toothpastes and mouthwashes (Bhargava and Leonard 1996). However, its effect against different bacterial species is variable. Also, due to it's non-ionic nature, triclosan does not bind to the oral surfaces for more than a few hours and may need to be used at relatively high concentrations (Davies 2007), which has also been shown in this study. As a result, it often has to be formulated with a co-polymer, such as Gantrez, to increase its substantivity and enhance its antimicrobial efficacy (Nudera et al. 2007; Zambon et al. 1990). This makes the production of triclosan-containing dentifrices more complex and expensive.

There has been limited research on the efficacy of triclosan against endodontic pathogens, as the main focus of its use has been on dental plaque and caries bacteria. Investigations into its efficacy against bacteria found in the pulpal chamber has been conducted *in vitro* on bacteria grown in suspension (Nudera et al. 2007), which may not reflect an accurate MIC for organisms *in vivo*; as they are more resistant to biocides when grown in a biofilm. There have been recent developments into the incorporation of a triclosan-containing composite which is proposed for use in restorative resins and has demonstrated some antibacterial activity against common oral bacteria (Rathke et al. 2010). As triclosan has previously been shown to have anti-inflammatory properties (Mustafa et al. 1998), in addition to its effectiveness against oral bacteria, this makes it an attractive biocide for potential use in endodontic

treatments as it may promote the natural repair processes of the tooth, as previously described (**1.2.3**). However, the results from this study have shown that triclosan alone is not effective at complete eradication of SAG bacteria, which could lead to problems in endodontic treatment. This is due to the high levels of failure in endodontically treated teeth in which bacteria survive and are able to re-infect the root canal. Despite these disadvantages, the model presented here provides a system which could be used to investigate the use of triclosan on bacterial biofilms of the dentine-pulp complex. In particular, it may be of use in assisting the development of triclosan containing resin composites and testing of co-polymers which enhance the antimicrobial efficacy of triclosan. Such developments could produce a triclosan containing product that would be useful in future treatments of pulpal infection.

Chapter 6: General Discussion

6.1 General discussion

The aim of this thesis was to develop an alternative model system to those currently available which could be used to investigate pulpal infection by SAG bacteria. To achieve this, the culture conditions and media of an existing tooth slice model system were modified to support the co-culture of tooth slices and SAG bacteria. This provided a novel *ex-vivo* system which was used to assess the effect of SAG bacteria on the tissue structure and cell viability of an organotypic tooth slice model. This enabled identification of a pattern of attachment of the bacteria to the pulpal tissues which has not previously been observed. Using this model, it was also possible to assess the initial inflammatory response of the pulpal tissues to the attachment of the bacteria and demonstrate the effect of supernatants in the absence of bacterial attachment. These findings are important in identifying the role of SAG bacteria in pulpal infection, and the development of the model system provides a basis for the testing the efficacy of new antimicrobials.

It is widely accepted in the dental community that the main reason for endodontic treatment failure is viable bacteria remaining in the root canal (Abou-Rass and Bogen 1998; de Paz et al. 2005; Siqueira and Rocas 2008). Although SAG bacteria have been identified as common pulpal pathogens that have a role in primary infection and are routinely isolated from abscesses, there has been limited research into how these bacteria behave in association with dental tissues or their susceptibility to common biocides. One of the major reasons for this could be the lack of an available model which allows reproducible results to be produced whilst limiting the costs and complications associated with *in vivo* models.

The major hurdle to overcome in the development of this model was the identification of a set of conditions which would support the growth of the fastidious SAG bacteria whilst maintaining the viability of the established tooth slice model. The previously published uses of the tooth slice model demonstrated the ability to culture the tooth slice at the liquid-gas interface (Dhopatkar et al. 2005; Sloan et al. 1998; Smith et al.

2010). This involves the tooth slices being embedded in a semi-solid mix of DMEM culture medium and agarose and floated on liquid DMEM through the use of a plastic support and a Millipore filter. In order to culture the tooth slice in contact with bacterial suspension this was modified in the model presented in this study, with the tooth slices cultured on the base of a tissue culture dish and the inoculated media then added to surround the slice. Histomorphometric analysis and viability staining was used to confirm that the tooth slices could be successfully cultured in this way at no detriment to the cells prior to using this method in co-culture. Further to this, the media for the co-culture of the bacteria and the tooth slice together needed to be modified to support the growth of both cell types. This was an important step in the development of the model and was a major focus of this study, as each proposed media composition had to be tested to ensure its suitability as a culture medium for both SAG bacteria and tooth slices. Development of a suitable medium was achieved by creating a combination of tissue culture medium and bacterial growth medium, the exact composition of which was decided by assessing the effects of possible combinations on the growth of both bacterial and mammalian cells.

As the growth conditions of the established tooth slice system had been modified for use in this model, any effects these changes may have had on the tissues needed to be assessed. This was necessary to obtain an accurate representation of how healthy tooth slices grown in this novel model would appear, in order to use this for comparison with tooth slices that had been infected with SAG bacteria. Also, as the SAG bacteria were to be cultured in a previously unused media and under different gaseous conditions from how they would generally be incubated, their growth under the modified culture conditions was also assessed. This was necessary to ensure that the bacteria were able to reach sufficient yields for use in the model and also to represent an active infection as it may be found *in vivo.* The success of the modified medium in supporting the culture of the tooth slices was assessed using histological analysis and viability staining which demonstrated that there was no significant decrease in cell number in the modified system and that the cells remained viable. The appearance of the tooth slices cultured in the novel medium were also comparable to those which have been used in similar models which have previously been published. Whilst the growth rates of *S. anginosus* and *S. constellatus* strains grown in the modified medium did appear to be altered slightly when compared to growth in BHI

this was not considered to be detrimental. This was based on the findings that whilst the growth appeared slower, the final bacterial yield supported by the medium was higher. However, *S. intermedius* strains were not able to reach sufficient yields for use in the model when grown in the modified culture medium. Further supplementation of the medium with haemin did not improve the growth and as such the *S. intermedius* strains were excluded from this study. The differences in growth requirements between the different species of the SAG bacteria was an interesting observation that warrants further study as this may influence their ability to establish pulpal infections.

Following validation of the model conditions, SAG bacteria were co-cultured with the tooth slices to mimic the events that occur in a pulpal infection following exposure of the pulp to the oral cavity. The co-culture model demonstrated that SAG bacteria appear to preferentially attach to the centre of the pulp in focal points and that these focal points of attachment are associated with tissue damage and cell death. This was characterised by a significant decrease in pulpal cell number and a decrease in the amount of staining surrounding the cells. These findings highlight the importance of SAG bacteria in pulpal infection, as such tissue breakdown and cell death as seen in the model will result in an impaired ability for repair in the *in vivo* situation as the cell death extends into the odontoblast layer. Odontoblast cell death was particularly pronounced in experiments where bacterial supernatants were applied to the tooth slices in the absence of bacterial attachment, which suggests the importance of these bacteria in cell destruction prior to their colonisation of the pulp. This is representative of the clinical situation where bacteria reside within the dentinal tubules and their supernatants are able to diffuse into the pulp through the dentinal fluid. As these bacteria are normally regarded as a problem associated mainly with abscesses, these novel findings highlight that they may also be affecting the pulpal tissues before they have established a pulpal infection, which may not previously have been considered. Also, their ability to attach directly to the pulp is an interesting revelation as it has previously been noted that many other bacterial species are able to attach to the surface of SAG bacteria. As such, the confirmation that these bacteria preferentially attach to the central areas of the pulp is important as it may create a pathway for other species to attach and infect the pulpal chamber (Love and Jenkinson 2002).

Extraction of the RNA from tooth slices that had been cultured in the presence or absence of SAG bacteria revealed that the tooth slices responded to infection by up-regulating the expression of the pro-inflammatory cytokines TNF-α and IL-1β. As previous *in vivo* studies have shown that this is a response of infected pulpal fibroblasts, this confirms that the tooth slice is responding in a way which is representative of the *in vivo* situation (Bletsa et al. 2006; Coil et al. 2004; Kokkas et al. 2007; McLachlan et al. 2004). The model may therefore be useful for assessing the efficacy of antimicrobials that have immunomodulatory properties such as some AMPs and triclosan, as it has already been acknowledged that an antimicrobial with anti-inflammatory properties would be of use in promoting the natural repair processes of the tooth. This model, which has no complications from circulating inflammatory factors, could be used to assess the direct effect of such antimicrobials on the inflammatory response of the pulpal tissues.

Previously, the susceptibility of SAG bacteria to commonly used biocides such as chlorhexidine and triclosan had not been investigated. In this study, the MIC of triclosan for two SAG strains was calculated at 125μg/ml when using a triclosan solution dissolved in β-cyclodextrin. Following this, the effect of exposure to MIC and sub-MIC concentrations of triclosan on SAG growth was investigated, along with exposure to commonly used concentrations of chlorhexidine. These experiments utilised a Bioscreen C, which enables study of the bacterial growth curves following exposure to biocides. The findings from the Bioscreen method were also compared to a direct plate count method which was used to calculate decrease in viable bacterial numbers following exposure to the biocide, but did not give any information about changes to the growth characteristics. The effect of biocides and disinfectants on the growth of bacteria which remain viable following exposure is of particular importance when considering bacteria which play a role in endodontic infection. This is due to the high incidence of endodontic treatment failure that occurs as a result of bacteria which survive disinfection of the root canal. The findings from the experiments using the Bioscreen suggest that triclosan may not be a suitable biocide for use in endodontic disinfection at the concentrations used in this study. Further testing of triclosan at higher concentrations is needed to identify if this will enable complete eradication of SAG bacteria. This model may also be used to test the development of biocide and co-

polymer complexes which may increase the efficacy of triclosan, as has previously been seen with Gantrez (Andrade Acevedo et al. 2009; Zambon et al. 1990).

The findings from the experiments in this study have demonstrated that this model provides a novel culture system for investigation of the processes involved in SAG infection of the pulp. In contrast to previous investigations into the role of SAG in pulpal infection and abscess formation (Nagashima et al. 1999; Okayama et al. 2005), this model does not require the inoculation of live animals with bacteria and therefore reduces the associated costs and ethical implications. The model allows culture of the dental tissues in the spatial arrangement seen *in vivo,* which has allowed the pattern of SAG bacterial attachment to be identified for the first time. SAG bacteria showed preferential attachment to the central areas of the pulp rather than the odontoblast layer, and excessive breakdown of the pulpal matrix following incubation times in excess of 24 h. This suggests that in the clinical situation these bacteria will also attach to the pulpal matrix, and that their removal from the pulpal chamber will require extraction of the pulp or extensive disinfection of the tissues.

As has been previously outlined, all available culture models have limitations. With this model, there is no circulatory system. As such, any investigations into the inflammatory response of the tooth to invading bacteria can only take into account the effect if immune cells present in the pulp. Also, the model is currently limited to studying the effect of co-culture over a maximum of 24 h. To improve the model the infection period could be extended to study the inflammatory responses at different time periods and to study the changes in histology with increased incubation time. As the investigation using the supernatants has shown that the odontoblasts are affected after 24 h, an increased exposure time to viable bacteria is needed to see if this effect is mimicked at longer incubation times. Identification of the cytokines expressed as a response to supernatant exposure could also be compared to that which occurs during contact with the bacteria in the co-culture model. Further to this, Western blot analysis to identify the proteins that are present in the supernatant of the different SAG species is required. This would then allow the role of the proteins in tissue breakdown to be further investigated and allow identification of specific proteins that are essential to pulpal infection in necrosis. Identification of these proteins may assist

in the development of more successful treatment methods than those currently available.

The model presented here can be used for further investigation into the processes involved in pulpal infection. Research into the immune response of the tooth slices as a reaction to invading bacteria may be elucidated to determine the events that cause abscess formation by these bacteria. Different bacterial species that are associated with pulpal infection may also be used to co-infect the tooth slice to further investigate the synergistic relationships between the different species. Some work has already been done in this area but a suitable model has not been previously available (Kuriyama et al. 2000; Nagashima et al. 1999). Identification of the main causes of pulpal infection that cause endodontic treatment failure and abscesses may allow for use of antimicrobials that are specifically targeted at problem organisms.

The major focus of this study was on the development of the model by identification of suitable culture conditions and validating their effects on the components of the model. Following its development there are a number of applications for which the model can be used. One of the major advantages of the model is its potential use for screening novel antimicrobials. In particular, as the model has been shown to have some inflammatory response, the model can be used to investigate novel antimicrobials that also have anti-inflammatory properties, such as AMPs and triclosan. AMPs are costly and difficult to investigate in *in vivo* models due to the innate expression of AMPs in the oral cavity. The *ex vivo* co-culture model presented here allows for smaller amounts of AMP to be used and provides a condition where there are fewer influences from naturally expressed proteins. This can be achieved by modifying previously published methods of delivering specific products to the tooth slice using agarose beads (Sloan and Smith 1999). This allows for smaller volumes of AMP or other products to be used as the beads can be soaked in the product prior to application to the tooth slice, rather than adding the solution directly to the tooth slice or the surrounding media. The small size of the agarose beads also enables application to specific areas of the tooth.

The development of this co-culture model has allowed co-culture of SAG bacteria and an organotypic tooth slice in an *ex-vivo* system for the first time. This has revealed a

number of characteristics of SAG infection which have not previously been seen. This model can now be used in further studies to elucidate the events that occur during abscess formation and can be used to test the efficacy of novel antimicrobials in preventing such infections. It is hoped that this model will allow for a reduction in the number of animals required in such experiments whilst providing an accurate representation of the clinical situation and will improve the currently available endodontic treatments.

6.2 Conclusions

The aim of the work contained in this thesis was to develop a model system which allowed the co-culture of SAG bacteria with organotypic tooth slices. The development of this model has further expanded the understanding of SAG growth characteristics and the processes that occur during pulpal infection. The key findings and their relevance to future work are outlined below:

- The different SAG species have varied growth requirements with *S. intermedius* species being the most fastidious organisms of the group. As a result of this, whilst it was possibly to culture *S. anginosus* and *S. constellatus* strains in a modified culture medium consisting of DMEM supplemented with BHI, *S. intermedius* strains could not be successfully cultured in any media except for BHI. These differences in growth requirements may also affect the ability of the different bacterial strains to colonise the pulp *in vivo.* Further research into the different growth requirements of the SAG bacteria may therefore be beneficial in fully understanding their role in pulpal infection.

- Co-culture of SAG bacteria and organotypic tooth slices resulted in attachment of the bacteria to the pulpal region of the tooth slices in small foci of bacteria which appear to penetrate into the tooth slice causing tissue damage. This damage is visible as cell death and breakdown of the pulpal matrix. These visible signs of damage increased with increasing periods of incubation with the invading bacteria. The cell death caused by the SAG bacteria was quantified using Image ProPlus software. This model can therefore be used to compare the relative amounts of cell death caused by the different SAG bacteria and may allow the protective abilities of different compounds to be studied in future work.

- Culture of the tooth slices with bacterial supernatants in the absence of the viable bacteria resulted in similar patterns of cell death and tissue damage as seen in the co-culture system. This indicated that these effects may be a result of soluble factors produced by the bacteria and are not entirely dependent on the physical attachment of the bacteria to the tooth slice. This is significant to

the clinical treatment of SAG infections as it emphasises the importance of early intervention and treatment as the bacteria may not need to colonise the pulp in order to cause cell death and damage if their products are able to diffuse to the pulpal chamber through the dentinal tubules.

- The efficacy of the biocides CHX and triclosan against the SAG bacteria after different periods of exposure was tested. These experiments revealed that commercially available forms of CHX were the most efficient at reducing bacterial numbers, whilst triclosan allowed significant bacterial numbers to remain viable in suspension and continue to grow after the biocide was removed. This data supports the use of CHX in endodontic disinfection and emphasises the importance of using biocides which eliminate the bacterial load, thus preventing re-growth of the bacteria after removal of the biocide. Introduction of these biocides into the co-culture model would provide further information on the efficacy of these antimicrobial formulations in an environment which is more representative of the *in vivo* situation. Such experimentation may enable the development of novel antimicrobials for use in endodontic treatments.

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