A Fluoride Releasing Dental Prosthesis Copolymer for Oral Biofilm Control

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Abstract

Microbial biofilms on removable oral appliances can act as reservoirs for pathogens that contribute to oral diseases such as candidiasis and root caries. In this study, we evaluated the efficacy of a new fluoride-releasing copolymer on biofilms formed by three acidogenic microorganisms, *C. albicans, S. mutans* and *L. casei*.

In all experiments, two materials were studied: chemically-activated copolymers of methyl methacrylate and 2-hydroxyethyl methacrylate with polymethyl methacrylate powder. For one group, 30% NaF was added at the expense of some of the PMMA. The effect that autoclaving had on important parameters such as chemical composition, roughness and fluoride release was analysed. Samples were fitted into two polysulfone Modified Robbins Devices. Single- and mixed-species biofilms were cultured for 48 h, harvested, and microorganisms were quantified by total viable counts and quantitative PCR. The pH was monitored during mixed species biofilm formation.

Autoclaving did not alter the chemical composition of either material, as measured by infra-red spectroscopy. However, it did cause a significant increase in roughness of the fluoridated materials (T-test, $p<0.05$). Fluoride release was unaffected by autoclaving, and high rates of fluoride (>300 μg/cm^2.day) were released from fluoridated samples for up to 6 days, followed by low levels of release for 6 months.

The inclusion of fluoride within the copolymer significantly reduced colonisation by *C. albicans, S. mutans* and *L. casei* in mixed-species biofilms (T-test, $p<0.05$). However, when microorganisms were grown in single-species biofilms, all the three organisms were not significantly (T-test, $p>0.05$) reduced. Fluoride suppressed the acidogenicity of biofilms for up to 24 h.

In conclusion, a fluoride-releasing copolymer has been developed to inhibit the growth of acidogenic oral biofilms *in vitro*. The copolymer potentially has major benefits for oral healthcare in wearers of removable oral appliances.

List of abbreviations

ADP	Adenosine diphosphate.
AFM	Atomic Force Microscopy.
Al^{3+}	Aluminium ion.
AmF	Amine fluoride.
ATP	Adenosine triphosphate.
ATR-FTIR	Attenuated total reflectance- Fourier transform infrared Spectroscopy.
Be^{2+}	Beryllium ion.
°C	Celsius, also known as centigrade.
C=0	Carbonyl group.
Ca	Calcium.
CaF_2	Calcium fluoride.
CBD	Calgary Biofilm Device.
CDFF	Constant Depth Film Fermenter.
CFU	Colony Forming Unit.
CLSM	Confocal Laser Scanning Microscopy.
CO_2	Carbon dioxide.
ConA	Concanavalin A.
CuF_2	Cupper fluoride.
CW	Calcofluor White.
3D	Three dimensions.
DAPI	4′,6-diamidino-2-phenylindole.
DNA	Deoxyribonucleic acid.
EDTA	Ethylenediaminetetraacetic acid.
EPS	Extracellular polymeric substances.
F	Fluoride.
F^-	Fluoride ion.
FTIR	Fourier Transform Infrared Spectroscopy.

G-6-P	Glucose-6-Phosphate.
h	Hour.
H^+-ATPase	Proton-translocating ATPase
HCl	Hydrochloric acid.
HEMA	Hydroxyethylmethacrylate.
IC	Ion Chromatography.
IPS	Intracellular Polysaccharides Storage Molecule.
IR	Infrared.
ISE	Ion Selective Electrode.
kg	Kilogram.
L	Litre.
LB	Luria Bertani.
mg/dL	Miligram /decilitre.
Mg^{2+}	Magnesium ion.
min	Minute.
mm	Millimetre.
mM	Millimolar.
MMA	Methylmethacrylate.
MRD	Modified Robbins Device.
mV	Millivolt.
Na^+	Sodium ion.
NaF	Sodium fluoride.
NCBI	National Centre for Biotechnology Information.
NH_4F	Ammonium fluoride.
OD	Optical density.
2-PGA	2-Phosphoglycerate.
PBS	Phosphate buffered saline.
PCR	Polymerase Chain Reaction.

PEP	Phosphoenolpyruvate.
PI	Propidium iodide.
PMMA	Polymethylmethacrylate.
PO_4^{3-}	Phosphate ion.
ppm	Parts per million.
PTS	Phosphotransferase System.
R_a	Roughness average.
rpm	Revolutions per minute.
R_q	Root mean square roughness.
SDA	Saboraud dextrose agar.
SD	Standard deviation.
Sec	Second.
SEM	Scanning Electron Microscoy.
SnF_2	Stannous fluoride.
Spp.	Species.
TISAB	Total ionic strength adjustment buffer.
UK	United Kingdom.
USA	United States of America.
UV light	Ultraviolet light.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
ΔP	Proton Motive Force.
ΔPH	Transmembrane chemical gradient of H^+ ion.
Δψ	Transmembrane electrical gradient which induces ΔP.
θ	Angle of incidence.
µL	Microlitre.
µm	Micrometre.
µM	Micromolar.

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Chapter 1 . Introduction

Middle-aged populations experience enhanced health care and long life expectancy when compared to previous generations, with the number of people aged over 65 likely to increase continuously (Shay, 2000). The widespread use of fluoride in oral healthcare products has dramatically improved the dental health of the population, and therefore there is strong hope that the incidence of edentulism may continue to decline (Dye *et al.*, 2007). However, there is still a significant proportion of the population who require dentures. Douglass *et al* (2002) estimated that the adult population in need of 1 or 2 dentures in the United States would increase from 33.6 million in 1991 to 37.9 million in 2020. The 2009 Adult Dental Health Survey revealed that 20% of the UK adult population wears a dental prosthesis, increasing to 70% in the over 75 year olds. Thus, at least in the near future there will be a growing need for prosthetic dentures (Steele *et al.*, 2011). At the same time, this will increase the chances of patients exposed to denture related diseases such as denture stomatitis and root caries.

Polymethyl methacrylate (PMMA) is a widely used denture base material utilized for manufacturing different oral appliances including partial and complete dentures (Chen *et al.*, 2001). This material continues to be used as it has good working characteristics, good aesthetics, and is inexpensive (John *et al.*, 2001). Despite its long clinical success, PMMA has a number of problems in use. One of these problems is that plaque forms on the surface of the denture in a similar manner to natural teeth (Sheen and Harrison, 2000) and this can lead to a variety of plaque related diseases.

Avon *et al* (2007) placed denture acrylic discs into complete maxillary dentures and microscopically evaluated microbial colonization. They observed numerous bacteria, spores, hyphae and germ tubes in an isolated pattern within one hour and this developed to a mature denture biofilm within two days. Microorganisms in denture plaque biofilms participate in a range of different disorders including denture

stomatitis, caries, periodontitis and gingivitis and a variety of extra-oral diseases (Coulthwaite and Verran, 2007; Preshaw *et al.*, 2011). Different anti-plaque agents have been assessed for their ability to minimise the adhesion of oral microorganisms to dentures, by interfering with bacterial co-aggregation and/or by reducing bacterial vitality thereby decreasing bacterial growth and biofilm formation (Baehni and Takeuchi, 2003). A number of previous studies have tried to reduce biofilm formation on dentures by using different antimicrobials or antifungals that can be incorporated into dentures, denture liners or tissue conditioners to minimize biofilm formation on the acrylic prosthesis (Addy and Thaw, 1982; Schneid, 1992; Edgerton *et al.*, 1995; Geerts *et al.*, 2008; Cao *et al.*, 2010; Salim *et al.*, 2012).

Fluoride is commonly used to protect against caries, and there is evidence that it can inhibit the growth of micro-organisms (Hamilton, 1990; van Loveren, 2001; Marquis *et al.*, 2003). Different methods have been used to deliver fluoride into the oral cavity, such as fluoride-containing dentifrices, fluoride mouth-rinses, topical fluoride (varnishes), and fluoride-releasing restorative materials (Kamijo *et al.*, 2009). Many of these studies did not investigate the impact of fluoride on mixed species biofilms that formed on teeth and related oral appliances. In this study, it is proposed to develop a novel antimicrobial denture lining material by incorporating fluoride with a copolymer of MMA and HEMA. The inhibitory properties of this material against mixed-species biofilms of acidogenic micro-organisms will be assessed using a flow-through biofilm model system.

1.1 Acrylic resin dental material

PMMA was introduced as a material for dental prosthesis construction by Wright in 1937 (John *et al.*, 2001). This material received approval from dental professionals by 1946 (Chen *et al.*, 2001). The initial use of polymers in dentistry was in the formation of dental appliances such as denture bases, artificial teeth, orthodontic appliances, obturators for cleft palates and sports mouth guards (Craig and Powers, 2002).

PMMA was one of the first polymers used for the fabrication of dental prostheses. This material continues to be the most popular dental resin for denture base purposes and is basically unchanged from those first introduced, except for slight modifications and refinements in its properties (Rueggeberg, 2002), and it has been estimated that about 98% of all denture prosthesis are constructed of PMMA (Anusavice, 1996; Chen *et al.*, 2001). This material continues to be used because it has been found to have satisfactory qualities including appearance, accurate fit, low cost and a simple procedure for processing the denture. The patient is usually pleased with the colour, characteristics, and function of the dentures produced from this material. However despite its popularity in satisfying aesthetic demands, PMMA is still far from fulfilling other important requirements of an ideal dental prostheses such as preventing food stagnation and controlling the accumulation of microorganisms (John *et al.*, 2001).

1.2 The composition of acrylic denture base resin

There are differences in the chemistry of denture materials that are based on PMMA chemistry. Some of the material manufacturers rely on high concentrations of cross linking agents and heat activated initiators to enhance the physical properties (e.g. dimensional accuracy, thermal conductivity) of the processed materials. Other companies add rubbers to modify PMMA to increase shock resistance and improve strength (Meng and Latta, 2005), while others add different minerals and fillers to reduce microbial activity on dentures (Casemiro *et al.*, 2008). In general, most denture materials are supplied as a powder and liquid (McCabe and Walls, 2008).

1.2.1 The powder (Polymethyl methacrylate)

The powder of denture acrylic essentially consists of particles or beads of PMMA with diameters up to 100 μm. These beads are produced by suspension polymerization in which methyl methacrylate with initiator (typically benzoyl peroxide) is suspended as droplets in water. The peroxide is decomposed by increasing the temperature to polymerize the methyl methacrylate and form the PMMA beads which are then dried to form a free flowing powder at room temperature (Brown *et al.*, 1981; McCabe *et al.*, 2002).

Initiators such as benzoyl peroxide or di-isobutylazonitrile are also added to the powder to start the polymerization when it is mixed with the monomer. Inorganic pigments (salts of cadmium or iron oxide) are usually added to denture base polymers. Characterizing fibres may be added to the powder to mimic the minute blood vessels of the oral mucosa (Winkler and Vernon, 1978). Plasticizers such dibutylphthalate can be added to the powder or liquid to increase the resiliance of the final polymer (Craig and Powers, 2002).

1.2.2 The liquid (Monomer)

The liquid component of powder-liquid type of denture materials is a mixture that comprises mostly of methyl methacrylate. This monomer is a clear, colourless liquid with a boiling point of 100.3°C (Craig, 1997; Ray, 1998). Small amounts (0.003-0.1%) of inhibitors such as hydroquinone are commonly added to the liquid to prevent premature polymerization and prolong the shelf life (Craig, 1997). In addition, the monomer usually contains a cross-linking agent such as ethylene glycol dimethacrylate (~10 %) to enhance the physical properties of the set material (McCabe, 1985).

In chemically cured acrylic resin, chemical accelerators such as tertiary amines, sulfonic acids, or the more stable salts of sulfonic acid, are added to the liquid to accelerate the benzoyl peroxide decomposition and enable the polymerization of the monomer at room temperature (Craig, 1997).

1.3 Polymer and copolymer

The polymer is prepared when the monomer unit is chemically interconnected to produce high molecular weight molecules in a process known by polymerization. This process occurs by two basic mechanisms, addition polymerization and condensation polymerization which are described below (Craig, 1997).

In addition polymerization, the reaction occurs without any by-products in four phases called the activation, initiation, propagation, and termination. This type of polymerization can be accelerated by heat; light and tiny amounts of peroxides as well as trialkyl borone. Most acrylic resin materials are produced by addition polymerization reactions (McCabe, 1985; Yau *et al.*, 2002). While in condensation polymerization, the reactions result in polymerization and the production of low-molecular weight by-products. This type of reaction can be seen in some impression materials such as polysulphide (Craig, 1997).

A copolymer is the product derived from the simultaneous polymerization of two or more materials. It is not a mix of separate polymers but a compound that has properties that are distinct from either polymer alone (Craig, 1997). As copolymers comprise of two units, they can be classified depending on how these units are arranged in the chain. The arrangements can be block, alternating or random. Block copolymers are formed from two or more homopolymer subunits joined by covalent bonds. In alternating copolymers, there is a regular alternating between the two homopolymers, while random copolymers show properties that approximate the weighted average of the two forms of monomer units. Copolymers can be produced both by addition polymerization and condensation polymerization (Ratner *et al.*, 1996).

In general, polymeric denture base materials can be classified into different groups and the most commonly used ones in dental prostheses construction are the heat cured and chemical cured acrylic resins.

1.3.1 Heat activated (Heat cured) polymethyl methacrylate

Heat activated acrylic resins are the most commonly used materials for complete and partial denture construction. These materials are processed by compression moulding or conventional pressure packing techniques. The polymerization of heat cured PMMA can usually be done using a water bath, or less frequently by the use of a microwave oven (Young, 2010). The boiling point of monomer is about 100.3°C, and if the temperature is raised above this limit, the monomer will boil and will lead to bubble formation that will produce either internal or external porosity on the surface of the denture (McCabe and Walls, 2008).

1.3.2 Chemically cured polymethyl methacrylate

The terms cold cured, self-cured acrylic and auto polymerized resins all represent chemically cured PMMA (Tandon *et al.*, 2010), in which the reaction starts as soon as the powder and liquid are mixed together at room temperature. The chemical activation is usually accomplished through the addition of a tertiary amine (Young, 2010). Once the polymerization reaction has started, it is the same reaction process as for heat cured acrylic resin (Greener *et al.*, 1972).

Chemically cured resins are most commonly used for repairing and relining dentures (McCabe and Walls, 2008), orthodontic removable appliances, and obturators for certain maxillofacial defects (Patil, 2011). A number of studies have reported attempts to improve the mechanical properties and porosity of chemically activated PMMA. Chee *et al* (1988) found that the use of a pressure device decreased porosity in auto polymerized resin samples. More recently, Alves *et al* (2007) and Goncalves *et al* (2008) found that the creation of smooth surface of chemically cured acrylic by mechanical polishing played an important part in reducing the subsequent attachment of microorganisms.

It is difficult to achieve an adequate degree of polymerization (maximum monomer to polymer transformation) with auto-polymerizing PMMA, resulting in inferior mechanical and surface properties (e.g. roughness) than heat cured resin (Oliveira *et al.*, 2008). Therefore, different cross linking agents (i.e. ethylene glycol

dimethacrylate, 1,4-butanediol dimethacrylate, bimethyrolplopane trimethacrylate, 1,6-hexanediol dimethacrylate, urethane dimethacrylate, methacrylated dendrimer) were developed to enhance its mechanical properties (Arima *et al.*, 1995a; Ferracane, 2006). These cross-linking agents have been added to the liquid monomer and they found that craze resistance, stiffness, transverse bending, water absorption, flexural modulus, and surface hardness were improved (Arima *et al.*, 1995b; Kawaguchi *et al.*, 2011).

1.4 Hydrophilic acrylic dental materials

PMMA is a hydrophobic, linear chain polymer that is glassy at room temperature. This material can be modified to become more hydrophilic by the addition of 2- hydroxyl ethyl methacrylate (HEMA) monomer to form a copolymer with high water absorption capability. HEMA is prepared in a single step by the addition of -CH_2OH group to the methyl methacrylate side group and can simply polymerize like most methacrylic by-products (Figure 1.1) (Montheard *et al.*, 1992). It is widely used in the biomedical fields including dentistry as it is highly biocompatible material. A copolymer of PMMA and HEMA was previously used in the construction of a controlled release therapeutic system inside the oral cavity to release fluoride, antibiotic and chlorhexidine (Mirth, 1987; Mirth *et al.*, 1989). In addition, it is incorporated in the production of soft and hard denture liners (Yoshida *et al.*, 2013), denture coating materials (Kodkeaw *et al.*, 2010), orthodontic adhesives (Su *et al.*, 2010) and fissure sealant (Zahroon, 2014).

(a (b

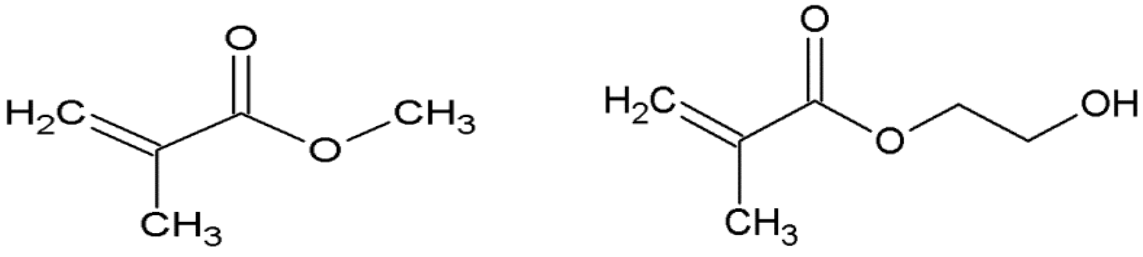


Figure 1.1 Structure of (a) MMA and (b) HEMA

1.5 Lining of oral appliances

Different types of autopolymerizing acrylic resin denture relining materials have become available in recent years. Depending on the purpose that they are used for, there are basically two different types of denture relines. Soft lining materials are also referred to as tissue conditioning materials, tissue treating materials, and relining materials (Park *et al.*, 2003). These materials are usually made from soft plasticized acrylic resin which applied to the tissue surface of dentures to achieve a more equal force distribution thereby reducing local point pressures and to enhance denture retention (Polyzois and Frangou, 2001). The other type of denture liner is a hard permanent reline which is also known as hard chairside reline resin. This material is most commonly made from polymer and contains different of methacrylate monomers and polymers such as MMA and PMMA (Murata *et al.*, 2007) that are stable for a several-year period of service (Matsumura *et al.*, 2001). Several previous studies have tried to introduce antimicrobial releasing lining materials to treat denture related diseases (Addy and Thaw, 1982; Lefebvre *et al.*, 2001; Nam, 2011; Salim *et al.*, 2012). However, these antimicrobials may have unwanted side effects and there are concerns over the development of antibacterial drug resistance, which has become a common global problem (Meurman *et al.*, 2006). Recently, fluoride-releasing lining materials have been developed. Hayakawa and co-workers (2006) introduced denture lining material containing a fluoroalkyl methacrylate polymer. Furthermore, Kodkeaw *et al.* (2010) introduced light cured denture-coating materials that contain NaF and CaF_2 within their ingredients. In both of these studies, the effects of fluoride on oral microbial biofilms have not been established. Therefore, further work is needed on fluoride releasing reline materials and their impact on micrsobial biofilms to decrease oral appliance related diseases.

1.6 Oral health of the denture-wearing population

As a result of improvements in health and medical care of people, with the increase in life expectancy of elderly population, there is an increase in the use of oral appliances (Morris *et al.*, 2001). Removable prostheses are a good option to replace missing teeth

(dentures), can be used to improve speech, aesthetic and may be used after surgical procedures (coverplates). A national survey in 2009 showed that fifteen million individuals wear oral prostheses in the UK, representing a significant consumer base and a special healthcare consideration (Steele *et al.*, 2011)

An unhygienic denture can lead to a number of different plaque related diseases such as gingivitis, periodontitis, root caries and denture induced stomatitis. These diseases are also associated with systemic diseases such bacterial endocarditis, (Krespi *et al.*, 2006), aspiration pneumonia (Abe *et al.*, 2006), gastrointestinal infections (Sumi *et al.*, 2003) and chronic obstructive pulmonary disease (Scannapieco, 1999).

Elderly people can encounter problems keeping their prosthesis adequately clean (Scannapieco, 1999). The medical community has historically put little emphasis on daily oral care, even though the oral cavity might be the starting place for different systemic diseases. A lack of attention to denture hygiene leads to an increase in the mass and complexity of denture plaque, which might provide a suitable environment for the colonization of pathogenic microbes.

1.7 Microbial biofilm

Bacteria are found in at least two distinct states, either planktonic or sessile cells. Planktonic cells are defined as free flowing bacteria in suspension which are different from the sessile state that known as biofilm. Biofilms are a very common mode of microbial growth. In natural environments it is estimated that 95-99% of microorganisms are present in the form of biofilms (Nikolaev and Plakunov, 2007). Biofilm formation starts when planktonic primary colonizers attach to an inert surface, forming a sessile monolayer. Growth of attached cells and further attachment by later colonizing strains leads to the development of a complex biofilm (Whitchurch *et al.*, 2002). Biofilms may develop into a range of structures, such as columnar, mushroom shaped multi-bacterial extensions into the lumen of the solution which is separated by channels that are apparently empty or filled with extracellular polysaccharide (Figure 1.2) (Costerton *et al.*, 1995).

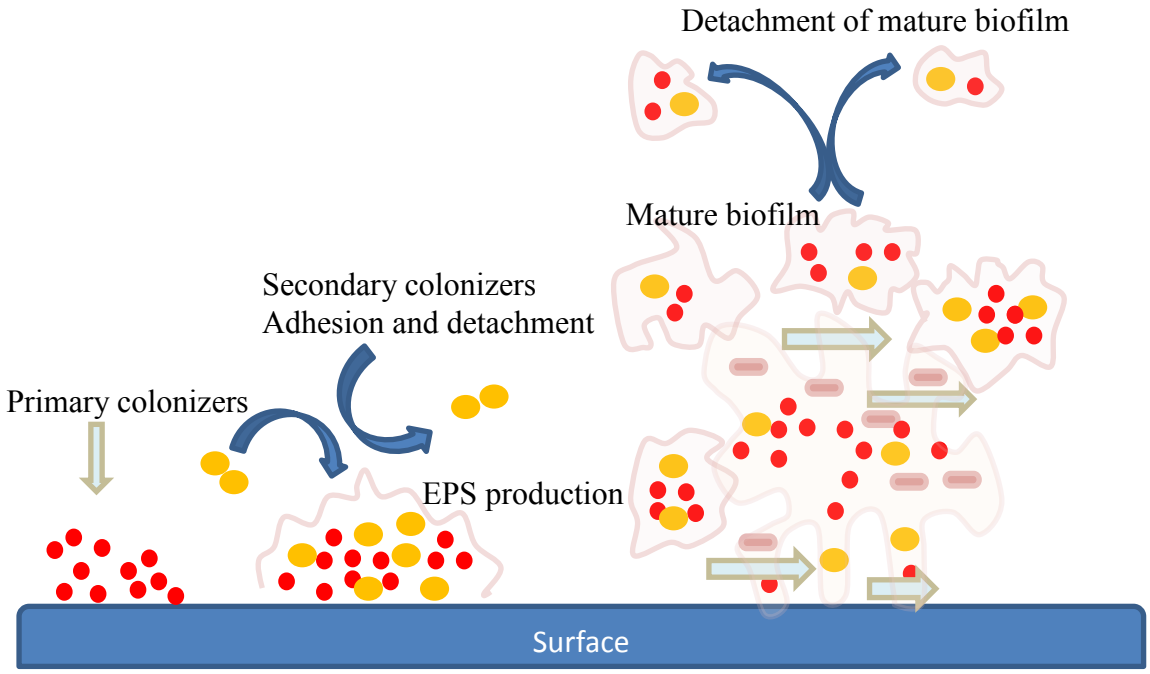


Figure 1.2 Schematic representation of the key stages in biofilm development. Planktonic cells attach to a surface, forming a monolayer. More cells attach and produce extracellular polymeric substances (EPS) to initiate the biofilm matrix. As the biofilm matures, planktonic bacterial cells spread again to colonize new surfaces.

The microorganisms in biofilms live in a matrix of hydrated extracellular polymeric substances (EPS) which construct their immediate environment. This matrix is generally composed of polysaccharides, proteins, nucleic acids and lipids which provide the mechanical stability and adhesion to surfaces. Furthermore, this matrix works as the external digestive system as it keeps extracellular enzymes close to the cells, allowing them to metabolize dissolved, colloidal and solid biopolymers (Flemming and Wingender, 2010).

EPS is different between biofilms, depending on the temperature, nutrient availability and microorganisms present. In most biofilms, the microorganisms account for less than 10% of the dry mass, while the matrix can account for over 90% (Wingender *et al.*, 2001). Different types of extracellular enzymes can be found in biofilms, some of which are capable of catalyzing the degradation of biopolymers to carbon and energy

sources. The substrates of these enzymes may be water–soluble polymers such as polysaccharides, proteins and nucleic acids, or water–insoluble complexes such as cellulose, chitin and lipids in addition to the trapped organic particles (Wingender *et al.*, 1999). However, the complete degradation of all EPS components requires a wide range of enzymes. Clearly, EPS are essential for biofilm construction and create a lifestyle that is very different from the planktonic state.

1.8 Denture plaque

There is significantly less published research investigating denture plaque microbiology than dental plaque. The majority of the studies on denture plaque have focussed on the aetiological agent of denture-related stomatitis, which is thought to be primarily associated with *Candida albicans* and related species (Coulthwaite and Verran, 2007). A number of light and electron microscopic studies have demonstrated that denture plaque has essentially the same structure as dental plaque (Budtz-Jörgensen *et al.*, 1981; Morris *et al.*, 1987). There is a general consensus that the composition of denture plaque is widely similar to that of supragingival dental plaque (Theilade *et al.*, 1983). Gram positive cocci and short rods are most commonly found, while Gram negative rods are observed less frequently (Budtz-Jörgensen *et al.*, 1981; Budtz-Jörgensen and Theilade, 1983; Koopmans *et al.*, 1988).

In general, the plaque microflora is different between individuals and within individuals, between sites in the mouth and on the denture (Peltola *et al.*, 2004). The commonly cultivatable denture plaque microorganisms that can be seen include *Streptococcus* species (for example, *S. sanguinis, S. salivarius, S. anginosus, S. oralis*), *Staphylococcus* species (*S. epidermidis, S. aureus*), Gram-positive rods such as *Actinomyces* species (*A. odontolyticus, A. naeslundii, A. israelii*), *Lactobacillus* spp., Gram negative anaerobic cocci such as *Veillonella* species (including *Veillonella parvula*), Gram negative rods and yeasts (Gusberti *et al.*, 1985; Theilade and Budtz-Jörgensen, 1988; Budtz-Jörgensen 2000; Coulthwaite and Verran, 2005).

In (2009), Matzourani and co-workers investigated denture biofilm by using sterile microbiological 5-µl loop and running it across the fitting surface of maxillary

complete dentures. The plaque samples were dispersed and plated on selective media. This study showed that *Bifidobacterium* species (*B. breve, B. scardovii and B. longum*) and caries associated microorganisms (mutans streptococci, lactobacilli) were found within denture plaque at levels similar to those found in carious infections. This study concluded that even though previous studies have found a loss of cariogenic species such as *Streptococcus mutans* after a complete dental clearance (Carlsson *et al.*, 1969; Theilade *et al.*, 1983), these bacteria can apparently re-colonize the mouth after the introduction of dentures (Mantzourani *et al.*, 2010).

Other microorganisms that are commonly seen in oral biofilms and especially denture plaque are *Candida* species. Previous studies have shown the interaction of *Candida* cells with many bacterial types inside the oral cavity (O'Sullivan *et al.*, 2000; Bilhan *et al.*, 2009; Mantzourani *et al.*, 2010). Thus, *Candida albicans* and acidogenic bacterial cells such as streptococci and lactobacilli can be considered the main colonisers that are responsible for denture plaque related diseases. Therefore, in this research we have chosen *C. albicans* and two other acidogenic species, *L .casei and S. mutans* to study their interactions and biofilm formation.

1.8.1 Candida albicans

Candida albicans is a dimorphic fungus that commonly lives in warm-blooded animals including humans. These microorganisms colonise the mucosal tissues and are capable of causing different types of diseases depending on the host defensive system. It lacks a sexual cycle and is a diploid microorganism which has made it challenging to manipulate genetically. One of the characteristic features of *C. albicans* is its ability to grow in two different ways; multiplying by budding or growth in a hyphal form that can split and form new mycelia or yeast like cells (Molero *et al.*, 1998). The ability to switch between forms contributes to the prevalence of *C. albicans* in fungal infections (Ten Cate *et al.*, 2009). *In vitro*, temperature, pH or various compounds such as N-acetylglucosamine or proline can modulate the transitions between these phenotypes which characterize *C. albicans*. Common morphological features of *C. albicans* are budding yeast cells (blastospores, blastoconidia), pseudohyphae, true hyphae and clamydospores (Molero *et al.*, 1998). Biofilms formed by *C. albicans* in *in vivo* systems

models are similar in appearance to those formed *in vitro* (Andes *et al.*, 2004), although *in vivo* biofilms tend to mature faster and become thicker than those grown *in vitro*. *In vitro*, the thickness of the biofilm can range from 25-45 μm (Ramage *et al.*, 2001), while *in vivo* it usually exceeds100μm (Andes *et al.*, 2004).

C. albicans in comparison with other *Candida* species such as *Candida tropicalis, Candida glabrata, Candida parapsilosis,* and *Candida krusei*, was shown to have a very high occurrence inside the oral cavity (Bilhan *et al.*, 2009). A previous study reported 76% of upper denture wearers' who they investigated were contaminated with *Candida*. The highly isolated species were *C. albicans* , then *C. glabrata* and *C. tropicalis* (Vanden Abbeele *et al.*, 2008). *C. albicans* has been isolated from dentures, dental plaque and from sites of periodontal diseases. Moreover, *C. albicans* has been shown to be capable of adhering to oral surfaces such as buccal oral mucosa, teeth, and dental prostheses through its interaction with bacterial biofilms (O'Sullivan *et al.*, 2000).

Salivary proteins and glycoproteins can work as receptors for attachment of *C. albicans* to enamel (Cannon *et al.*, 1995) and denture acrylic surfaces (Vasilas *et al.*, 1992; Edgerton *et al.*, 1993; Nikawa *et al.*, 1993). Furthermore, salivary proteins enhance co-adherence of oral microbiota such as streptococci, actinomyces and *Candida* spp.*,* which is believed to be an important factor in the early development of oral plaque (Lamont and Rosan, 1990; Holmes *et al.*, 1995). *In vitro*, *C. albicans* forms complex biofilms that are covered by dense matrix composed of extracellular polymeric material and hyphal elements (Kuhn *et al.*, 2002) that are most commonly found on the tissue contact surface of the denture rather than on or in the inflamed mucosa (Bilhan *et al.*, 2009).

The development of *C. albicans* biofilm *in vitro* has been observed by many researchers and organized into three developmental phases. Phase I (Early 1-11 hours): Adhesion of microorganism to a surface: *C. albicans* biofilms form after free floating *Candida* cells attach to a substrate. At this point, many of the *C. albicans* cells appear as blastospores (Chandra *et al.*, 2001a). After that, adhesive components such as glycoproteins (agglutinin, CaEap1) are attached to facilitate stronger adhesion. At three to four hours, microcolonies appear and *C. albicans* accumulations can be found

on the substratum, particularly in areas where surfaces irregularities are present, by eleven hours (Chandra *et al.*, 2001a). Phase II is a developmental stage that lasts from 12-30 hours: The *Candida* biofilm structure progresses into a bilayer composed of yeasts, germ tubes and young hyphae with a matrix of EPS (Seneviratne *et al.*, 2008). The EPS at this stage is largely composed of polysaccharides produced by the microbial components, and the cells at this point have relatively low metabolic activity (Ten Cate *et al.*, 2009). Phase III which is known as the maturation phase lasts from 38-72 hours: extracellular material increases with incubation time, until *C. albicans* yeasts, pseudohyphae and hyphae are completely embedded in a matrix (Ramage *et al.*, 2001). Hyphae are considered the essential elements that provide the structural integrity and multilayered architecture characteristic of fully developed biofilms (Baillie and Douglas, 1999).

1.8.2 Lactobacillus casei

L. casei is gram-positive, fastidious, non-motile, non-spore forming species of bacterium that produces short or long straight rods that form chains. The length of these chains depends on various factors including the constituents of the culture medium. *L. casei* can grow in temperatures from 10°C to 40°C. *L. casei* is one type of lactic acid bacteria that are used widely for the fermentation of dairy and vegetable products due to its capability to metabolize carbohydrates to lactic acid (Hansen and Lessel, 1971; Thompson *et al.*, 2008; Douillard *et al.*, 2013). It also has the ability to synthesize extracellular polysaccharides. The total yield of the extracellular polysaccharide is affected by the medium, especially the carbon source from sugar fermentation and the growth conditions, including temperature and incubation time (Mozzi *et al.*, 1996). *L. casei* naturally is found on the mucosal tissues of the oral cavity and gastrointestinal tract (Douillard *et al.*, 2013). It is well-known lactic acid producer (acidogenic) is acid-tolerant (aciduric) and most commonly isolated from caries active sites inside the mouth (Caufield *et al.*, 2006). It is considered as a secondary invader in the caries disease process, and generally appears in high numbers only once the favourable conditions of low pH have been generated by other bacteria in the biofilm (Tanzer *et al.*, 2001). In addition to its ability to co-aggregate with streptococci to

induce caries lesions (Willcox *et al.*, 1993), lactobacilli have also been found to co-aggregate with yeasts, actinomyces and streptococci during the formation of oral appliance-related diseases (Koopmans *et al.*, 1988; Theilade and Budtz-Jörgensen, 1988; Bilhan *et al.*, 2009).

1.8.3 Streptococcus mutans

S. mutans was originally isolated from caries in human teeth by Clarke (1924). The name '*mutans*' was given because of the change in coccal morphology to rod-like bacilli in acidic environments. The primary description of *S. mutans* was Gram positive, aerobic and facultatively anaerobic that cannot grow at 22°C. In glucose containing medium, *S. mutans* gives greyish white small colonies that become embedded in the medium after 24 hours (Clarke, 1924). Although *S. mutans* had been isolated from a carious lesion, no-one carried out any further studies on the species for several decades. In the late 1950s early 1960s, with the development of germ-free animals, it was shown that dental caries could be experimentally-induced by the presence of *S. mutans* (Fitzgerald, 1968). However, it should be noted that many other microorganisms (i.e. *Streptococcus sobrinus, Actinomyces viscosus,* and *Streptococcus salivarius*) were also found able to induce caries in these models (Krasse and Carlsson, 1970; Nagaoka *et al.*, 1995)

S. mutans is considered one of the main pathogens in the progression of different oral diseases including dental caries, periodontal and denture related diseases (Loesche *et al.*, 1985; Baena-Monroy *et al.*, 2005). It is one of the most strongly acidogenic microorganisms commonly found in the oral cavity (Takahashi and Nyvad, 2008). As such, it lowers the surrounding pH within dental or denture plaque biofilms and potentially outcompetes non-aciduric species. The integration of *S. mutans* may be enhanced by co-aggregation with other species (Bowden and Hamilton, 1998). This type of bacteria has the ability to colonize different sites inside the oral cavity depending on the variable conditions that are suitable for their growth (Gibbons, 1984). The ability *of S. mutans* to ferment different types of carbohydrates is well recognized (Hamada and Slade, 1980). The generation of acid from carbohydrate metabolism is one of the important virulence factors of *S. mutans*. This microorganism

is hetero-fermentative, generating both lactic acid and other acids. For intermediary metabolism, it can ferment a wide range of mono-, di-, or oligo-saccharides (Russell, 1990). The amount of energy obtained from glucose metabolism gives *S. mutans* a substantial growth advantage over other bacteria (Simmonds *et al.*, 2000). This property of *S. mutans* is related to the multitude of enzyme systems that it expresses for the transport and metabolism of sugars (Loesche, 1986).

In addition to the role is progression of dental caries*, S. mutans* can colonize the oral mucosa of patients wearing dental prostheses in higher numbers than *C. albicans* (Carlsson *et al.*, 1969; Baena-Monroy *et al.*, 2005). Firm adhesion of *S. mutans* to acrylic surfaces has been noted in the presence of sucrose (Branting *et al.*, 1989). In addition to the cell interaction between *C. albicans* and *S. mutans,* it was found that *S. mutans* synthesizes water insoluble glucans in concomitance with the adhesion of both these microorganism*s*. Therefore, *S. mutans* may contribute to denture stomatitis through its interactions with *C. albicans* (Shinada *et al.*, 1995)*.*

1.9 Problems associated with denture plaque

Denture wearers and especially elderly populations are at a heightened risk of oral mucosal diseases because increased age is associated with an increased number of medications, many of which lead to hyposalivation. In addition, with increased age the oral mucosa becomes more permeable to noxious agents and more susceptible to mechanical trauma (Narhi *et al.*, 1993). Therefore, denture users are often at increased risk of fungal-bacterial infections and other oral and systemic diseases (Silverglade and Stablein, 1988). Budtz-Jörgensen (1974) confirmed that the deterioration of general health compromises the immune defence system in the elderly and potentially leads to pathogenic colonization by opportunistic fungi, including *Candida* sp., and oral bacteria. Continued swallowing or aspiration of microorganisms exposes denture wearers to numerous infections. Another study by Sumi *et al* (2003) showed that the predominant microorganisms found on dentures in 50 patients were *Streptococcus* spp. and *Candida* spp. They concluded that denture plaque can be a reservoir for colonization and subsequent aspiration pneumonia.

1.9.1 Oral Candidiasis and Denture Stomatitis

Denture associated stomatitis (DAS) and chronic atrophic candidiasis, are common clinical conditions affecting two-thirds or more individuals who wear removable dentures, patients with orthodontic appliance or obturators for cleft palate (Lamfon *et al.*, 2005). Candidiasis tends to occur in people predisposed by illness, debility, or those who have a reduced host response to an overgrowth of their own yeast flora (Odds, 1988). The main source of *Candida* species in humans is endogenous*. Candida* species are characterized by their ability to adhere to surfaces, and their adhesion can be modulated by a variety of host factors including saliva, pH, and oral bacteria (Nair and Samaranayake, 1996). Adhesion is the first step in the process leading to colonization and infection (Coulthwaite and Verran, 2005). This disease is associated with biofilm formation on many implanted medical devices. The ability of *C. albicans* to form biofilms on these devices has a profound impact on its capacity to cause this type of disease (Nett and Andes, 2006). Therefore, as the name of this disease suggests, it is most commonly related with the overgrowth of fungal cells and especially *C. albicans* on oral mucosa and appliances used inside the oral cavity.

Denture induced stomatitis can be defined as a common inflammatory process that mainly involves the palatal mucosa when it is covered by complete dentures (Bilhan *et al.*, 2009). This disorder is now recognized as a complex process which follows the development of plaque material on the denture (Raj and Dentino, 2013). This plaque demonstrates an oral biofilm with multiple organisms, including *Candida*. Many previous studies have presented a relationship between *Candida* species (especially *C. albicans*) and several oral bacteria assuming that these bacteria promote the adhesion of *Candida* cells in the oral epithelium and acrylic surfaces. This adherence is improved *in vitro* when *Candida* is cultured together with *S. mutans, S. sanguinis, S. salivarius* (Baena-Monroy *et al.*, 2005) or some other bacteria such as *Lactobacillus* and *Fusobacterium* (Theilade and Budtz-Jörgensen, 1988; Bilhan *et al.*, 2009).

C. albicans is the most prevalent *Candida* species that can be seen in DAS patients; other species of *Candida* such as *C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis* are found at lower prevalence than *C. albicans*. The hyphal forms are most commonly

present within patients with denture stomatitis and they appear to have a greater pathological activity (Webb *et al.*, 1998).

The aetiology of denture stomatitis is considered multifactorial, and has a number of related factors rather than a single cause (Gendreau and Loewy, 2011). Poor oral hygiene, eukaryotic-prokaryotic infection, increasing age of the denture, poorly fitting appliances, and wearing of dentures during the night seem to be the main risk factors for denture stomatitis (Gendreau and Loewy, 2011). Therefore, it is important to reduce the risk for development of these kinds of diseases. Many previous studies found antifungal medications to be an effective treatment of DAS (Urban *et al.*, 2006; Geerts *et al.*, 2008; Redding *et al.*, 2009). However, a recent meta-analysis conducted by Emami *et al.* (2014) to compare the efficacy of antifungal therapy found no statistically significant difference between antifungal medications and placebo on the clinical outcome. This may be explained by the increase in the microbial resistance and especially *Candida* to these types of medications (Theilade and Budtz-Jörgensen, 1988; Chandra *et al.*, 2001b), making the treatment of *C. albicans* biofilm-related oral infections difficult (Baena-Monroy *et al.*, 2005). Therefore, many researchers have suggested substitutions such as antiseptic mouthwashes, microwave disinfection and photodynamic therapy (Bakhshi *et al.*, 2012; Mima *et al.*, 2012; Silva *et al.*, 2012; Emami *et al.*, 2014). However, there are occasions where these substitute measures may not be suitable, including the disabled, older adults, and in some surgical cases where oral appliances must be worn continuously for a period of time. Therefore, finding a material with antimicrobial property and minimum side effects that has the ability to control these kinds of diseases is the aim of this study.

1.9.2 Root caries

Removable dental appliances can adversely influence the condition of the remaining natural teeth. Different studies have shown that the amount of dental plaque is higher among people who wear removable appliances, and they found wearing of these appliances can nearly double the probability of new root caries lesions being present (Steele *et al.*, 1997). Appliances are anchored by abutment teeth, and therefore these

teeth specifically are more likely to be affected by caries and periodontal diseases than any other teeth because of bacterial and food stagnation (Mukai *et al.*, 2009).

Previous studies have reported an increased possibility of root caries when wearing removable partial dentures. A study by Jepson *et al* (2001) on 60 patients requiring restorative treatment with removable appliances, found that 51 of these patients developed new root caries lesions within a period of two years. Another study introduced by Budtz-Jörgensen and Isidor (1990) found that patients treated with a mandibular partial removable appliance had six times higher caries incidence than patients who received fixed appliances in a follow up study over a five year period.

The prevalence of cariogenic bacteria in patients wearing removable prostheses has generally been investigated by selective culture methods. Mutans streptococci (*S. mutans, S. sobrinus*) and lactobacilli are most frequently studied as these organisms show a positive relationship with caries (Bergman *et al.*, 1977; Spratt, 2008; Nordlund *et al.*, 2009). Beighton *et al* (1990) found the levels of *Streptococcus mutans* in saliva were slightly higher in patients with partial or full dentures than in persons with solely their own teeth. This study was supported by another longitudinal study by Rocha *et al* (2003) who found increased levels of salivary mutans streptococci in partial denture wearers four to six months after fitting the appliance. *Streptococcus mutans* was suggested to gain benefit from the insertion of removable partial dentures in the mouth and there is a possibility that these organisms multiply on the denture materials (Preshaw *et al.*, 2011).

Plaque acidogenicity is also affected by the wearing of oral prostheses. The presence of partial dentures increases the likelihood of plaque buildup and caries on the remaining teeth (Nikawa *et al.*, 1998). The reduction in pH may also increase the pathogenicity of *C. albicans*, since low pH causes the activation of *Candida* acid proteases, phospholipases and collagenases which advance tissue damage and can promote later *Candida* adherence and invasion (Odds, 1988).

Ainamo *et al* (1993) found high counts of mutans streptococci, lactobacilli and yeasts in association with large numbers of root caries lesions in elderly people. Nyvad and Kilian (1990) and Brown *et al* (1986) concluded that mutans streptococci may play an

important part in the initial development of root surface caries due to the higher isolation frequency and/or higher proportion of these species on carious root surfaces compared with advanced root caries.

Mantzourani *et al.* (2009) examined the microbiota of root caries lesions in denture wearers, and they evaluated the amount of bifidobacteria that associated with the clinical severity of root caries. *B. dentium* was the predominant bifidobacterium on carious roots, and the quantities of mutans streptococci, lactobacilli, bifidobacteria and *Candida* that were found in root caries were similar to those found in the denture plaque (Mantzourani *et al.*, 2010). Thus, it can be concluded that dentures or oral appliances may be reservoirs for many oral microorganisms including cariogenic species. This may explain the higher incidence of root caries in people who wear removable appliances.

1.10 Dietary sugars metabolism by oral microorganisms

Many species of microorganism have the capability to metabolise dietary sugars into one or more polysaccharides (intracellular glycogen, extracellular fructans or glucans). Sucrose has been shown to be the most important fermentable carbohydrate and the sole substrate for extracellular fructosyltransferases (FTF) and glucosyltransferases (GTF) enzymes that are released by Mutans streptococci. The resulting polysaccharides are glucans and fructans which are then metabolized by endogenous and exogenous hydrolases, generating a continuous source of energy in dental plaque. Insoluble glucans are known by mutans while soluble glucans are known as dextrans. Mutan is thought to be responsible for sucrose–dependent adherence to hard surfaces, while dextran helps in plaque aggregation (Colby and Russell, 1997). Intracellular polysaccharides are also generated which act as carbohydrate supplements that can be transformed to acid during periods when dietary carbohydrates become limited. This property enables the bacteria to continue their growth and acid production beyond the time at which carbohydrates are consumed by the host (Simmonds *et al.*, 2000). The fructans and glucans polymers synthesized by FTF and GTF, are important for the overall plaque structure by serving

as energy stocks, changing the permeability of the plaque, and stabilizing the biofilm through bacteria-bacteria and bacteria-surface adhesion (Colby and Russell, 1997).

Glucan synthesizing enzymes in *Lactobacillus* species have also been reported by Kralj and co-workers (2004). On sequence alignment with streptococcal GTF enzymes, most characteristic features and distinct domains were present. Therefore it may be assumed that the GTFs in lactobacilli have a similar mechanism of action to those from *S. mutans*.

Sugar metabolism by eukaryotic microorganisms is more complicated than in bacteria. Glucose works as a morphogen stimulating the yeast to hyphal transformation in *C. albicans*. It also plays a major role as a carbon and energy source. The sensing and response networks to glucose are greatly developed and precisely regulated in yeast cells, which is reflected in the large number of hexose transporters they possess (up to 20 in *C. albicans*, for example) (Brown *et al.*, 2006). Therefore, it is expected that *C. albicans*, which prefers respiration to fermentation, can obtain up to 38 ATPs per glucose molecule that it metabolizes (Brown *et al.*, 2006; Sabina and Brown, 2009).

1.11 Saliva

Studies about saliva and its functional properties and compositions started in the early Twentieth Century. These studies identified many of the key components of saliva and demonstrated antimicrobial characteristics of saliva, as well as the potential for salivary constituents to modulate microbial attachment, buffering, lubrication, tissue coating, taste, and mineralization (Levine, 1993).

Saliva is secreted by three paired major glands, the parotid, submandibular and sublingual glands, and minor salivary glands. In addition, subgingival biofilms are bathed in gingival crevicular fluid (Edger *et al.*, 2012). Saliva is a colourless and opalescent viscous watery fluid (Bell *et al.*, 1959). It helps to keep a comparatively neutral pH in the mouth (Mandel, 1987), and it has an important role in controlling the oral micro-flora by the specific and non-specific defence mechanisms (Edger *et al.*,

2012). Saliva is mainly composed of organic and inorganic components; the latter is primarily phosphate, calcium, sodium, potassium, chloride, magnesium, fluoride, and sulphur. In addition, bicarbonate is the most important buffer under physiological conditions (Thylstrup and Fejerskov, 1994). Each one of these elements has a specific effect inside the oral cavity. Organic components include protein such as mucins which are important glycoprotein that are present as alkaline salts and it is important in lubrication, pellicle formation, bacterial agglutination and clearance. Urea is also present in a normal concentration of 30 mg/dL (Macpherson and Dawes, 1991) and provides a nitrogen source for certain oral bacteria that produce urease (Speirs, 1984). In addition, salivary enzymes such as carbonic anhydrase, amylase and lysozyme have important jobs in the maintenance of pH homeostasis (Lenander-Lumikari and Loimaranta, 2000). Further components such as sialin, vitamins, lipids, carbohydrate, leukocytes, and many different bacterial species are also included (Lenander-Lumikari and Loimaranta, 2000).

One of the important points is that saliva changes its composition after leaving the oral cavity (Speirs, 1984), upon standing and storing or through exposure to air, bacterial activity, enzymatic reactions (Lazzari and Eugene, 1968) and loss of CO_2 (Muhler, 1964). Thus, natural saliva appears to be an extremely complex system that contains different constituents and has an unstable nature. Therefore, artificial saliva has been developed in an attempt to replace natural saliva for *in vitro* experiments. The application of artificial saliva for studies related to dental materials was reported as early as the 1930s (Leung and Darvell, 1997). A large number of recipes were introduced that tried to simulate the nature and composition of natural saliva. Nevertheless, most of the available artificial saliva still has several deficiencies and does not fully mimic the properties of natural saliva (Levine, 1993).

1.12 Biofilm model systems

A variety of different model systems have been employed for the study of biofilms. Most research has been conducted *in vitro* (Palmer, 2010), using models that range in complexity from a simple bacterial colony that grows directly on solid nutrient

medium such as agar to complex systems such as continuous flow-through devices in a variety of different formats. Model systems need to be designed to most closely mimic the type of biofilm under study, but taking into account factors such as ease of use, cost, and compatibility with analysis systems, particularly microscopy techniques. Therefore, there is no ideal model system that can be used for all investigations (McBain, 2009). The model systems that are used for biofilm investigations can be categorized according to the manner in which the biofilm comes in contact with the growth medium: by flow of the medium across the biomass or by simple diffusion in a static system (Palmer, 2010).

One of the most commonly used static biofilm model systems is based on 96-well microtitre plates. These systems can be used for simple and quantitative investigations of bacterial adherence and /or biofilm formation. For example, they are useful in molecular genetic studies to screen large numbers of mutants for their ability to form biofilms. The microtiter plate has the advantage of allowing researchers to rapidly examine adhesion or biofilm formation under different growth conditions or using different bacterial strains within each experiment (Djordjevic *et al.*, 2002). However, disadvantages of this model include the fact that it is a 'closed' system and there is no replenishment of nutrients, and that biofilms are not easily visualized microscopically.

Ceri et al (1999) developed a versatile model system which is known as the Calgary Biofilm Device (CBD) (Figure 1.3 A). This system consists of a two-part reaction container. The upper part forms a cap that has 96 pegs which are sealed on the top, allowing the pegs to be removed without opening the container and exposure to contamination. The pegs are manufactured to sit in the channel of the lower component of the container and to fit into the wells of a standard 96-well plate. The plate is shaken to allow the growth medium to flow around each peg so that all sides are exposed to similar shear forces, in order to form equal biofilms all around the peg. Therefore, the CBD can test multiple equivalent biofilms at the same time through an easy method that requires no pumps or tubing, and minimises the risks of contamination.

Surfaces inside the oral cavity are continually exposed to fluid flow and shear stress. Therefore, *in vitro* models for mimicking microbial biofilms, the oral environment should ideally involve fluid flow conditions (Morgan and Wilson, 2000). A commonly used and relatively simple flowing system is the flow cell in which the bacterial suspension is flowed within a closed channel (Figure 1.3 B). At least one side of this channel is transparent and amenable to microscopy (usually a microscope cover slip). After inoculation, the medium is run through the channel without interruption. In this state, the biofilm cells remain adhered and are supplied with continuous fresh medium, whilst concurrently biological waste and unattached cells are removed (Palmer, 2010). The flow cell is often used for short-term adhesion and colonisation studies, as non –destructive biofilm development can be visualised microscopically (Spratt and Pratten, 2003).

Other experimental models have been developed that include recessed surfaces for bacterial adherence. One of these models is the constant depth film fermenter (CDFF) which operates by drip-fed biofilm formation (Figure 1.3 C). The CDFF consists of a number of plugs that are recessed to an exact depth within a pan, and biofilms are grown to the thickness of the recess. The excess biofilm is scraped off by a scraper that also distributes the nutrients evenly over the pans. The growth conditions within the CDFF can be selected to be either aerobic or anaerobic (Ten Cate, 2006). The CDFF is particularly suited to study the maturation of biofilms, the effects of antimicrobials, endodontic microleakage, or the influence of substratum material on biofilm growth (Matharu *et al.*, 2001; McBain, 2009).

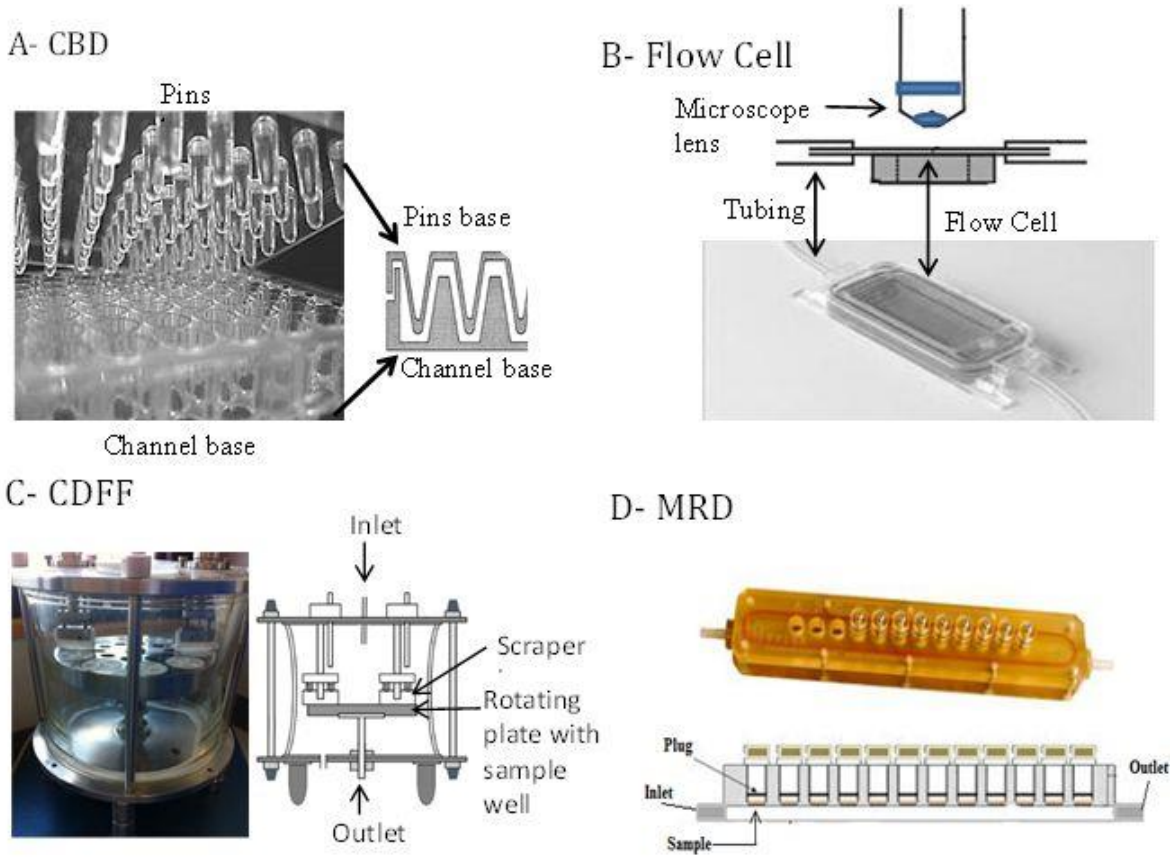


Figure 1.3 Biofilm model systems. (A) Calgary biofilm device (CBD)(B) Flow cell. (C) Constant depth film fermentor (CDFF), (D) Modified Robbins device (MRD).

Another flow model is Modified Robbins Device (MRD) which permits simultaneous biofilm development by different micro-organisms, in different growth media and / or on different substrata (Figure 1.3 D). This system consists of a plastic or metal tube that contains a number of individual ports (pegs or plugs) that can be inserted inside the tube, flush against the tube wall. The MRD is fed continuously with culture fluid which submerges the substrata. One drawback of the system is that the same fluid passes from the first pegs to the last, potentially resulting in differences between biofilms on different plugs along the device. Biofilms are formed on the pegs and can be removed separately later for sampling (McBain, 2009). Furthermore, discs containing the microbial biofilms can be transferred from the MRD to a different growth media without cells loss. As with the CDFF, examination of the anti-biofilm activity of disinfectants can be achieved offline with MRD, which means that different

procedures or products can be examined simultaneously (Coenye *et al.*, 2008). Like other flowing devices, the MRD is relatively cumbersome and is not ideal for rapid antibiotic vulnerability testing in a clinical laboratory setting (Ceri *et al.*, 1999). However, MRD are very useful system to investigate the effect of different flow rates in relation to the maximum microbial growth rates (Bagge *et al.*, 2001). In addition, the MRD system is well-suited for studies that involve surfaces that release chemicals as multiple MRDs can be assembled in parallel for comparisons with controls. It has advantages over static models for oral biofilms, because it is an 'open' system where medium is constantly replenished, just as it is in the mouth.

1.13 Fluoride role inside the oral cavity

Fluoride is the anion (F^-) of fluorine, which is an element of the halogen family and the most reactive of the non-metals. It is the 13th most abundant element in the earth and its components can be seen in earth soil, plants and freshwater. Over the last fifty years, there has been increased research related to the use of fluoride in preventive dentistry. A significant decline in dental caries has been seen since the incorporation of fluoride in different supplements (Toumba, 2001).

Different strategies have been used to deliver fluoride inside the oral cavity to reduce microbial plaque and dental caries. Different methods have been cited in the literature, and the most common are discussed below.

Since the 1930s, more than one hundred studies have reported the relationship between the fluoride content of drinking water and decreased caries prevalence from different communities over the world. As a governmental procedure, the fluoridation of drinking water with 1 ppm fluoride has been reliably showed a reduction in caries prevalence (WHO, 1994). Many previous studies have monitored the prevalence of dental caries and the effect of fluoridated water in its reduction. Water fluoridation has been shown to reduce the prevalence of dental caries of 40-49% in primary dentition and 50-59% in permanent dentition (Brunelle and Carlos, 1990; Limeback, 1999). Fluoridated milk was also another substitution to decrease dental caries. A previous *in vitro* study by Pratten *et al.* (2000) using CDFF with artificial saliva, found

that introducing 200 ml/30 min/day fluoridated milk (5 ppm) for one month was effective in reducing microbial biofilms formed by streptococci and especially *S. mutans*, and higher pH values were identified (5) in compared with people who gave normal milk (4.5). Arnold *et al.* (2006) found that fluoridated milk induced a beneficial effect in the reduction of *S. sobrinus* biofilms on enamel samples when visualised by scanning electron microscopy.

Fluoridated toothpastes are one of the most common ways to introduce fluoride inside the oral cavity. Studies regarding the effectiveness of fluoride-containing toothpastes date back as far as 1945. A variety of different forms of fluoride have been added to toothpastes to combat caries including sodium fluoride, stannous fluoride, acidulated phosphate fluoride and amine fluoride. The results of many previous studies demonstrated the significant reduction in caries prevalence when fluoridated toothpastes were used (Holt and Murray, 1997; ten Cate and van Loveren, 1999). The incorporation of fluoride in toothpastes is considered one of the important means for the topical application of fluoride. Evidence about the reduction in dental caries was recorded in most industrialized countries when fluoridated toothpastes utilized (Haugejorden *et al.*, 1997). Herbals incorporation with fluoride have been found to induce a boosting effect against micoorganisms. Adwan *et al.* (2012) investigated nine toothpastes containing sodium fluoride, sodium monofluorophosphate and herbal extracts against 45 orally isolated *C. albicans* using a standard agar well deffusion method. These containg herbals with fluoride were more potent against *C. albicans* in comparing with others that containing fluoride only. Twice-daily treatment for one-minute exposure of sodium fluoride with myricetin (flavonoid), tt-farnesol (terpenoid), were able to suppress *S. mutans* biofilm formation on saliva coated Hydroxyapatite (Jeon *et al.*, 2009).

Fluoride rinses have also become a common approach for introducing fluoride due to the simplicity and effectiveness of this prophylactic method especially in low fluoride areas (Toumba, 2001). The efficacy of fluoridated mouthwashes has been investigated in many clinical trials such as acidified and unbuffered NaF, SnF_2, and amine fluoride (Hind, 1999). The efficacy of fluoridated mouth rinses was estimated at about a 30%

decrease in caries induction (Horowitz, 1980). The use of AmF/SnF_2 toothpaste and rinse were more effective than using each agent separately in the reduction of total amount of DNA of the 39 major plaque species measured *in vivo* using checkerboard DNA:DNA hybridization (van Loveren *et al.*, 2009).

Another way of topical fluoride application is by using fluoridated gels. Sodium fluoride gel of 2% is most popularly used for 3-4 min following oral prophylaxis with children and high caries susceptible people. One of the beneficial aims of using this way of delivery is the immersion of the whole dentition using a tray for only single application (van Rijkom *et al.*, 1998). Fluoridated tablets are one of the alternative dietary supplements to deliver fluoride for children. When introduced in early childhood (before the age of 2 years), fluoride tablets lead to 40-80% caries reduction in primary and permanent teeth (Riordan, 1999; Liu *et al.*, 2013). Fluoridated varnishes are also one of the products that may exert an efficient prophylactic effect against dental caries. A recent study has shown that fluoride varnishes are clinically effective in the prevention of dental caries. Four types of commercially available fluoridated varnishes were topically applied to hydroxyapatite discs to investigate the change in *S. mutans* biofilm accumulation during subsequent biofilm formation. These varnishes showed >75% reduction in the biofilm adhesion, acid production and water-insoluble extracellular polysaccharides production for up to 70 h, then their effects started to decrease with the increasing age of the biofilm (Chau *et al.*, 2014).

Nowadays, there are numerous fluoride-containing dental restoratives including glass-ionomers (GIC), resin modified glass-ionomer cements, composites, fissure sealant, polyacid-modified composites, bonding agents, and adhesives. Due to their different matrices and setting mechanisms, the products vary in their ability to release fluoride. However, it is assumed that the antibacterial and cariostatic properties of restoratives are often associated with the amount of fluoride released. A number of previous studies have attempted to demonstrate anticaries-antimicrobial properties of fluoride when incorporated into these restorative materials. Two commercially available resin composites, one of them containing fluoride, were used to assess the level of acid production and acid tolerance of *S .mutans* biofilms that were grown for

94 h (Pandit *et al.*, 2011a). This study reported that fluoride releasing resin composites might contribute to the decrease in cariogenic composition of *S. mutans* biofilms if an appropriate amount of fluoride is released in the early stages of biofilm formation. GIC is a well-known material that has the ability to release fluoride. Hengtrakool *et al.* (2006) studied the effect of two commercially available fluoridated and non-fluoridated GICs on biofilms of *S. sanguinis* using a CDFF. There was a greater reduction in biofilm accumulation on the surface of the fluoride containing GIC than non-fluoridated material. Fluoridated bonding materials are considered desirable in orthodontics because of their ability to release fluoride irrespective of the patient's cooperation, with the aim of reducing plaque accumulation and demineralization around brackets. Three fluoride containing bonding materials (Transbond™ XT, Transbond™PLUS colour and Fuji Ortho™ LC) have been investigated using an inoculation medium of *S. mutans, L. casei* and *C. albicans*. The percentage of growth inhibition was evaluated quantitatively by spectrophotometer and qualitatively by scanning electron microscopy. Despite Fuji material was the highest fluoride releasing during the 14 days of measurement, the PLUS group presented the highest percentage of microbial inhibition and the most contamination-free surface (Caldeira *et al.*, 2013).

Fluoride releasing devices are one of the professional methods used to release fluoride intraorally. Different types of devices have been introduced to control caries. The pharmacokinetic concept of these devices is aimed at achieving persistent elevation of the fluoride level in the saliva, which has important bacteriostatic potentials and caries control (Ekstrand *et al.*, 1990). A copolymer type design for fluoride-releasing intra-oral devices was first developed in the United States (Gambhir *et al.*, 2012). It was made from a membrane-controlled reservoir with an inner core made from HEMA /MMA copolymer (50/50 ratio) and surrounded by another membrane of HEMA/MMA copolymer (30/70 ratio) which regulates the amount of fluoride that can be released from the inner core. Depending on the amount of fluoride that is uploaded in the inner core, the amount could be between 0.02- 1.0 mg fluoride per day with a duration range of 30-180 days (Toumba, 2001). Mirth and co-workers in (1982) introduced devices that can attach to the teeth surface and release fluoride

for up to six months. Several other different types of fluoride-releasing devices have also been investigated, including sodium fluoride encapsulated in an acrylic polymer which releases fluoride at a rate of approximately 0.04 mg/day for 4 months (Marini *et al.*, 2000), micro-encapsulated NaF in an aerosol delivery system (Williams *et al.*, 1982) and NaF film coating on orthodontic band (Friedman, 1980).

Despite the widespread use of fluoridated toothpastes and fluoride rinses all over the world, the prevalence of caries lesions is still high (Bagramian *et al.*, 2009). Fluoride release devices seem to be an appropriate method to target high caries risk groups including people with special needs, those living in care home, and handicapped, through its sustained release of fluoride.

1.13.1 Anti-caries role of Fluoride

Fluoride is currently one of the most potent and active chemicals for the prevention and treatment of dental caries. The therapeutic effects of fluoride can be achieved when an optimal concentration is available within and around dental plaque, tooth surface and the caries lesion (Ekstrand *et al.*, 1990).

The effects of fluoride on oral bacteria are many-fold (van Loveren, 2001). The antibacterial activities against cariogenic bacteria have been studied in the greatest detail (Shani *et al.*, 2000). Fluoride appears to act in preventing caries by modifying the dynamics of the carious process (Burke *et al.*, 2006). *In vitro* and *in situ* experiments have shown that the acidogenic bacteria that invade the tooth structure cause the release of calcium and phosphate ions from hydroxyapatite. The presence of fluoride leads to the formation of CaF_2 crystals that deposit on the tooth surface. When the pH rises in the oral environment, these crystals attract fluoride and calcium to form fluoroapatite crystals, and to promote remineralization (Ten Cate *et al.*, 1995).

In addition to its role in the hydroxyapatite crystal structure, fluoride also helps to control the activity of oral bacteria. Fluoride ions interfere with initial adhesion, aggregation, and metabolism of bacteria (Maltz and Emilson, 1982), and interrupt

their ability to generate acids (Phan *et al.*, 2000). This role of fluoride will be discussed in more details in the next section.

Many previous studies have tried to investigate the anticaries properties of fluoride. A recent study by Mei *et al.* (2013) used a computer-controlled artificial mouth containing 38% Silver diamine fluoride coated human dentin blocks, to study biofilm formation by five common cariogenic bacteria (*Streptococcus mutans, Streptococcus sobrinus, Lactobacillus acidophilus, Lactobacillus rhamnosus* and *Actinomyces naeslundii*). The results of this study showed fluoride to reduce the demineralization process through the inhibition of the biofilm formation by these species after an incubation period of 21 days. Maltz and Emilson in (1982) studied the effect of metal fluoride salts (CuF_2, SnF_2, NaF and NH_4F) on the bacteria that are most commonly found within caries lesions (*S. mutans, S. sanguinis, S. salivarius, Lactobacillus* spp. and *Actinomyces* spp.). The reduction in bacterial amount was observed most clearly with SnF_2 and CuF_2. Furthermore, the effects of fluoride combined with a range of other materials has also been investigated by many researchers. Coated hydroxyapatite discs with sodium fluoride (250ppm), apigenin and tt-farnesol, which are anti-caries agents, were found to be effective in reducing *S. mutans* biofilm growth with less biomass and insoluble polysacharides produced by bacteria (Koo *et al.*, 2005). In (2010), Murata and co-workers prepared a treatment solution to disrupt biofilm development and pathogenicity of *S. mutans in vivo* using a rodent model of dental caries. This solution was made of fluoride and 7-Epiclusianone (7-epi), a novel naturally occurring compound isolated from *Rheedia brasiliensis* plant. They concluded that 7-epi doubled the cariostatic effects of fluoride by disturbing sugar metabolism of *S. mutans*.

Thus, fluoride appears to act as bacteriostatic and cariostatic agent by modifying the dynamics of hydroxyapatite dissolution and by directly affecting acid production by different bacteria. However, high concentration of fluoride is also undesirable as it may cause diseases such as osteoporosis, osteosclerosis (Shivashankara *et al.*, 2000). It is a significant challenge to maintain an appropriate non-toxic, but anti-caries concentration of fluoride in the mouth.

1.13.2 Anti-microbial role of fluoride

Although fluoride is widely used as an anti-caries agent and to influence the remineralization process around the tooth surface area, fluoride also has an effect on the oral bacteria and their activity inside the mouth. It has an effect on bacterial stability through its ability to prevent pH reductions that are caused by bacterial carbohydrate metabolism. For example, a study by Marsh and Bradshaw (1990) found that pulsing with a combination of fluoride and glucose to a mixed culture in a chemostat system, resulted in delayed microbial acid production compared with glucose alone. The resultant pH after 10 uninterrupted days was 4.49, compared with 3.83 in the absence of fluoride. The mechanism by which fluoride interacts with bacterial cells starts when the microorganisms try to keep their intracellular pH close to neutrality when the surrounding environmental pH is reduced. The differences between intra- and extracellular pH (ΔpH) will rise as the extracellular pH falls following carbohydrate metabolism. This will lead to transport of fluoride that accumulated outside the cell, in the form of HF inside the bacterial cell which is rapidly dissociated into H^+ and F^- if the cytoplasmic pH is more alkaline than that outside the cell (Figure 1.4, reaction 1 and 2) (Hamilton, 1990).

Different chemical techniques have been used for cell extraction to differentiate three forms of fluoride inside the bacterial cytoplasm: ionizable (loosely) bound, tightly bound, and free fluoride ions. The last of these is commonly less than 1% of the total amount of F present (Tatevossian, 1980). Therefore, the dissociated F- ion is either bound in the loosely bound ionizable fraction (Figure 1.4, L) or in the smaller tightly-bound part (Figure 1.4, T). These two reactions cause a direct inhibitory action of F on bacterial enzyme activity. At the same time, the protons that are introduced by this process acidify the cytoplasm. This will ultimately lead to the fall in the internal pH below the optimum pH for both biosynthetic and catabolic enzymes. Following the acidification of the cytoplasm (Figure 1.4, reaction 3), fluoride is also effluxed from the cell (Figure 1.4, reaction 4) unless it is bound irreversibly with cellular constituents (Hamilton, 1990).

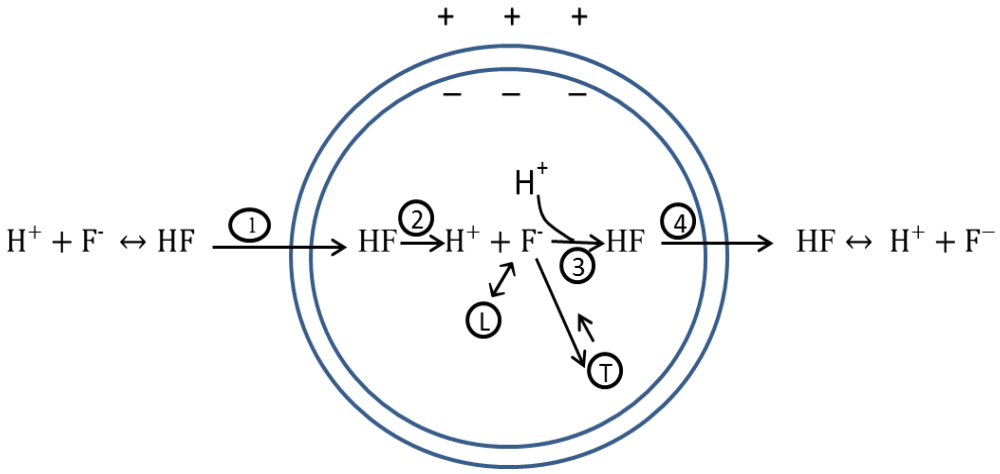


Figure 1.4 The accumulation, distribution and efflux of fluoride from bacterial cell adapted from Hamilton (1990). Fluoride enters microbial cells after pH changes in the form of HF (1) which then dissociates intracellularly into F and H ions (2) and the antimicrobial effects of fluoride on cell metabolism are started. After the reduction of ΔpH, F and H ions are reunited (3) and extruded (4) outside the cell. (L) loosely attached fluoride that can easily detach, (T) represents the tightly bound fluoride within cytoplasm which is less likely to be detached and removed from the cell.

The biochemical and physiological effects of fluoride on bacterial growth are most clearly demonstrated represented by its interaction with bacterial enzymes and its metabolic effects which will be discussed in details below:

Glycolysis: enolase enzyme is considered one of the main targets for fluoride in the bacterial cell. This enzyme converts the 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway (Figure 1.5, reaction 1) (Marsh *et al.*, 2009). The inhibition of enolase was first indicated by experiments designed to identify the antimicrobial effects of F on *S. salivarius* (Hamilton, 1977). The addition of 46 ppm NaF at pH 5.8 resulted in an immediate increase in the concentration of 2-PGA with a decrease in PEP. This led to a reduction in the cellular content of ATP because of the reduced flow of PEP to pyruvate by pyruvate kinase (Figure 1.5, reaction 2). Thus, fluoride has not only an effect on PEP but also on ATP (Hamilton, 1990; Marquis, 1995). The inhibition of glycolysis by low concentrations of fluoride has not been broadly investigated. Nevertheless, a previous study by Kashket and co-workers (1977) detected significant inhibition of glycolysis by 1-2 ppm fluoride. In addition,

significant inhibition of glycolysis was also observed with *S. salivarius* when fluoride concentrations of 0.15-0.25 ppm/mg dry weight of cell were administrated (Kanapka and Hamilton, 1971). Curran *et al*., (1994) found that fluoride has the capability to inhibit enolase in *S. mutans* GS-5 and fungi when 1ppm/L of NaF is introduced. In (2011) Takahashi and Washio found that 225 ppm of sodium fluoride was enough to inhibit enolase formation by plaque biofilm *in vivo* when they collected supragingival plaques and studied them by metabolome analyses. However, the association of fluoride with other components such as Mg^{2+} or PO_4^{3-}, might also produce a more potent and effective inhibitor of enolase enzyme than fluoride alone (Lebioda *et al.*, 1993).

Construction and degradation of polysaccharides: intra- and extracellular polysaccharides can be synthesized by certain species of oral bacteria, and provide an energy store that can be utilized to extend metabolic activity when the extracellular nutrients are depleted. In addition, extracellular polysaccharides promote adhesion and colonization of oral bacteria as previously described. Fluoride can inhibit bacterial attachment by reducing the production of water insoluble extracellular polysaccharides and reducing the molecular weight of bacterial polysaccharide (Shimura and Onisi, 1978). Koo *et al.* (2006) studied the co-operative inhibition by NaF and zinc on glucosyl transferase production and polysaccharide synthesis by *S. mutans* biofilm grown in fed-batch culture using saliva-coated hydroxylapatite discs for 5 days with sucrose. The combined effect of these agents showed a greater reduction in levels of alkali-soluble glucans and intracellular polysaccharides in the *S. mutans* biofilm than either agent alone. Intracellular polysaccharides (IPS) are commonly formed by plaque bacteria especially streptococci, lactobacilli, and actinomyces (Bowden *et al.*, 1979; Hamada and Slade, 1980; Marsh *et al.*, 2009; Zijnge *et al.*, 2010). It is likely that the IPS inhibition was by an indirect effect of fluoride (Figure 1.5, reaction 3) because of the unavailability of both ATP and glucose-6-phosphate (G-6-P) that are necessary for IPS formation (Hamilton, 1990).

Sugar transport: it is clear that there are major changes in levels of glycolytic intermediates in response to fluoride. Among these is glucose-6-phosphate and fructose-1,6-biphosphate. This means, the supply of sugar to the cell by phosphotransferase system (PTS) is decreased or inhibited in the presence of fluoride (Phan *et al.*, 2002). Different acidogenic bacteria such as, *Streptococcus* spp., *Lactobacillus* spp. and *Actinomyces* spp., have been shown to transport different mono and disaccharides using this system (Hamilton, 1990; Deutscher *et al.*, 2006). Therefore, the reduction in sugar transport can be interpreted by the inhibition of the PTS as the PEP supply restricted due to enolase inhibition. An alternative interpretation is that the PTS itself is not sensitive to fluoride (Hamilton, 1977) but it is sensitive to cytoplasmic acidification (Belli and Marquis, 1994) whereas the fluoride plays a role in the acidification of the cytoplasm. Recently, The effects of fluoride on acid adaptation were tested by exposing biofilm bacteria that had been grown in a mini-flow cell system under static conditions, to pH 5.5 overnight in the presence 20 ppm NaF. The results showed that fluoride significantly reduced the acid tolerance of the plaque biofilm (Neilands *et al.*, 2014).

Proton motive force (Δp): In bacteria, metabolic energy within the cell is preserved by an electrochemical proton gradient across the cytoplasmic membrane (Harold, 1972; Konings *et al.*, 2002). This gradient is composed of the transmembrane chemical gradient of H^+ ions (ΔpH) and a transmembrane electrical gradient (Δψ) which induces a proton motive force (Kashket *et al.*, 1977; Baker-Austin and Dopson, 2007). Proton gradients can be created in bacteria by the ejection of protons from the cells by proton extruding ATPase (H^+/ATPase) at the expense of ATP (Wu *et al.*, 2012). The transport of different soluble materials, specifically amino acids and sugar (Figure 1.5, reaction 4), is known to be coupled to proton electro-chemical gradients (Hamilton, 1990). Oral streptococci including *S. mutans* have been shown to transport glucose and different amino acids by using mechanisms based on electro-chemical gradients (Keevil *et al.*, 1986; Poolman, 1987). As this mechanism is energy-dependent, two factors are involved in the reduction of proton expulsion by fluoride. The first is the inhibition of glycolysis by fluoride and its indirect effect on transport via the PTS, and

the second factor is the direct inhibition of the H^+/ATPase by fluoride (Figure 1.5, reaction 5) (Marquis *et al.*, 2003).

H^+-ATPase: Proton-translocating ATPases in acidogenic and aciduric bacteria is considered essential for the maintenance of pH homeostasis inside the cell during growth and metabolism in acidic environments (Padan *et al.*, 2005; Wu *et al.*, 2012). The extrusion of protons from the cell at the expense of ATP confirms that the cytoplasmic pH will be above of the external medium and more in maintaining with the pH necessities of the biosynthetic and catabolic enzymes inside the cell. The antimicrobial effect of fluoride involves significant disruption of cellular pH homeostasis. The pH homeostasis from the other side is affected by the inhibition of ATP generation through its action on enolase, and a direct inhibition of the proton-pumping H^+/ATPases (Sutton *et al.*, 1987; Bradshaw *et al.*, 2002).

The effects of fluoride on this enzyme have not been fully elucidated. For example, it is not clear whether the fluoride itself has an inhibitory effect or whether inhibition is mediated by the metal ions (e.g. Al^{+3}, Be^{+2}) that are introduced with fluoride as salts (Sutton *et al.*, 1987; Lunardi *et al.*, 1988; Sturr and Marquis, 1990; Marquis *et al.*, 2003; Suwalsky *et al.*, 2004). However, Pandit *et al.* (2013) used a wide range of NaF (0-2000 ppm) to investigate fluoride effect on 75 h old *S. mutans* biofilms formed on saliva-coated hydroxyapatite discs using 24-well plate. This study found that a range of 10-100 ppm NaF was effective in reducing the structural integrity, proton permeability and H^+-ATPase activity. They suggested that most appropriate concentration of fluoride to reduce the virulence factors is approximately 100 ppm F^-; above that inhibitory effects of fluoride on growth were apparent. Using the same approach for biofilm growth as outlined above, Pandit *et al.* (2012) found that *Polygonum cuspidatum* plant extract in combination with 10 ppm NaF was effective in supressing acid production and H^+-ATPase activity of *S. mutans* in biofilm. Therefore, it can be observed that sodium fluoride alone or in a combination with other agents can reduce H^+-ATPase activity.

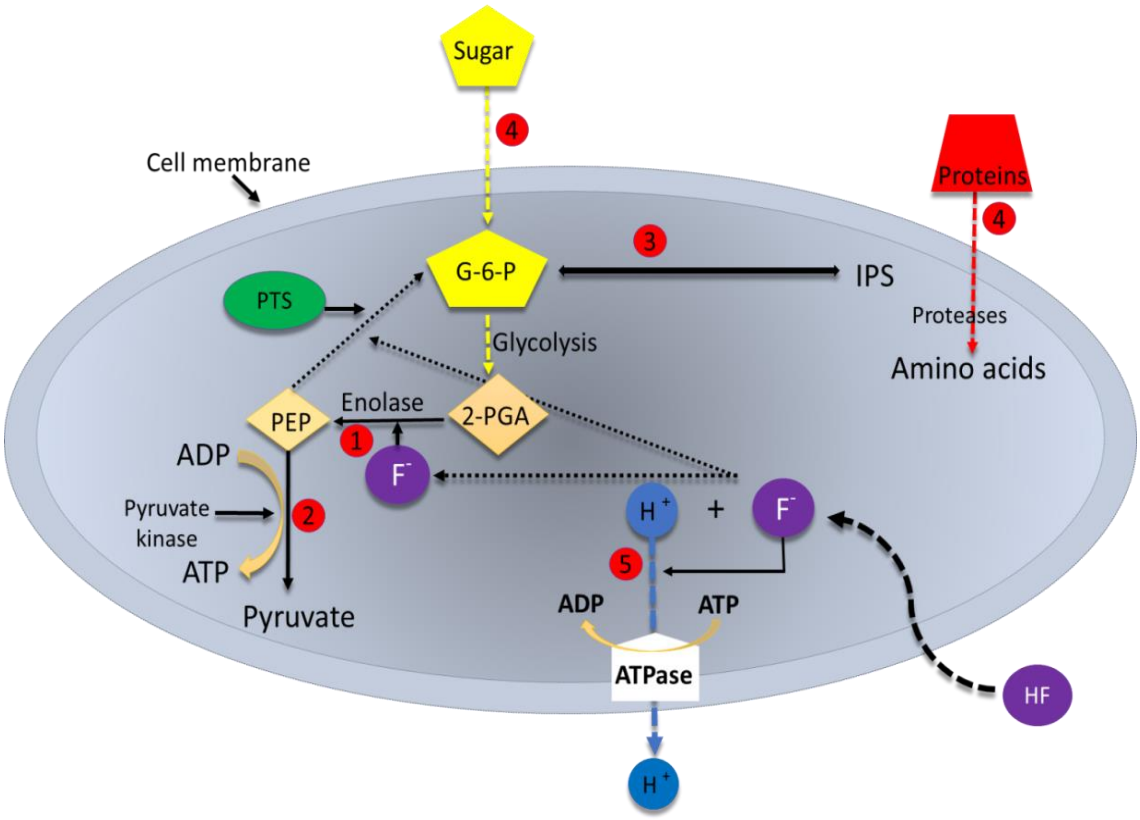


Figure 1.5 Fluoride-microbial cell reactions. 1) inhibition of glycolysis through direct effect on enolase enzyme, 2) indirect fluoride effect on the reduction ADP to ATP formation because of the reduced flow of PEP to pyruvate by pyruvate kinase, 3) reduction in sugar transport as the reduction in PEP cause inhibition of glycogen formation, 4) amino acids and sugar transport inside cell by utilizing mechanisms based on electro-chemical gradients, these mechanisms are energy consumptive which already depleted by fluoride, 5) direct inhibition of the H^+/ATPase by fluoride.

Other processes and enzymes: Fluoride is remarkable in terms of its broad effects on many enzymes. Most of these enzymes need a higher inhibitory concentration of fluoride when compared with those available in the cell cytoplasm. For example, *S. mutans* DSM enzymes such as phosphoglycerate mutase and pyruvate kinase can be inhibited by a fluoride concentrations that are 10-100 times more than those needed for enolase inhibition (Marquis, 1995). Pyrophosphatases also have an essential role in many bacterial metabolic processes through the recycling of pyrophosphate produced in biosynthetic reactions. Fluoride inhibition of these enzymes is achieved through the replacing of Mg^{2+} in the active site of the enzyme (Pohjanjoki *et al.*, 2001). Many metalloenzymes such as phosphorylases, phosphatases, and polyphosphatases,

need magnesium for activity. Fluoride has been found to be a potent inhibitor the action of these enzymes (Luoma, 1980; Bonting *et al.*, 1999; Meyer-Fernandes *et al.*, 1999). Fluoride has many other effects and many of them related to its effects on proton permeability. It has been shown its ability to decrease the cellular content of peptidoglycan by augmenting its turnover and in many cases leads to autolysis of oral bacteria that usually have highly active autolytic systems (Lesher *et al.*, 1977; Koo, 2008).

In summary, it seems that the antimicrobial and anticaries effects of fluoride are important in reducing the acid tolerance and glycolysis of intact, harmful bacteria in oral biofilms. Fluoride has a powerful direct inhibitory effect on different enzymes particularly in acidic environments such as enolase and H^+-ATPase to reduce microbial virulence which in turn indirectly affects many other mechanisms such as sugar transport and proton expulsion from the cell. However, whilst the most well-known processes and enzymes were discussed in this review there are still many others that fluoride will influence and that are not yet fully understood.

1.14 Properties of dental acrylic resin which may influence denture plaque

Several difficulties exist in producing satisfactory acrylic resin materials. Some properties of these materials may have a major role in facilitating or inhibiting the growth of pathogens on its surfaces, in particular the roughness of the surface.

1.14.1 Roughness

During the fabrication of a dental prosthesis, care is needed to produce smooth surfaces that minimize the initial adherence and accumulation of oral microorganisms. Thus, external surfaces of these appliances need to be adequately polished and finished in regard to the clinical restorative protocol.

In dentistry, the most common parameters used to quantify roughness are roughness average (R_a) and the root mean square roughness (R_q) (McCabe and Walls, 2008). These parameters measure the deviations of the profile height from the mean line

across a profile. R_a indicates the arithmetic mean roughness whereas R_q means the geometric mean roughness. Both use the same individual height measurements of a profile peaks and valleys with larger deviations from an idealized surface form meaning rougher surfaces. R_a measures the average deviations from the mean line, while R_q measures the root mean of the square of those deviations (Field *et al.*, 2013). The way in which these parameters are measured can lead to a misinterpretation of surface features. Some profiles differ clearly with respect to their actual surfaces, but still may have the same average roughness (Field *et al.*, 2013).

Previous light and electron microscopic studies have shown that these deviations or irregularities in the surfaces serve as preferential places for the initial attachment of bacteria providing niches where pathogens are protected from shear forces. This is expected to permit the microbial cell to attach irreversibly to the surfaces (Bollen *et al.*, 1997). Small increases in R_a (0.04-1.24 μm) have been shown to significantly increase bacterial attachment to PMMA (Taylor *et al.*, 1998).

Many different species of pathogens reside in the oral cavity and rely on two mechanisms, selective adhesion and stagnation to ensure a continued colonization (Newman, 1980). The roughness of the denture may help to determine the extent of colonization by different microorganisms. Repeated aggressive brushing or poorly polished denture surfaces will leave scratches and rough surfaces (Oliveira *et al.*, 2008) that harbor potential pathogens such as *Streptococcus sanguinis, Porphyromonas gingivalis* and *C. albicans* (Yamauchi *et al.*, 1990). *C. albicans* colonization is significantly increased on dentures with increased roughness (Verran *et al.*, 1991). Achieving a smooth surface on the internal (fitting) surface of dentures is particularly troublesome, since these surfaces are not polished.

Different techniques can be used to measure the surface profile such as stylus profilometry, laser profilometry, and atomic force microscopy. Stylus profilometry involves crossing the surface with a diamond-tipped stylus, pressed into contact with the surface with a loading weight on the stylus that can range from 0.05-100mg (Stachowiak and Batchelor, 2004). The tip is usually of a fixed radius 1.5-5μm (Stachowiak and Batchelor, 2004), nevertheless, the shape of the tip may differ

(Robbins *et al.*, 2001). Chisel-point (0.25µmx2.5µm) tips may be used for detecting raised areas in a surface whereas conical tips are almost exclusively used for micro-roughness measurements (Field, 2012). One of the points that needs to be considered while using stylus profilometry is the finite tip radius, which is not able to record detail of concave radii smaller than the tip. Therefore, fine details of the surface may be filtered out (Robbins *et al.*, 2001).

Laser profilometry is another technique used to measure roughness. This system may use either a triangulation laser sensor which detects the deflection of a laser beam on a CCD camera or it may use a white light , typically less than 100µm in diameter, to record the surface topography using the confocal principle (McBride and Christian, 2004). Many identified drawbacks of this technique are that the measurement is influenced by microgeometry, reflectivity and inclination of the surface of the sample (Wieland *et al.*, 2001). Furthermore, laser profilometry relies on light at a specific wavelength, which can be absorbed or reflected by the sample in different ways depending upon the colour and transparency of the material (Heintze *et al.*, 2006). This gives fallacious results more related to the colour and transparency of the materials than to real differences in roughness (Rodriguez *et al.*, 2009).

Another powerful and versatile tool uses for measuring surface profile with a very high resolution form, is atomic force microscopy (AFM) (Sedin and Rowlen, 2001). AFM consists of a cantilever that is used to scan the specimen surface in a similar way to that used in stylus profilometry. It has an advantage over stylus profilometry in that the tip can be raster-scanned across the surface enabling a topographical image of the specimen surface to be obtained, rather than the two-dimensional line scans obtained by stylus profilometry. Sample surface or profile preparation is not required but the image quality can be effected by contamination of the tip or by its blunting during scanning (Quate, 1994; Marshall *et al.*, 2004).

Therefore, each technique has its specific use. Stylus profilometry appears to have considerable merit. By careful choice of stylus radius and load conditions for the particular surface, one might guarantee that there is no permanent distortion and therefore propose that one is recording more accurate profile, although elastic deformations can still occur.

1.14.2 Fluoride release

Fluoridated dental materials show obvious variation in their fluoride release and uptake characteristics. The amount and duration of fluoride release from dental materials is related to different factors such as matrices, fillers, setting mechanisms, fluoride content and environmental conditions. It is assumed that the antibacterial and cariostatic properties of restorative materials are often associated with the amount of fluoride released. Fluoride-releasing materials can work as reservoir which may control the fluoride level in saliva, plaque and dental hard tissues (Wiegand *et al.*, 2007). There are commonly two general methods used to release therapeutic agents from dental biomaterials: - 1) those that supply a release rate which slowly decreases with time. 2) Those that release agent in a steady linear rate. The first type is the most common in which either a degradable or non-degradable materials can be used (Rawls, 1991).

A limited number of studies have studied fluoride release from dental appliances. In (1981), Zitz and his co-workers studied the effect of incorporated CaF_2 (2.04%), NaF (2.27%) and amine fluoride (0.71 %) inside acrylic resin plates, on the release of fluoride after immersion and rinsing samples in artificial saliva. A high fluoride release took place from NaF-containing samples, while the lowest, and most prolonged, release was from CaF_2. These observations might be related to the relative solubility of the different compounds. Another experiment were carried out by Gedalia *et al* (1977) to reduce the formation of plaque by releasing of 2% NaF from dental appliances. A significant decrease in plaque was detected in patients during two weeks.

The process of fluoride release from resin materials depends on the absorption capability of the resin, generally following the rule that the more water assimilation, the more fluoride release (Preston *et al.*, 2003). Furthermore, the size and composition of filler particles incorporated with the resin powder may have a role in fluoride release dynamics. The smaller the particle size the greater the surface area which can increase fluoride release (Xu and Burgess, 2003). The fluoride release capability of materials may be restored by the application of topical fluoride, and

materials that show high initial fluoride release rates are the easiest to recharge (Xu and Burgess, 2003).

Many attempts have been made to determine the best methods for measuring fluoride release. The most commonly used method is the ion selective electrode (ISE) which is simply utilized to measure total fluoride ions released (free and complexed) at concentrations less than 0.1 ppm after adding the acetic buffer solution (total ionic strength adjustment buffer) (TISAB) to adjust the pH of sample solution and decomplex the complex fluoride ions to free ions (McCabe *et al.*, 2002; Itota *et al.*, 2004). The electrode is covered with a lanthanum fluoride membrane, which allows the permeability of fluoride ions only (Rajković and Novaković, 2007). At low level of fluoride, hydroxide ions might interfere with fluoride ions which also can be affected by acidic solution; therefore adjustment of sample solution is necessary while using ISE (Tyler and Comer, 1985; Rajković and Novaković, 2007)

Ion chromatography (IC) has also been used to measure the concentration of free fluoride ions above 0.001 ppm (Itota *et al.*, 2004), which can be considered clinically pertinent as it is able to detect low levels of free ions (McCabe *et al.*, 2002), but it can only detect the free fluoride ions unlike the ISE which measures total ions of fluoride (van den Hoop *et al.*, 1996). Thus, the level of fluoride measured by IC is generally lower than that measured by ISE (McCabe et al., 2002b).

1.15 Sterilization techniques of biomaterials

Sterilization can be defined as the destruction of all living microorganisms including bacterial and fungal spores, which might not eliminate by disinfection methods. Disinfection is a lower grade of sterilization that involves only the removal of organisms in vegetative state (Block, 2001). Sterilization can be accomplished through different methods. The most common are dry heat, autoclaving, irradiation, microwave and gaseous chemical. Dry heat sterilization usually uses on metal packages with no risk of corrosion, leaving the instruments dry after completion. Nevertheless, it requires a long sterilization cycle at 160˚C for one to two hours.

Furthermore, this cycle could be interrupted if the oven door is opened before completion (Fais *et al.*, 2009).

Autoclaving is one of the most effective methods used for sterilization of medical devices (Fais *et al.*, 2009). Autoclaving utilizes saturated steam to permit lower temperatures and shorter times in comparison to dry heat. By autoclaving, the steam penetrates into the product to reach all the surfaces of the items need to be sterilized. The temperatures and times used for autoclaving differ depending on the particular cycle chose (lower temperatures must be held for longer times), but it is common for the temperature to be around 121^{o}C. The suitability of item for autoclaving will depend on the material, the size of the item, the wall thickness of the item and the contents (Block, 2001).

Some materials lose structural integrity at the temperatures used for autoclaving. Therefore, the effect of multiple sterilization cycles needs to be considered to prevent cumulative effects of the treatment on the plastic (Block, 2001). A previous study by Avon *et al* (2007) used autoclaving at 121^{o}C for 15 minutes to sterilize chemically cured acrylic discs to study biofilm growth *in vivo*. Therefore autoclaving is one of the main reliable methods can be used to obtain complete sterilization (Chang and Merritt, 1991; Nevzatoğlu *et al.*, 2007), despite its effect on some properties of plastic materials.

UV light irradiation is also used to sterilize metal and plastic items but in combination with chemical disinfectants. This method has been previously used to sterilize heat cured acrylic samples with 95% ethanol for 1 hr (Li *et al.*, 2010). Disinfection with UV light might significantly reduce bacterial contamination but it is not enough without chemical disinfection (Bhola *et al.*, 2010).

Sterilization by microwave is claimed to be useful in decontamination of microbiological laboratory materials and dental instruments. Hard chairside relining resin microwaved for 6 minutes at 650W showed consistent sterilization of all microorganisms (*S. aureus, P. aeruginosa, C. albicans,* and *B. subtilis*) on agar plates and no growth was seen even after seven days incubation at 37°C (Neppelenbroek *et al.*, 2003). Resin dentures contaminated with individual suspensions of four aerobic

bacteria and one fungus showed eradication of all microorganisms after 10 minutes of microwave irradiation (720 W) (Rohrer and Bulard, 1985). Placing the specimens in water during microwave exposure provided uniform heating of the specimens. This was considered to be adequate to kill organisms even within the pores of the materials (Neppelenbroek *et al.*, 2003).

Gamma radiation sterilization destroys bacteria by breaking down their DNA and inhibits bacterial division. An e-beam is mostly used for the sterilization. Gamma radiation brings a certain dose that can take a period of time from minutes to hours depending on the thickness and the volume of the material. This sterilization method affects the chemical compositions in different ways. Therefore, the method of sterilization selected should be compatible with the item to be sterilized to prevent damage (Takehisa *et al.*, 1998).

PMMA can be sterilized by gamma irradiation at dose of 25 kGy and used in absorbed dose measurements in intense radiation fields. Many reactions and volatiles products such as $HCOOCH_3$, CO, CO_2, $HCOCH_3$ and CH_4, can be accounted from this method of sterilization that cause the degradation of PMMA (Da Silva Aquino, 2012). In addition, it can cause the decrease in the fracture and fatigue resistances of PMMA cement material (Graham *et al.*, 2000). A common visible side effect of irradiation sterilization is that many plastics will discolour or yellow as a result of the processing (Da Silva Aquino, 2012).

Gaseous chemicals such as ethylene oxide are also one of the powerful methods of sterilization. The effectiveness of this sterilization depends on many variables such as time, temperature, gas concentration and humidity (important to produce moisture environment to insure destruction of all microorganisms). Most of plastics are unaffected by ethylene oxide sterilization, but some can absorb it, therefore, it should be eliminated before use (Block, 2001).

In conclusion, there is more than one single sterilization process for the medical materials and devices. It is difficult to evaluate the best sterilization method since every method has some advantages and disadvantages. Consequently, sterilization can be chosen according to the chemical and physical properties of the material. The

absolute eradication of these microorganisms is essential to the safety of the medical products. The sterilization procedure should be validated to confirm that it efficiently and consistently kills any germs that might be existed on the pre-sterilized product.

1.16 Summary

PMMA is a material commonly used in the construction of oral dental appliances. This material is characterized by acceptable mechanical, physical and chemical properties. Incorporation of hydrophilic chemicals such as HEMA leads to the formation of a hydrophilic copolymer, with potentially enhanced antimicrobial release. The oral cavity contains a multitude of microorganisms, which may adhere to removable appliances and initiate the formation of denture biofilm, potentially leading to the development different oral diseases such as denture induced stomatitis and root caries.

Different mechanical and chemical cleansing agents have been used to reduce bacterial colonization of denture surfaces. An effective denture cleanser must have the ability to remove microbial plaque formed and prevent its rebuilding. There is a need to develop anti-plaque formulations that will help patients maintain a healthy oral environment. Anti-microbial releasing appliances are also one of the methods that are used to treat and prevent oral diseases. In this study, it is proposed to develop a copolymer material that is capable of releasing fluoride to the oral cavity to obtain a better biological response with reduced risk of infection. A Modified Robbins Device has been chosen as the main biofilm model in this study with 3 microorganisms that are commonly found on the surfaces of dentures and oral appliances.

Chapter 2 . Aims and programme of work

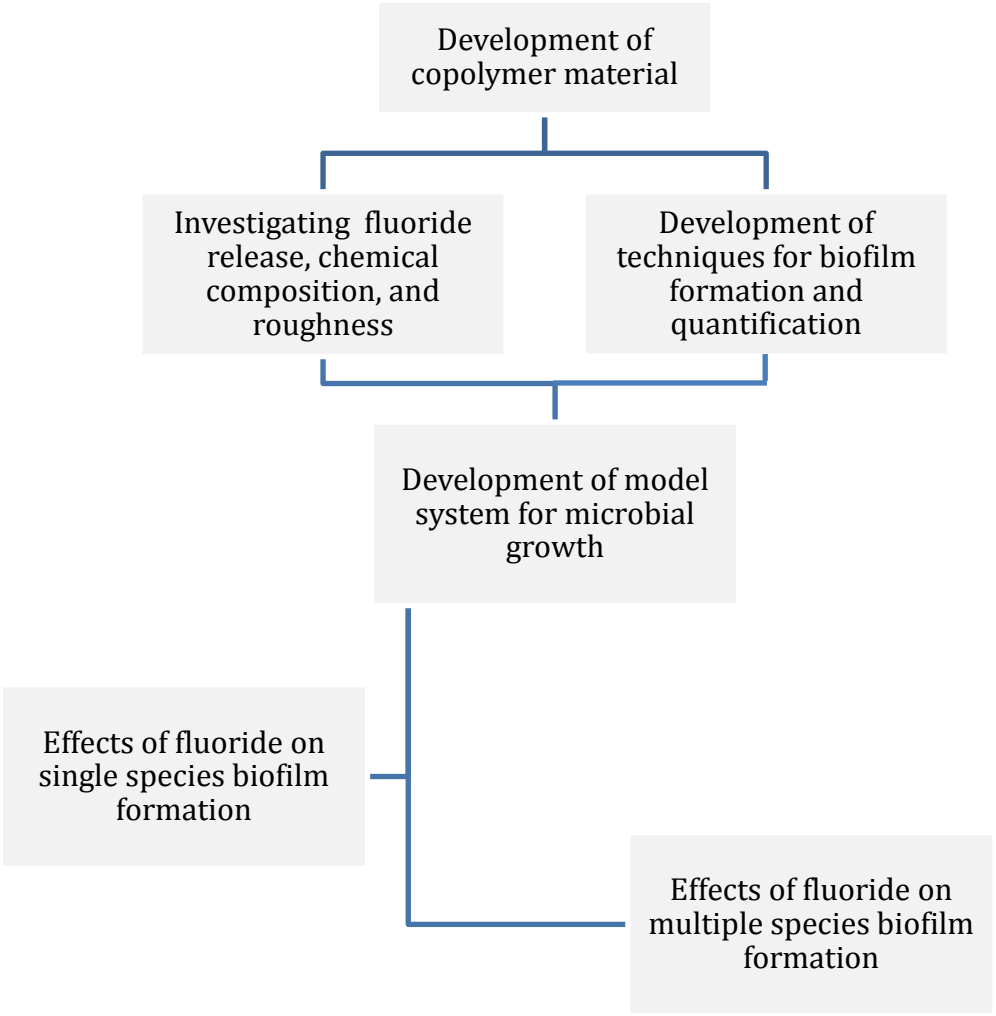
2.1 Aims

Removable oral appliances can act as sites for colonization by oral micro-organisms, with the underlying mucosa also experiencing a greatly decreased degree of mechanical cleansing from the tongue and a reduced flow of saliva (Budtz-Jörgensen 2000). Consequently, the wearing of removable oral appliances can potentially lead to a number of oral and general diseases. With the increase in the number of people who wear removable oral appliances all over the world (Douglass *et al.*, 2002; Zitzmann *et al.*, 2007; Steele *et al.*, 2011), it is clear that there is a need for improved materials for the treatment of these diseases. Recently, our group has developed a novel fluoride-releasing copolymer of methyl methacrylate and 2-hydroxyethyl methacrylate that may be suitable for bonding orthodontic appliances (Su *et al.*, 2010). A similar material, with a higher rate of fluoride release, could be highly effective for situations where the control of acidogenic biofilms is important such as denture liners, obturators or surgical coverplates. Therefore the primary aim of this study is to develop a modified fluoride-releasing copolymer and assess its potential for controlling the growth of acidogenic biofilms. The objectives were as follows:

1- Produce a copolymer containing NaF and evaluate the key physical and chemical properties (fluoride release, chemical composition, and roughness) of the material.
2- Develop a biofilm model system for culturing acidogenic microorganisms on the surface of the material under flowing conditions.
3- Evaluate single species biofilm growth by three different acidogenic microorganisms (*Candida albicans*, *Lactobacillus casei* and *Streptococcus mutans*) on fluoridated and non-fluoridated materials.
4- Evaluate mixed species biofilm growth on fluoridated and non-fluoridated materials.
5- Evaluate the amount of fluoride released from the samples and the pH during mixed species biofilm formation in the model system.

2.2 Programme of work

The plan of study was designed to be followed as below:



Chapter 3 . Characteristics of prepared copolymer samples

3.1 Introduction

Fluoride has been incorporated into different types of polymeric materials to investigate the impact of fluoride inside the oral cavity (Mayanagi *et al.*, 2014). A copolymer of MMA and HEMA has been used to produce devices that can release fluoride inside the oral cavity to reduce bacterial invasion and dental decay (Cowsar *et al.*, 1976; Mirth *et al.*, 1989). The hydroxyl group of HEMA makes the material hydrophilic, which is helpful in fluoride release, but a reduction in the physical and mechanical properties of the material occurs due to water absorption (Arima *et al.*, 1995a).

A denture base-resin that releases fluoride has shown potential in caries prevention (Mukai *et al.*, 2009). Wearing a partial denture with this property in the oral cavity can help to raise the local fluoride concentration, especially in an environment with limited access for saliva and where large amounts of bacterial and fungal debris may stagnate (Mukai *et al.*, 2009). This type of materials could not only prevent caries in geriatric patients and those with poor oral hygiene, but may also be useful in the construction of orthodontic retainers, night guards or other appliances worn long term, where the release of fluoride could reduce microbial inhabitation in these appliances. Studying the effects of fluoride released from these appliances on different microbial growth, is one of the features need to be investigated.

Prior to investigate microbial growth on resins *in vitro*, a suitable sterilization method is needed to produce a decontaminated environment before running experiments. The choice of the sterilization method is altered depending on the material to be sterilized, to reduce the potential for material damage. The effectiveness of any sterilization method is also dependent upon other factors such as the type of microorganisms present as some microorganisms are very difficult to kill, the number of microorganisms present, and the surface characteristics of the material with cracks and crevices providing good protective harbors for microorganisms. Therefore,

efficient techniques of sterilization are required to ensure complete microbial destruction (Fais *et al.*, 2009; Da Silva Aquino, 2012).

There is a correlation between some types of sterilization, such as microwave and changes in the surface topography of denture base material (Sartori *et al.*, 2006; Machado *et al.*, 2009). The surface properties of any denture base material are of particular concern (Abuzar *et al.*, 2010). Most oral microorganisms, especially those responsible for caries, stomatitis and periodontitis can only survive inside the mouth when they adhere to non-shedding surfaces and begin to form colonies (Morgan and Wilson, 2001). Studies on denture materials have shown a direct link between roughness, the accumulation of plaque and adherence of *Candida albicans* (Radford *et al.*, 1998; Bahrani *et al.*, 2012). Furthermore, Rougher surfaces can cause discolouration of the prosthesis, be a source of discomfort to patients in addition to the microbial colonization and biofilm formation (Abuzar *et al.*, 2010). Therefore, it is necessary that the roughness of materials used for dental prostheses is measured.

The composition of the materials may be affected by exposure to high temperature. For example, the degree of polymerization of PMMA might be altered by heating (Craig, 1997). One of the techniques used to measure the composition of acrylic resin materials is Fourier Transform Infrared Spectroscopy (FTIR) (Wei *et al.*, 2011). It can be used to investigate materials in the solid, liquid and gaseous phases. A spectrum for a material is characterised by the vibrations of the constituent atoms within the material when excited by an infrared laser. As the frequency of the vibrations is dependent upon, amongst other things, the mass of the atom, each atomic group has a characteristic peak, at a specific wavenumber (Wendl *et al.*, 2004; Moraes *et al.*, 2008). Infrared spectroscopy involves some precautions such as using standard or calibration curve during sample preparation and the experiment to obtain accurate spectral data and precise results. Spectra can be acquired by transmission or reflection methods. The transmission method is obtained by transmitting light through the sample while the reflection method by reflecting light off the sample (Moraes *et al.*, 2008). Attenuated total reflectance (ATR-FTIR) spectroscopy is appropriate method for the reflection mode to analyse the sample surfaces (Wendl *et al.*, 2004).

Studying a fluoride releasing dental material makes it difficult to select an appropriate method of sterilization, because it is important that the sterilization process has only a minimal effect on the fluoride release. Sterilization should also have a minimal effect on the materials physical and chemical properties.

3.2 Aims

In this chapter, a series of experiments have undertaken to try and optimize the sterilization method of fluoride releasing acrylic resins.

The aim was to select a suitable method to sterilize fluoridated copolymer samples with minimal fluoride release, or impact on the chemical composition and surface topography of the fluoridated copolymer material.

3.3 Materials and Methods

3.3.1 Effect of sterilization methods on fluoride release

-Sample preparation

PMMA (John Winter & Co Ltd, UK) and NaF (Sigma –Aldrich, Inc. USA) powders were mixed according to the formula in (Table 3.1) using a digital scale (Mettler- Toledo Ltd., Switzerland). MMA (John winter & Co Ltd, UK) to HEMA (Sigma –Aldrich, Inc. USA) ratio (Table 3.1) was prepared in a dark glass container. Following manufacturer instruction, liquid/powder ratio of (7 vol. /10 wt.) was added and all the materials mixed manually using a plastic spatula.

Teflon moulds were used to prepare discs of 10 mm diameter and 1 mm thickness (Figure 3.1). Samples were fixed on dental die-stone (GC FUJIROCK, Alsip, USA) prepared base that was coated with separating medium liquid (Metrodent, Ltd. UK). Another prepared dental die-stone coated base was placed over the samples and were compressed using a 3 kg weight for 20-30 min. The diameter and thickness of the samples were measured by digital caliper with accuracy of 0.02 mm (Mitutoyo Digimatic, Japan) after excess material was cleaned from the sample borders. The samples were stored in an incubator at 37°C before use.

Table 3.1 The formulations of acrylic resin that were used to prepare the non-fluoridated and fluoridated samples.

Groups	Powder		Liquid	
	PMMA wt%	NaF wt%	MMA vol%	HEMA vol%
PMMA/NaF (non-fluoridated)	100	0	60	40
PMMA/NaF (fluoridated)	70	30	60	40

-Procedure

Twenty-five specimens of fluoridated material were polymerised and then divided randomly into 5 groups (n=5):

Group 1: Specimens were sterilized for 2 h at 160°C in a Memmert U40 drying oven (Memmert, Schwabach, Germany).

Group 2: Specimens were placed in a sealed container (universal glass bottle of 28 mL, Fisher Scientific UK Ltd, Loughborough, UK) and sterilised using an autoclave for 15 min at 121°C

Group 3: Specimens were placed in distilled water in a sealed container and sterilised using an autoclave for 15 min at 121°C

Group 4: Specimens were placed in an open container and sterilised using an autoclave for 15 min at 121°C

Group 5: A control group, which was not sterilised.

The control group was not sterilized but stored inside the incubator at 37°C until fluoride measurements commenced. All the samples were placed obliquely into plastic cylindrical vial (12 ml, VWR international Ltd., UK) containing 5ml of deionized water to allow full immersion of the samples in the storage water whilst maintaining minimal contact with the container walls (Figure 3.1). Samples were stored at 37°C for 24h before fluoride release was measured.

The fluoride release measurements were carried out on day 1, 2, 3, 4, 5, 6, 7, 14, 21, 30, 60, 90, 120, 150 and 180. At the time of fluoride measurement, each sample was removed from its container and the storage solution decanted for analysis. After the first week, fluoride was measured weekly, then monthly basis and storage water was changed a day before the measurement day. The samples were dried using a paper towel and replaced in a clean container with fresh 5 ml deionized water, and storage was continued.

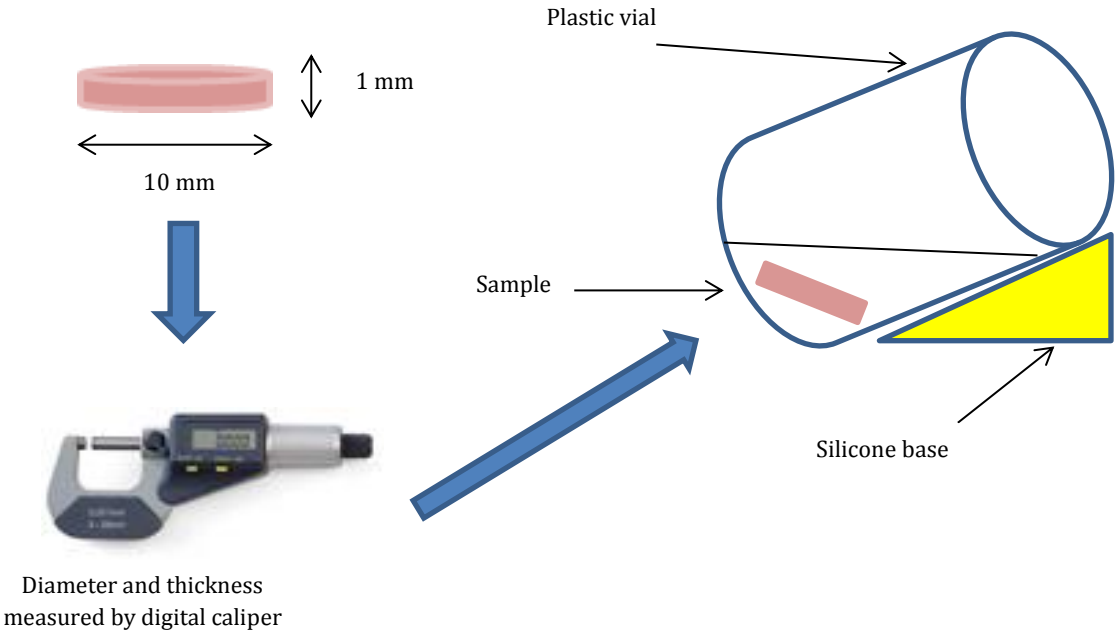


Figure 3.1 A diagram showing the prepared sample with an inner diameter of ≃10mm, and a depth of ≃1.0mm measured by digital Vernier calliper.

-Measurement of fluoride using ion selective electrode (ISE)

An acetic buffer solution (TISAB III, Orion Research, Thermo Scientific, MA, USA) of 500 µl was added to each storage solution. Fluoride concentration was measured using a calibrated ion selective electrode (Model 720A, Silver/Sulphide combination ISE, Orion Research, Thermo Scientific, MA, USA) attached to an ion meter (Bench top pH/ISE meter, Thermo Scientific, MA, USA) as shown in Figure 3.2. Standard solutions of fluoride (Thermo Scientific, MA, USA) with different concentrations (100, 10, 1, 0.1, 0.01 ppm F^-) were used for calibration every two hours. The solution being measured was stirred throughout the test using a magnetic stirrer (VWR international Ltd.) and the electrode immersed. Between measurements, the electrode was rinsed with de-ionized distilled water and blotted dry. When not in use, the electrode was immersed in a standard fluoride solution as instructed by manufacturer.

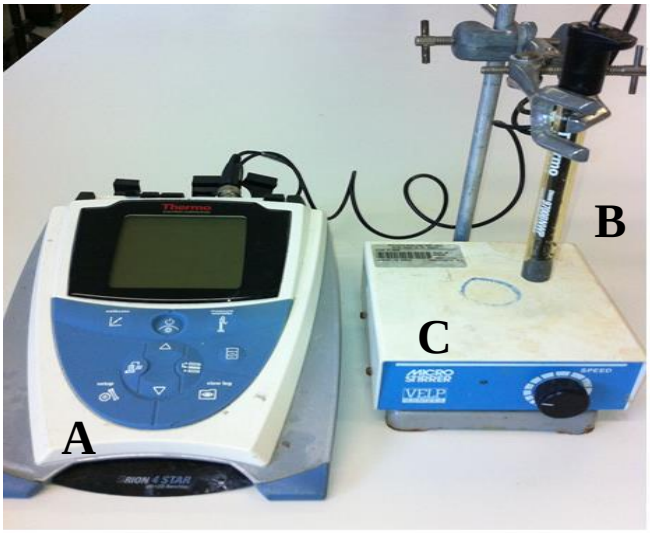


Figure 3.2 The ion selective electrode measures fluoride release in millivolts; a) Ion meter, b) Ion selective electrode, c) Magnetic stirrer.

All readings, in milliVolts (mV), were taken after 3 min immersion, to allow equilibration. The calibration curve determined from the standard solutions was used to define the fluoride release range of each sample. The readings recorded from the meter were converted from mV to ppm using the calibration curve and were then converted to (µg/cm^2) by dividing it by the surface area of the sample. Therefore, the results were presented as the rate of fluoride released per unit surface area of the sample per day (µg/cm^2.day).

3.3.2 Effect of sterilization method on chemical composition of prepared samples

-Sample preparation

Fluoridated and non-fluoridated samples were prepared using the same method described in section 3.3.1. Ten samples from each group were prepared in an isolated container for 24 hours inside incubator at 37°C before use.

-Procedure:

All of the measurements were made using an FTIR (Spectrum One, Perkin Elmer, UK) equipped with a single reflection diamond attenuated total reflectance (ATR) accessory (Figure 3.3). All spectra were measured between (2000 cm^{-1} - 750 cm^{-1})

using a diamond sensing element at 4 cm^{-1} spectral resolution and analyzed using Spectrum Time Base software (Perkin–Elmer). Prior to analysis of each specimen a background spectrum was taken. Next, the specimen was placed onto the diamond sensor and spectrum acquisition commenced. Data were analyzed using Microsoft Excel software 2010 (Microsoft office 14) and the standard baseline method was utilized to evaluate peak height (Rueggeberg *et al.*, 1990).

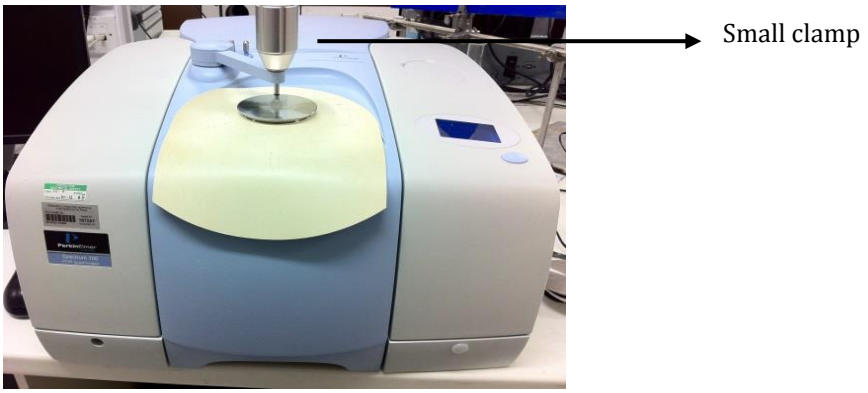


Figure 3.3 Fourier transform infrared spectroscopy (FTIR). A diamond crystal element is positioned in the middle of a sample plate with a clamp arm to hold the sample.

3.3.3 Effect of sterilization method on roughness measurement

-Sample preparation

Fluoridated and non-fluoridated samples were prepared using the formulation described in section 3.3.1. Teflon moulds were used to prepare discs of 15 mm diameter and 3 mm thickness. Ten samples from each group have prepared and were kept in an isolated container for 24 hours inside an incubator at 37°C before use.

-Procedure

On the surface of each sample, three straight lines were drawn using a permanent pen (Staedtler Lumocolour, Germany) to indicate the position of measurements to be taken before and after autoclaving (Figure 3.4, A). A stylus profilometer (Mitutoyo Surftest SV-2000) (Figure 3.4, B) and its associated software (Surfpak-SV Mitutoyo Corp V1.600) were used for sample profiling. The stylus is a diamond cone tip held at 90 degrees to the surface, with a 5 µm radius using a force of 4 mN. Calibration was

carried out at R_a 2.90 μm using a calibration grid. The stylus was run with speed of 1.0 mm/sec on evaluation length of 12.5 mm as the sample radius was 15mm. The R_a value was obtained from the 3 lines of each sample. Each profile was Gaussian filtered using the software. A final R_a average was then calculated for that specimen which was transferred to Microsoft Excel software 2010 (Microsoft office 14) for analysis.

3.3.4 Surface imaging by Scanning Electron Microscopy (SEM)

Four freshly made samples (non-fluoridated and fluoridated groups) were prepared to be visualized under SEM (Stereoscan S40, Cambridge Instruments, UK). Samples were imaged both before autoclaving and after autoclaving to visualize the impact of autoclaving on the profile of the material. Prior to SEM analysis, samples were dehydrated in a desiccator for 7 days, mounted on aluminum stub and coated using Achesons silver dag (Agar Scientific, Essex, UK). After drying overnight, samples were coated with gold (standard 15nm) using a Polaron SEM coating unit. A magnification power of $\simeq$ 50 was used to visualize the surface.

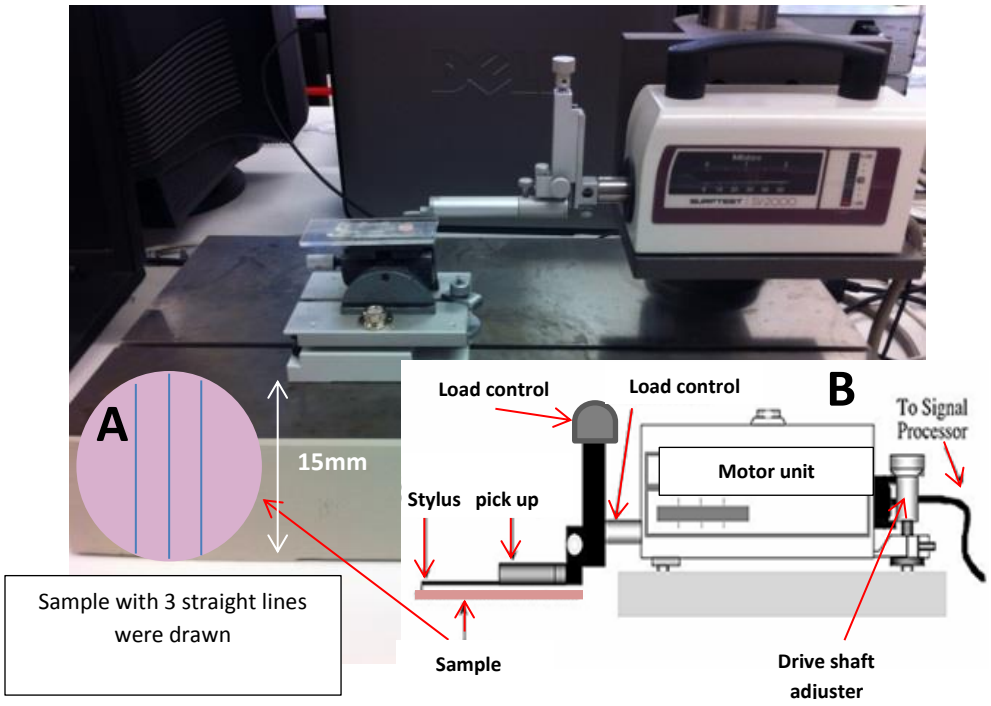


Figure 3.4 Stylus profilometry (Mitutoyo Surftest SV-2000). A) PMMA samples with 15mm in diameter with three lines that has marked on its surface, B) Schematic diagram shows the parts of the instrument.

3.4 Results

3.4.1 Effect of sterilization methods on fluoride release of prepared samples

The median amount of fluoride released from the five groups measured by ISE at each test point during this study, together with its interquartile range are listed in Table 3.2 and illustrated in Figure 3.5 below.

Table 3.2 the median values (and interquartile range) of five groups (n=25) during the six months in fluoride release measurement. Within rows, numbers with matching superscript letters are significantly different from each other (Kruskal Wallis Test, Mann-Whitney U Test $p<0.05$).

Group μg/cm².day / Days	Dry heat	Autoclaving in water	Autoclaving sealed cover	Autoclaving in steam	Control unsterilized
1	593.4 (125)	616.9 (77)	637.7(122)	568.9 (153)	655.7 (91)
2	559.0(210)[a]	450.1 (85)[b,c]	589.0(166[d]	405.0(109)[a,b,d,e]	585.3(93)[c,e]
3	476.6 (211)	308.2(91)[a,b,c]	475.0 (89)[a]	376.4 (128)[b]	454.1(190)[c]
4	399.2 (68)	261.9 (171)	429.6 (55)	360.1 (112)	402.0 (175)
5	348.4 (170)	249.7 (105)	304.0 (46)	271.5 (103)	260.1 (100)
6	343.5 (156)	235.0 (112)	294.8 (50)	265.4 (112)	267.4 (108)
7	100.0 (49)[a]	58.8(23)[a,b,c,d]	84.6 (4)[b]	123.5 (44)[c,e]	83.4 (29)[d,e]
14	81.0 (25)	57.3 (22)	52.9 (11)	70.3 (19)	62.0 (29)
21	44.6 (15)	37.8 (13)	29.9 (4)	39.0 (14)	33.2 (8)
30	28.1 (12)[a]	27.9 (6)[b,c]	20.6 (4)[b,d]	25.9 (8)[d,e]	22.9 (6)[a,c,e]
60	22.7 (10)[a,b,c]	18.6 (6)[d,e,f]	13.0 (2)[a,d]	14.7 (3)[b,e]	13.1(4)[c,f]
90	24.1(8)[a,b,c,d]	13.1(5)[a,e,f,g]	9.4 (2)[b,e,h]	10.9 (2)[c,f,h,i]	9.5 (2)[d,g,i]
120	16.1 (12)[a]	16.9 (2)[b]	11.9 (1)[a,b,c]	16.7 (2)[c]	15.5 (4)
150	14.3 (7)[a]	15.9 (2)[b,c]	10.7 (3)[a,b,d]	15.6 (3)[d]	14.5 (4)[c]
180	14.1 (7)[a]	15.3 (2)[b]	10.3 (3)[a,b,c]	15.2 (2)[c]	14.2 (4)

In general, the fluoride released from all groups showed the same pattern of release. On the first day, the highest release was observed from all experimental groups, which started to decrease gradually. A sustained amount of fluoride was seen released during the first six days, after which fluoride was released at a low rate for six months. Along the periods of measurement, there was a significant different ($p>0.05$) between groups in the days 2, 3, 7, 30, 60, 90, 120, 180.

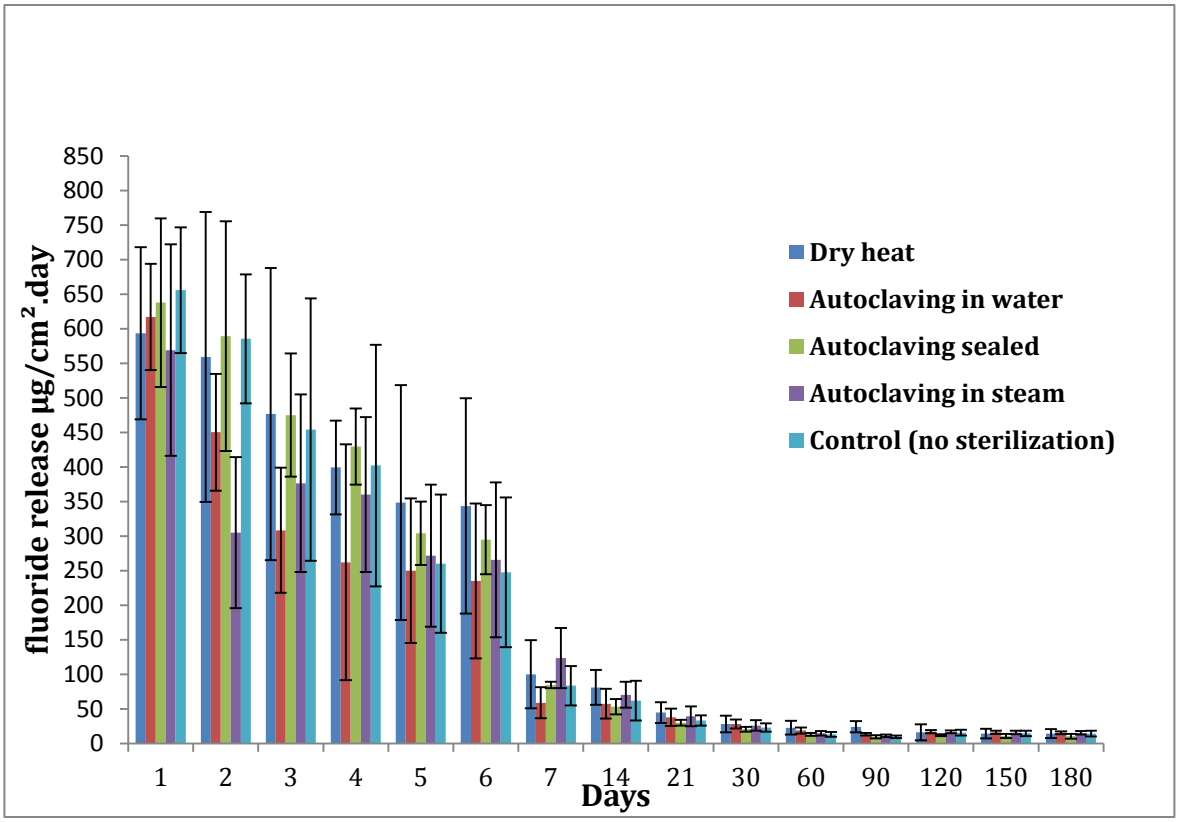


Figure 3.5 Rates of fluoride release from samples following incubation in deionized water for up to 6 months. Fluoride release was measured on specific days over the incubation period and the median and interquartile range from 5 different samples per group is shown. From 7 days to 180 days, the amount of fluoride released from all the groups was minimal in comparison with the first 6 days.

The results of six month fluoride release demonstrated that samples autoclaved in sealed containers were the only group that has no significant difference to the control group along this period. Therefore, this autoclaving method was chosen for further investigating its effect on the chemical composition and roughness of the materials.

3.4.2 Effect of sterilization method on chemical composition of prepared samples

FTIR analysis was used to evaluate the effect of autoclaving on the chemical composition of the material. Following autoclaving, new peaks were evident at 1600 cm^{-1} and 1550 cm^{-1} that were not present in the freshly prepared samples (Figure 3.6). So it was hypothesized that these peaks may be related to water sorption during autoclaving. Therefore, samples were stored in the incubator at 37°C for 7 days to reduce the water content. Following repeat FTIR testing one week later, these peaks were no longer evident, which confirmed that they are likely to be due to water (Figure 3.6).

The height of the peak at 1715 cm^{-1} which represents the carbonyl group C=O was chosen to normalize the graph to facilitate comparison, because it undergoes minimal change during setting, due to C=O not participating in the polymerization reaction (Duray *et al.*, 1997). The peaks of all groups were analyzed qualitatively depending on the results in Figure 3.6. Quantitative analysis was not undertaken as it was not thought to contribute to the results because all groups demonstrated similar peak heights and shapes apart from those previously identified at 1550 and 1600cm^{-1}.

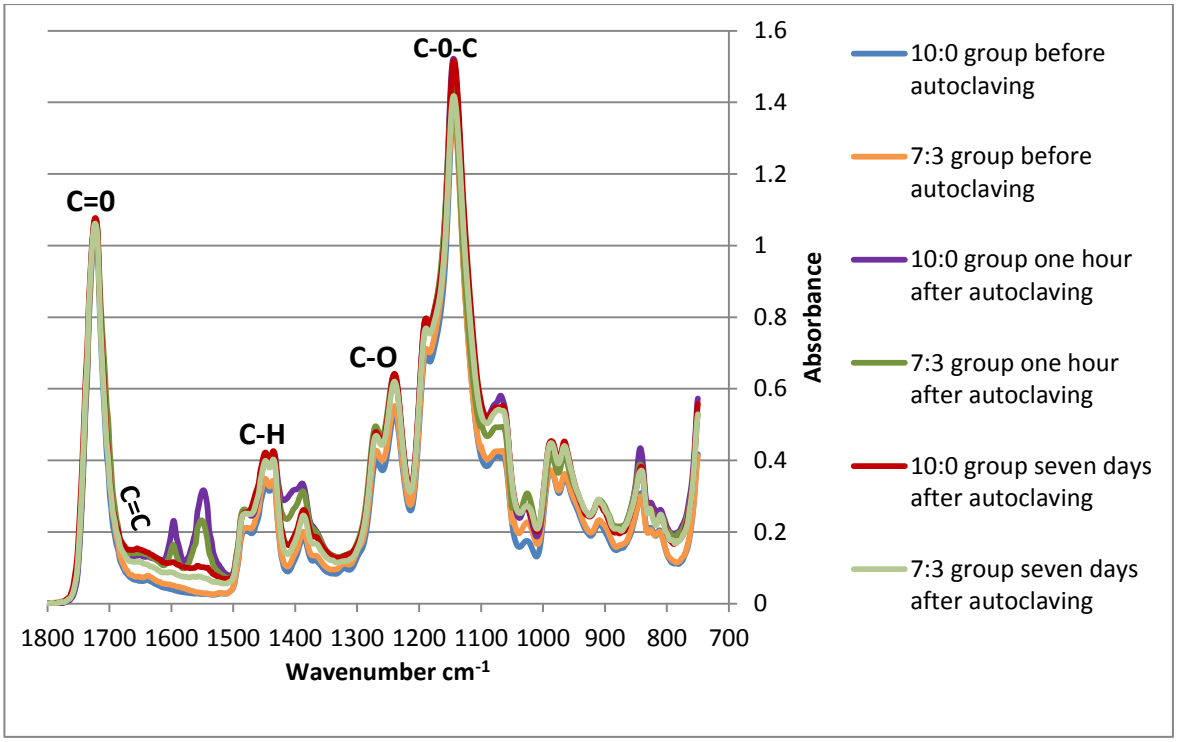


Figure 3.6 Effect of autoclaving on both non-fluoridated and fluoridated material. This figure represents the mean ATR-FTIR spectra of the groups (n=10). The peak at 1715 cm^{-1} was normalized to one after adjusting to zero at 1800 cm^{-1}.

3.4.3 Effect of sterilization method on roughness of prepared samples

The results of this experiment (Table 3.3) show the effect of autoclaving on the surface profile of fluoridated and non-fluoridated samples. The data collected from stylus profilometry illustrate the difference between the same group before and after sterilization in addition to the difference between the two groups before and after sterilization. There was no significant difference in roughness R_a ($p>0.05$, independent samples T-test) between the two groups before sterilization but there was a significant difference ($p<0.05$, independent samples T-test) between them after sterilization. To find out which group was affected by sterilization, paired samples T-test was used and data showed a significant difference ($p<0.05$) in the fluoridated group before and after sterilization while there was no significant difference ($p>0.05$) in the non-fluoridated group. Typical stylus profiles of both groups were shown in Figure 3.7.

Table 3.3 The mean and standard deviation of roughness measurement (R_a) of fluoridated and non-fluoridated groups before and after sterilization.

Method R_a (µm)	Groups	
	Non fluoridated Mean (SD)	Fluoridated Mean (SD)
Before	2.29 (0.39)	2.77 (0.91)
After	2.37 (0.64)	3.49 (0.9)*

*Significantly difference after autoclaving and also significantly difference compared with non-fluoridated group after autoclaving.

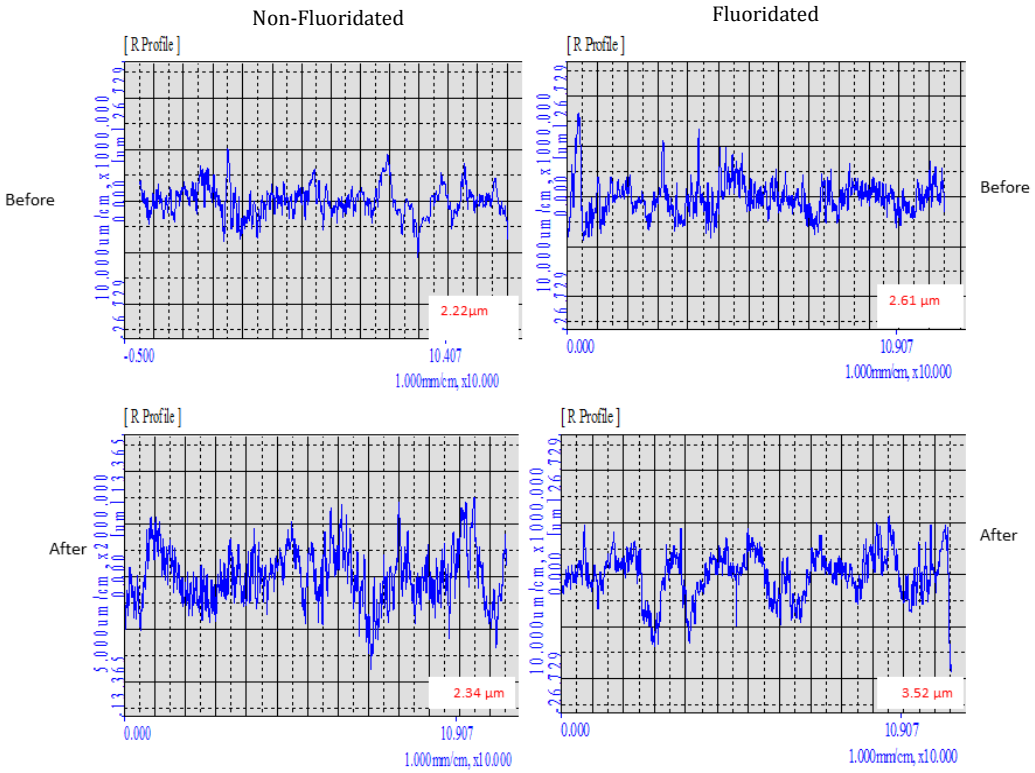


Figure 3.7 Typical stylus profilometry profiles of non-fluoridated and fluoridated materials and autoclaving effect. Differences in the peaks and valleys of both materials appeared after autoclaving with higher R_a value were found on fluoridated material after autoclaving.

3.4.4 Surface imaging by SEM

SEM images showed that the surfaces of both non-fluoridated and fluoridated materials were likely to be affected by autoclaving when visualized at 50x magnification (Figure 3.8). More defects were visualized on fluoridated material which means that autoclaving might induce minor changes in the surface topography of this material.

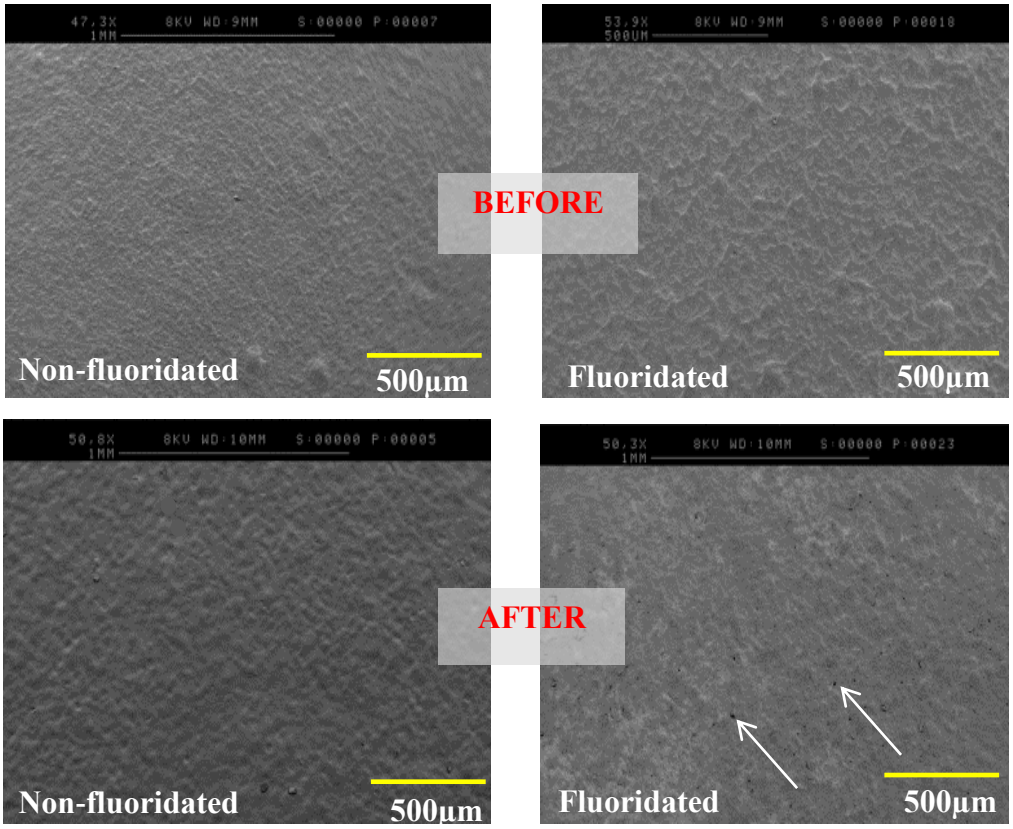


Figure 3.8 SEM images show the surface topography of non-fluoridated and fluoridated materials before and after autoclaving. Voids appeared in fluoridated samples after autoclaving (arrows).

3.5 Discussion

In this part of study, a number of experiments were carried out to investigate the effect of sterilization on the amount of fluoride released, chemical composition and roughness of the materials. The formula of the materials used to prepare the samples was chosen depending on previous studies (Zahroon, 2014). The ratio of fluoride concentration selected was the highest that used previously (Zahroon, 2014), because material properties (i.e. hardness and wear) were influenced negatively by increasing the fluoride content of the material (Zahroon, 2014). In addition, it was intended to investigate whether this concentration can decrease the rate of biofilm growth on the surface of the material. Sodium fluoride was chosen in this study as it can yield free F^- and Na^+ ions in the solution (Nakajo *et al.*, 2009). NaF solubility was found not to be influenced to the pH of the medium (Shen *et al.*, 2007), so it is more useful in conditions where the pH is fluctuant like in the oral cavity (Shen *et al.*, 2007). Many previous studies have investigated other fluoride compounds (i.e. AlF^-_4, SnF_2, BeF^-_3) and demonstrated that they were effective in suppressing the enzyme function of microbial cells (Sternweis and Gilman, 1982; Petsko, 2000; Embleton *et al.*, 2001; Meurman *et al.*, 2009). However in this study, it was preferable to investigate the specific effect of fluoride ions on impairing microbial colonization.

Developing a method to sterilize samples with minimal fluoride release was a challenge. As mentioned in the literature review, different methods are available to sterilize polymer materials but the issue in this study was to find a method or technique with minimal amount of fluoride loss from the material. Three of the methods were depended on autoclaving and they were suggested for better compatibility and ease of use with system models used for *in vitro* biofilm formation. Sterilization by dry heat was the last option if autoclaving would lead to high fluoride loss from the material. The protocol of daily fluoride measurement for the first week, then weekly for a month, followed by monthly measurement for six months was utilized previously by many researchers (McNeill *et al.*, 2001; Su *et al.*, 2010; Zahroon, 2014). The water containing the samples was changed 24 h before fluoride release measurements were taken to detect the amount of fluoride released in this period

rather than measuring cumulative fluoride release (Rix *et al.*, 2001). Deionized water was used to reduce the interference at the ion electrode surface that might be induced by ions, impurities and other ingredients of natural and artificial saliva.

The amount of fluoride released was measured by an electro analytic method using an ion selective electrode (ISE). This instrument is most commonly used to measure fluoride release as it is reliable, easy to use, and has great selectivity and specificity for fluoride ions (Rajković and Novaković, 2007). However, this instrument is highly sensitive to temperature and pH changes and its accuracy is affected in very low fluoride concentration. Therefore, a relatively long time is needed to stabilize the analyzer reading (McCabe *et al.*, 2002) by using the magnetic stirrer to optimise the analytic parameters of the electrode. In addition, stirring facilitates ion transmission through the electrode membrane by avoiding the saturation and accumulation of ions in the solution and around the electrode. TISAB was added to the solutions (standard and sample solutions) to de-complex fluoride ions and prevents interference from other ions complex such as hydroxide ions (McNeill *et al.*, 2001), as both these complexes have similar ionic charge and ion radius (McCabe *et al.*, 2002; Rajković and Novaković, 2007).

The results showed higher amount of fluoride released during the first six days which gradually decreased to become low but constant with time. These results were in agreement with Erdem *et al.* (2012) and Zahroon (2014). The way that fluoride was released supported the two phase diffusion theory which stated that the first process relates to early rapid surface elution of short term release. This is followed by bulk diffusion produced from prolonged slow elution and long term release from the core of the material (Verbeeck *et al.*, 1998). This can be interpreted that the high fluoride released at first was most likely due to fluoride 'wash-off' from the exposed surface of the material (Anusavice *et al.*, 2005). While the second phase of elution may be due to smaller volumes of ions released from the pores and cracks which induce the slow and prolonged release by bulk diffusion of the material (Anusavice *et al.*, 2005). Furthermore, the addition of HEMA facilitated sustained fluoride release by promoting diffusion of water (Su *et al.*, 2010). Whilst the presence of HEMA can affect the mechanical properties of the material, it will increase the water sorption into the

porosities rather than the matrix of the material. This phenomenon might enhance the reduction in the mechanical properties after water storage (McCabe and Rusby, 2004).

The amount of fluoride released by all groups was in the range of (10-15 μg/cm^2.day) after six months. These values are still high enough to inhibit acid production by microorganisms in dental plaque (Jenkins *et al.*, 1969; Bibby and Fu, 1986). Additionally, it has been found recently that this material has the capability to be recharged (Zahroon, 2014). Therefore recharging of the material could be another option to maintain high rate of fluoride available to decrease the growth chance of pathogens and occurrence of biofilm related diseases.

Evaluation of the effect of autoclaving on the chemical composition of the material was achieved by using FTIR. The FTIR is used to monitor the interactions between various constituents because of the differences in compositions and investigate autoclaving effects on the material. This technique can measure sorption into polymers. After autoclaving it was clear that there were new peaks apparent in the spectra. HEMA is a hydrophilic material, which could have absorbed some water during the autoclaving. Consequently, the specimens were left to dry for a week and tested by FTIR. After this time, the peaks had disappeared, allowing the conclusion that these peaks were due to absorbed water to be made. However, small changes in the absorption intensities were found between the two groups of materials tested. These inaccuracies may have been due to the methodology, rather than changes in the samples. For example, some irregularities might be present on prepared sample surface which can lead to imprecise contact with the diamond crystal. Furthermore, the amount of pressure applied by the physical clamp device may vary slightly, which could have an impact on the results obtained. The influence of this parameter on the peak intensities of the IR spectrum was previously introduced by (Carlsson and Wiles, 1970). These two parameters might induce changes in the angle of incidence (θ) which will be reflected in the shape of peaks that were initiated. Therefore, the carbonyl group C=O was chosen to normalize the graph and facilitate the comparison as it has minimal overlap with neighbouring peaks. Overall, it was found that there was minimal effect of autoclaving on the chemical composition of non- fluoridated and

fluoridated samples. Existing peaks remained relatively stable and no new peaks appeared after this method of sterilization.

The effect of autoclaving on the roughness of non-fluoridated and fluoridated materials was investigated using stylus profilometry. As mentioned in the literature review, there are many different instruments that can be used to measure the surface profile with specific advantages and disadvantages of each technique. In this study, stylus profilometry was used as it can measure samples with larger size and scale in comparison with AFM. Furthermore, sample visualization by AFM usually requires liquid immersion. This might influence the surface property of fluoridated group by fluoride release. Laser profilometry has not been tried due to the many drawbacks that identified of this technique (Poon and Bhushan, 1995; Wieland *et al.*, 2001; Heintze *et al.*, 2006; Rodriguez *et al.*, 2009). The position of sample measurement was determined by lines drawn on the surface of each sample. This method helped to ensure the same profile was measured before and after autoclaving. The results showed a significant increase in roughness of fluoridated samples after autoclaving. A likely explanation for this is that fluoride might leach out from the material as a normal behaviour when this copolymer material exposes to water. However, other possible explanation is that the high temperature reached during the autoclaving procedure could cause a breakdown of the surface layer, probably as a result of microcrazing of the surface, with loss of integrity (Machado *et al.*, 2009). In addition, the presence of HEMA will greatly increase the water absorption during autoclaving and this could induce NaF leaching out from the material leaving voids and pores on the surface of the material.

SEM is one of the common techniques used to visualize the shape and morphology of the materials surfaces (Perevyazko *et al.*, 2010). Surface imaging by SEM revealed slight changes in the surface topography of both non-fluoridated and fluoridated groups after autoclaving. The pores that were formed on the fluoridated group after autoclaving are not likely to be formed by air entrapment during setting, because they have not been found in fresh samples. Therefore, they are more possibly related to the released fluoride or surface defect as discussed previously.

From a clinical point of view, the method of sterilization that was chosen is not commonly used for sterilizing oral or dental appliances. There are many other disinfection methods (i.e. sonication, UV light) that are more commonly used to maintain appliance hygiene. Therefore, material properties are less likely to be affected. In summary, the results of these experiments revealed that fluoride release and the chemical composition of the material were not affected by the autoclaving method that was chosen. However, a slight variation in roughness was noticed in fluoridated samples after autoclaving.

Chapter 4 . Development of techniques for biofilm quantification

4.1 Introduction

Denture plaque is in many ways similar to plaque on tooth surfaces, and consists of many different species of microorganisms. As denture plaque ages, proportions of aciduric organisms, including mutans streptococci, lactobacilli, yeasts and particularly *Candida albicans* tend to increase (Budtz-Jörgensen, 1974; Theilade *et al.*, 1983; Koopmans *et al.*, 1988; Sumi *et al.*, 2003) *C. albicans* is associated with denture stomatitis that presents in approximately 65% of edentulous individuals (Chandra *et al.*, 2001a). Furthermore, the presence of *Candida* species (especially *C. albicans*) has been recognized on obturator prostheses, whether silicone or polymethyl methacrylate, in patients with maxillary defects, such as congenital malformation, tumours, or trauma, and on the mucosa adjacent to the prosthesis (Courtois, 2011). In addition to *Candida albicans*, previous studies found the participation of other bacterial species such as *S. mitis, S. oralis, S. sanguinis, S. mutans* and *L. casei* in the development of oral appliance-related diseases especially denture stomatitis and root caries (Sato *et al.*, 1997; Budtz-Jörgensen 2000; Morgan and Wilson, 2000; Preshaw *et al.*, 2011; Teles *et al.*, 2012).

Different methods have been employed for the identification and quantification of different species in oral biofilms. Traditionally, plate counting has been the method of choice for determination of viable cell numbers. DNA-based methods and especially qPCR have been used for the detection and quantification of cariogenic (Rupf *et al.*, 2003; Yoshida *et al.*, 2003; Kreth *et al.*, 2008) and periodontal bacteria (Paster *et al.*, 2001). While conventional polymerase chain reaction (PCR) involves laborious post-PCR analysis, modern real-time instruments, such as Taqman or LightCycler, offer a fast kinetic analysis of the amplification process (Morrison *et al.*, 1998). The release of the fluorescent dye during each round of amplification allows the rapid detection and quantification of DNA without the need for post-PCR processing, such as gel electrophoresis or hybridization (Heid *et al.*, 1996). In general, DNA quantification methods require microorganisms DNA extraction by a combination of physical and chemical methods, including breaking the cells down (cell lysis), and removing lipids,

proteins and RNA using specific detergents and enzymes (Sambrook *et al.*, 2001). Quantitative PCR is now widely used in bacterial recognition as it is more accurate than TVC's in some situations (Yano *et al.*, 2002). For qPCR quantification, plasmids DNA are usually used as standards. These plasmids are circular small double stranded DNA (thousand to hundreds of thousands base pairs) which are often carrying genes provide bacteria with genetic properties such as antibiotic resistance (Sambrook *et al.*, 2001). Therefore, both these techniques were selected to quantify and differentiate single and mixed species biofilms that will be produced in this study.

4.2 Aims

The aims of this chapter are to develop and validate methods for quantifying the different microorganisms. The specific objectives were as follows:

1- Identification of selective agar media for each microorganism.
2- To find a protocol for the efficient extraction of DNA from the three species.
3- To develop methods for enumerating cells in biofilms using viable counts and qPCR quantification.

4.3 Materials and Methods

4.3.1 Bacterial strains and growth conditions

Candida albicans VPSA1 (isolated and identified by Shakir *et al.* (2012)) was cultured in Sabouraud Dextrose Agar (SDA) (Oxoid Ltd, Hamshire, UK) containing 10 g/L Mycological peptone and 40 g/L dextrose, aerobically at 37°C.

Streptococcus mutans UA159 was cultured in Todd Hewitt (THYE) containing 36.4 g/L Todd Hewitt and 5 g/L yeast extract (Difco, Becton Dickson and Co.), in a candle jar at 37°C.

Lactobacillus casei ATCC334 was cultured in DeMan Rogosa Sharpe (MRS) (Sigma-Aldrich, Inc.) containing 2 g/L diammonium citrate, 2 g/L dipotassium hydrogen phosphate, 20 g/L glucose, 0.2 g/L magnesium sulfate heptahydrate, 0.05 g/L manganous sulfate tetrahydrate, 0.05 g/L manganous sulfate tetrahydrate, 10 g/L meat extract, 5 g/L sodium acetate trihydrate, 5 g/L yeast extract, pH 5.4±0.2, using candle jar at 37 °C.

E-coli TOP10F´ was used as a host for pCR2.1-based constructs (Invitrogen, Life Technologies Ltd, Paisley, UK). *E. coli* was grown in Luria Bertani (LB) Broth (Melford laboratories Ltd.) at 35°C, with shaking at 200 rpm in aerobic conditions. Alternatively, *E. coli* was grown aerobically on LB agar (LB broth plus 15 g/L Bacto agar) at 37°C. 100 µg/mL ampicillin (Amp) was added when needed.

For solidified media, 15 g/L of Bacto-Agar (Difco, Becton Dickson& Co.) was added prior to autoclaving.

For glycerol stocks, 20 ml of the appropriate growth medium were inoculated with a colony from an agar plate and incubated at 37°C for 20 h. Cells were harvested at 5,000 g for 10 min at 4°C. Pellets were resuspended in 1 ml of a 50% dilution of growth medium supplemented with 50% (v/v) glycerol. Glycerol stocks were stored at -80°C.

4.3.2 Preparation of selective agar media for each microorganism

The selection of agar media that permitted the selective growth of individual species (*C. albicans*, *S. mutans, and L. casei*) was required in order to enumerate viable counts of different species in mixed biofilms. Two of the most commonly used agar media were selected for each microorganism:

C. albicans was cultured in: 1) SDA agar (see section 4.3.1). (2) Yeast Extract-Peptone-Dextrose (YPD) Agar (Sigma-Aldrich, Inc.) containing 20 g/L bacteriological peptone, 10 g/L yeast extract, 20 g/L glucose, and 15 g/L agar, aerobically at 37°C.

Streptococcus mutans was cultured in: 1) Tryptone Yeast Cystine (TYCSB) Agar (LAB M Ltd., Bury, UK) containing 0.2 g/L L-cystine HCl monohydrate, 15 g/L Bacto peptone, 5 g/L yeast extract, 0.1 g/L sodium sulfite , 1 g/L sodium chloride, 0.8 g/L, disodium phosphate anhydrous, 2 g/L sodium bicarbonate, 12 g/L sodium acetate anhydrous, 50 g/L sucrose , 15 g/L agar, bacitracin (0.2 unit/ml) (Sigma-Aldrich, Inc.), natamycin (21.6 µg/ml) (Sigma-Aldrich, Inc.). (2) Mitis Salivarius Agar (MSB) (Difco) containing 6 g/L pancreatic digest of casein, 9 g/L proteose peptone No. 3, 1 g/L dextrose, 50 g/L saccharose, 4 g/L Dipotassium Phosphate, 75 mg/L Trypan Blue, 0.8 mg/L crystal violet, 15 g/L agar, potassium tellurite (1%) (Oxoid Ltd, Hamshire, UK), bacitracin (0.1 unit/ml), and natamycin (21.6 µg/ml). In both cases, cells were cultured in a candle jar at 37°C.

Lactobacillus casei was cultured in: 1) MRS agar (see section 4.3.1), and natamycin (21.6 µg/ml). (2) Rogosa Agar (Sigma-Aldrich, Inc.) containing 2 g/L ammonium citrate, 10 g/L casein peptone, 0.034 g/L ferrous sulphate, 20 g/L D(+)-glucose, 15 g/L agar, and natamycin (21.6 µg/ml), using candle jar at 37°C.

4.3.3 Total viable counts

For viable counts (TVC's), serial ten-fold dilutions of cell suspensions were prepared in phosphate buffer saline (PBS) at pH 7.4. Triplicate 20 µl spots of each dilution were transferred to solidified selective media for each microorganism (section 4.3.1) using

the method of Miles *et al,* (1938). After incubation at 37°C for 24-48 h aerobically or in candle jar, colonies were enumerated and the original number of colony forming units (CFU) ml^{-1} was calculated.

4.3.4 Preparation of stock cultures from mid-logarithmic phase cells

C. albicans, S. mutans, and *L. casei* were cultured for 20 h at 37°C in 20 ml SDA, THYE, and MRS, respectively. Cultures were diluted 1:20 in pre-warmed fresh broth and incubated at 37°C. The optical densities of cultures at 600 nm (OD_{600}) were read in a UV/Vis Spectrometer (Unicam UV 2, ATI, USA) every hour for 5-7 h until mid-logarithmic phase (OD_{600} ~ 0.5). Cells of each species were harvested by centrifugation at 3500 x g, 4°C for 10 min. A glycerol stock for each microorganism was stored at -80°C and viable counts were determined the following day.

4.3.5 Extraction of DNA from microorganisms

In order to quantify the different species by qPCR, it was important to develop a single extraction method that would work for both bacteria and *C. albicans*. Four different types of DNA extraction kits were tried: UltraClean microbial DNA isolation kit (MO BIO laboratories, Carlsbad, CA), MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, USA), MasterPure yeast DNA purification kit (Epicentre Biotechnologies), and ZR Fungal/Bacterial DNA extraction kit (Zymo Research Co., CA, USA).

For the UltraClean microbial DNA isolation kit, *S. mutans* or *C. albicans* was cultured for 20 h in the appropriate growth medium and 1.8 ml were centrifuged at 15,000 x g for 30 sec at 25°C. The cell pellet was re-suspended in 300 µl of microbead solution and DNA was extracted according to the manufacturer's instructions. For the MasterPure DNA purification kit, *S. mutans* was cultured for 20 h in the appropriate growth medium and 10 ml were centrifuged at 5000 x g for 10 min. The remaining steps were performed in accordance with the manufacturer's instructions. For the MasterPure yeast DNA purification kit, 1.5 ml from *C. albicans* overnight culture was centrifuged by microcentrifuge at 18000 x g for 3 min. Then 300 µl of yeast cell lysis

solution was added and DNA was extracted following the manufacturer's instructions. For the ZR Fungal/Bacterial DNA extraction kit, 50-100 mg (wet weight) of fresh cell pellet was prepared from 20 ml of an overnight culture after centrifugation at 5000 x g for 10 min. The pellet was re-suspended in 200 µl PBS and all the steps were followed in accordance with the manufacturer's instructions. The eluted DNA from each of the kits was stored at -20°C prior to analysis.

4.3.6 PCR and purification of amplified DNA fragments

PCR was performed in 50 µl reaction volumes containing: 5 µl (10 µM) forward primer, 5 µl (10 µM) reverse primer, 25 µl 2X ReddyMix PCR Master Mix (containing 1.5 mM $MgCl_2$, Thermo Scientific, MA, USA), 1 µl DNA template, and 14 µl dH_2O. PCR amplifications were carried out in a T100 Thermal Cycler (BioRad, Hertfordshire, UK), using the protocol: initial denaturation at 94˚C for 2min, 35 cycles at 94˚C for 10 s, 55-58˚C for 30 s, 68˚C for 60 s, elongation at 68˚C for 7 min, and a hold at 12°C. DNA fragments amplified by PCR were cleaned using the EZ-10 Spin Column PCR Products Purification Kit (NBS Biological, Huntingdon, UK) and eluted in 50 µl elution buffer. The concentration of DNA was determined using a ND-1000 NanoDrop spectrophotometer (Thermo Scientific). An estimation of DNA purity was made based on the ratio of absorbance readings at 260 nm to 280 nm. A ratio of ~1.8-2.0 is generally accepted to be acceptable for DNA preparations.

4.3.7 Agarose gel electrophoresis

Electrophoresis through agarose was used to estimate the size of DNA fragments. Agarose concentrations between 0.8 – 1 % were prepared by boiling as required in 60 ml Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0). The solution was allowed to cool for 20 min, and 2 µg/ml ethidium bromide was added. The agarose solution was then poured into gel trays, with gel combs, and allowed to solidify. The gel was placed in an electrophoresis tank (Horizontal Electrophoresis System, Hybaid) containing 1 x TAE buffer. DNA samples were mixed with 0.2 volumes of 5 x loading dye [70 % (v/v) glycerol, 0.25 % Bromophenol blue, 0.25 and Xylene Cyanol] before loading. For estimation of DNA

molecular weight, samples were compared against 5 µl of either Hyperladder™ one (1 kbp) or Hyperladder™ four (100 bp) (Bioline Reagents Ltd, LN, UK). The gel was run for 1 h at a constant voltage of 110 V, and then transferred to G:BOX Transilluminator (Sygene) to capture images at 5.5 Mpixel using GeneSnap software (Sygene).

4.3.8 Primer design

Different software programs such as Primer3Plus and ClustalW2 were used to design and check primers and probes for *C. albicans, S. mutans* and *L. casei*.

The primers and probe of Guiver *et al.* (2001) were checked for complementarity against the genome sequence of *Candida albicans* strain WO1 (available through NCBI Genbank Reference Sequence: L28817.1).

The primers and probe of Yoshida *et al.* (2003) were checked for complementarity against the genome sequence of *S. mutans* UA159 (available through NCBI Genbank Reference Sequence: NR_075139.1).

The primers and probe of Haarman and Knol (2006) were checked for complementarity against the genome sequence of *Lactobacillus casei* strain ATCC 334 (available through NCBI Genbank Reference Sequence: NC_008526.1).

Oligonucleotide primers were ordered and synthesised by Eurogentec (Eurogentec Ltd, Hampshire, UK.

4.3.9 Cloning of fragments to produce qPCR standards

The TOPO cloning kit (Life technologies Ltd, Paisley, UK) was employed for cloning PCR-amplified fragments into pCR2.1-TOPO vector to create standards for qPCR reactions. For each product, two different molar ratios (vector:insert) of 1:1 and 1:3 were used in separate ligation reactions. Reactions were set up containing: 1-3 µl fresh PCR product, 2 µl 5x T4 DNA ligase reaction buffer, 2 µl pCR2.1 vector, 1 µl ExpressLink T4 DNA Ligase, and distilled water to a total volume of 10 µl. Samples

were incubated at room temperature (~23°C) for 15 min. Competent *Escherichia coli* TOP10F´cells (Life technologies Ltd) were transformed using the standard protocol recommended by the manufacturer. Transformation always included pUC19 as a positive control. For blue/white screening, LB agar plates supplemented with 100 µg ampicillin/ml (LB/AMP) were spread with 40 µl of X-Gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside (from a stock of 40 mg of X-Gal in 1 ml of dimethylformamide)]. One hundred µl aliquots of cells were spread on LB agar plates and incubated for 20 h at 37°C. Plates were transferred to 4°C for 3-4 h to allow proper colour formation. White colonies, likely containing the plasmid with insert, were picked and sub-cultured three times on LB AMP agar plates. Four isolated colonies were sub-cultured in 5 ml LB/AMP broth and incubated overnight at 250 rpm, and 37°C. Plasmids were extracted from 1.5 ml using Spin Column Plasmid Mini Kit (NBS Biological, Huntingdon, UK), and the remainder of each culture was used to prepare glycerol stocks as described in section 4.3.1 above.

4.3.10 Analysis of plasmids

Restriction enzyme digestions using *Eco*RI (New England Biolabs, Ipswich, UK) were performed on plasmid DNA extracts to check for the correct insertion of gene fragments of interest. Digestion reactions were carried out with the following reagents: Plasmid DNA product (up to 1 µg), *EcoRI* buffer 1 µl [100 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.5 and 0.025 % Triton], *EcoRI* enzyme 1µl, and dH_2O to 10 µl total reaction volume. The reactions were carried out using a T100 Thermal Cycler (bioRad, Inc.), using the steps: enzyme activation at 37°C for 2 h, heat inactivation at 65°C for 20 min, then held at 4°C. DNA fragments were analysed by agarose gel electrophoresis.

4.3.11 Plasmid purification

Once the correct plasmids were identified, large scale preparations were made using the Qiagen plasmid midi kit (Qiagen). For this, 10 µl of -80°C cultures were transferred to universal bottles containing 5ml LB/Amp broth. The cultures were incubated at 37°C with shaking at 200 rpm for 8 h. Cells were harvested by centrifugation at 5,000

g for 10 min at 4°C, and plasmids were extracted according to the manufacturer's instructions. The purified plasmids were checked using the NanoDrop spectrophotometer and agarose gel electrophoresis and stored at -20°C.

4.3.12 DNA sequencing

DNA samples were sent to MWG Eurofins (Eurofins Genomics, Ebersberg, Germany) for DNA sequencing. Sample template concentration was measured using NanoDrop and the concentration was adjusted to the amount required (PCR products, 10 ng/µl; Plasmid DNA, 50-100 ng/µl). Templates of 15 µl were sent with sequencing primers at a concentration of 10 pmol/µl in a total volume of 15 µl to Eurofins.

4.3.13 Plasmid quantification by PicoGreen

Plasmid quantification was performed by using the Quant-iT™ PicoGreen dsDNA Kit (Invitrogen, Carlsbad, USA). Following the manufacturer's instructions, 1:20 dilutions of DNA samples were produced in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). A standard curve was made by diluting a λ DNA standard (2 µg/ml) in TE buffer from a range of 1,000 ng/ml to 0 ng/ml. The PicoGreen dye was diluted 200 fold in TE buffer and an equal volume was added to the λ DNA standards and DNA samples. Triplicate 50 µl standards or samples were aliquoted to wells of a 384-well polystyrene plate, and the fluorescence was measured at excitation 485 ± 10 nm/emission 528 ± 10 nm in a Synergy HT microplate reader (BioTek, Bedfordshire, UK). Concentrations of DNA samples were calculated from the DNA standard curve using Microsoft Excel 2010 (Microsoft Office 14).

4.3.14 Quantitative PCR

Amplification and detection of DNA by qPCR were performed with an Opticon 2 DNA Engine (Bio-Rad Laboratories Ltd., Hertfordshire, UK) using optical grade 96-well plates (Eurogentec, Belgium). Ten-fold serially diluted DNA concentrations of plasmids pTOPO-*Ca*, pTOPO-*Sm* and pTOPO-*Lc* were used as reference standards for absolute quantification. Sequences of primers and TaqMan probes used in qPCR are

listed in Table 4.1. Each probe was modified with a fluorescence reporter dye (FAM-6) at the 5' end and a fluorescence quencher (MGBNFQ) at the 3' end, which suppresses the fluorescence while the probe is intact. Quantitative PCR reactions were prepared, containing the following reagents in each well: 1.2 µl of 2.5 µM forward primer, 1.2 µl of 2.5 µM reverse primer , 0.6 µl of 2.5 µM probe, 7.5 µl of 2X Takara master mix (Takara Bio Europe, France), 3.3 µl of H_2O and 1.0 µl of template DNA. The thermocycling conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 95°C for 5 sec, annealing/extension at 60°C for 30 sec, plate read and the cycle was repeated 40 times. All the reactions were carried out in triplicate and the final analysis was based on the mean of the three reactions. The qPCR data analysis was carried out using Opticon Monitor 3 software (MJ Research).

The quantity of template DNA in each sample was calculated by reference to wells containing serial 10-fold dilutions of standards of known DNA concentration. The logarithm of the DNA concentration of the standard was plotted against the cycle threshold (C_t). This generated a linear relationship over at least six orders of magnitude. The lowest concentration of standard for each probe was equivalent to approximately 10^3-10^4 cells. Genes *ITS2* (*C. albicans*), *16S-23S/ L. casei, and gtfB* (*S. mutans*) were used as targets and corrections were made for gene copy number. Representative genome sequences of *C. albicans, L. casei,* and *S. mutans* have been determined, and the *C. albicans* genome contains 110 copies of *ITS2* (Jones *et al.*, 2004), the *L. casei* genome contains 3 copies of *16S-23S*, and *S. mutans* contains 1 copy of *gtfB* per genome.

During the development of qPCR, three different master mix kits were tried: Takara (Takara Bio Europe, France), DyNAmo flash qPCR kit (Thermo scientific Ltd), and Sensimix NoRef (Quantace, London, UK).

4.3.15 Primers and probes

Table 4.1 Oligonucleotide primers and probes used during this study

PCR primers			
Name	Oligonucleotide sequence (5'–3')	Target region (gene)/species	References
CaFP	5'-GGAACCGAGAAGCTGGTCAA-3'	*ITS2/ C. albicans*	This study
CaRP	5'-GTCATCTCATCGCACGGGAT-3'	*ITS2/ C. albicans*	This study
CaF1	5'-GGGTTTGCTTGAAAGACGGTA-3'	*ITS2/ C. albicans*	(Guiver *et al.*, 2001)
CaR1	5'-TGAAGATATACGTGGTAGACGTTAG-3'	*ITS2/ C. albicans*	This study
SmF1	5'-GCCTACAGCTCAGAGATGCTATTCT-3'	*gtfB/ S. mutans*	(Yoshida *et al.*, 2003)
SmR1	5'-GCCATACACCACTCATGAATTGA-3'	*gtfB/ S. mutans*	(Yoshida *et al.*, 2003)
LcF1	5'-TGGATGCCTTGGCACTAGGA-3'	*16S-23S/ L. casei*	(Haarman and Knol, 2006)
LcR1	5'-ATAAGTAAGCTTTGATCCGGAGATTT-3'	*16S-23S/ L. casei*	(Haarman and Knol, 2006)
M13 Reverse	5'-CAGGAAACAGCTATGAC-3'	*pCR2.1-TOPO*	Invitrogen
T7 promoter	5'-CCCTATAGTGAGTCGTATTA-3'	*pCR2.1-TOPO*	Invitrogen
Taqman probes *			
Ca-probe	5'-TGAAGATATACGTGGTAGACGTTAG-3'	*ITS2/ C. albicans*	(Guiver *et al.*, 2001)
Sm-probe	5'-TGGAAATGACGGTCGCCGTTATGAA-3'	*gtfB/ S. mutans*	(Yoshida *et al.*, 2003)
Lc-probe	5'-TATTAGTTCCGTCCTTCATC-3'	*16S-23S/ L. casei*	(Haarman and Knol, 2006)

***** Probes were modified with a fluorescence reporter dye (FAM-6) at the 5' end and a fluorescence quencher (MGBNFQ) at the 3' end.

4.4 Statistical analysis of microbiological studies

Experiments were done in triplicate as standard. Tables and graphs were made using Microsoft Excel 2010 (Microsoft Office 14). Results are displayed as the mean and ± standard deviation. IBM SPSS Statistics 19 software was used for statistical comparisons. The Mean values of two groups were compared for significant differences using the independent sample *t*-test. A *p*-value less than 0.05 was considered statistically significant.

4.5 Results

4.5.1 Identification of appropriate selective agar media

Species isolation for TVC's depends on the selection of specific agar media. YPD or SDA are most commonly used as an isolated media for *Candida* growth. In here (Table 4.2), it was found that YPD was able of completely inhibit other bacterial cells growth. For *S. mutans*, TYCSB was found more sensitive and selective media for its growth in comparison with MSB. Rogosa agar was found more selective for *L. casei* growth in comparison with MRS. These three media were chosen for the three species isolation in the multispecies biofilm quantification.

Table 4.2 Selective agar media for each microorganism. Highlighted media represented the ones that were chosen for quantification experiments.

C. albicans	*L. casei* ●	*S. mutans* ▲●
SDA	MRS	MSB
YPD	Rogosa	TYCS

▲Bacitracin ● Natamycin

4.5.2 Optimisation of DNA extraction from different microorganisms

In order to find an effective method for extraction of genomic DNA from three different microorganisms (*C. albicans*, *S. mutans* and *L. casei*), four commercial DNA extraction kits were used on monospecies fungal and bacterial cultures (Figure 4.1). The purity and yield of DNA extracted from these species is critical for accurate qPCR analysis. Initially, the four kits were tested on *C. albicans* and *S. mutans* to investigate whether they efficiently extracted DNA from both fungal and bacterial cells. The UltraClean Microbial DNA isolation kit (MO BIO) produced very little (1.3 ng μl^{-1}) DNA from *C. albicans*, and only a low yield from *S. mutans* (37 ng μl^{-1}). Using the MasterPure DNA purification kit (Epicentre), high concentrations of DNA were extracted from *S. mutans* (1400 ng μl^{-1}) but produced small amount of DNA (3.8 ng μl^{-1}) from *C. albicans*. To produce a good yield of DNA from *C. albicans*, a fungal-specific DNA extraction kit was tested (The MasterPure yeast DNA purification kit, Epicentre).

This kit was used as a positive control to ensure that it is possible to extract fungal DNA using an optimised kit. This gave a high yield of 3,300 ng μl^{-1} of DNA with low molecular weight band that is probably RNA (Figure 4.1, 8). Therefore, a fourth kit which was labelled for Bacterial/Fungal DNA extraction was tried. The ZR Fungal/Bacterial DNA extraction kit (Zymo Research) was tested with *C. albicans* and *S. mutans*. This produced clear bands of DNA from each organism, although the yield was a little lower than for the other kits (67.6 ng μl^{-1} and 786 ng μl^{-1} for *C. albicans* and *S. mutans*, respectively). The kit was also found to be appropriate for *L. casei* and yielded 817 ng μl^{-1} from this organism. The quality of the DNA extracts was assessed by agarose gel electrophoresis (Figure 4.1).

In all cases, the ZRFungal/Bacterial DNA Extraction kit produced sharp bands of chromosomal DNA with little smearing.

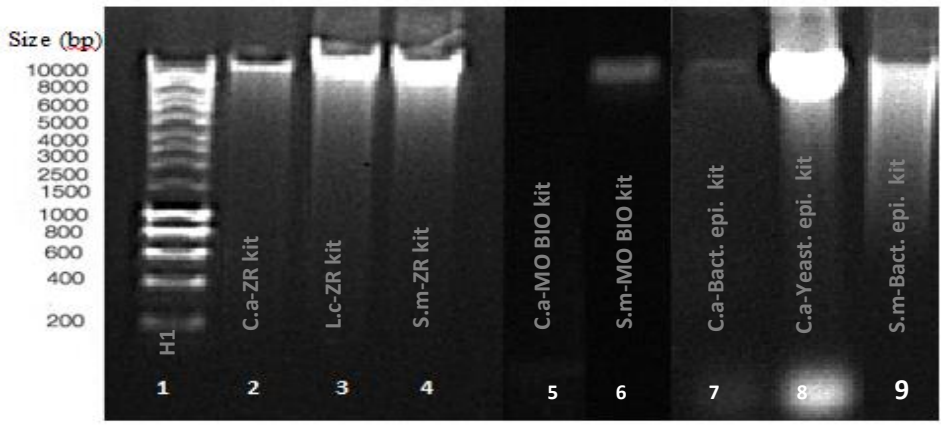


Figure 4.1 Gel electrophoresis of DNA concentrations extracted by different kit (1) Hyperladder one – a wide range standard DNA Molecular Weight Marker (2) Extracted DNA of *Candida albicans* (ZR kit) (3) Extracted DNA of *Lactobacillus casei* (ZR kit) (4) Extracted DNA of *Streptococcus mutans* (ZR kit) (5) Extracted DNA of *Candida albicans* (MO BIO kit) (6) Extracted DNA of *Streptococcus mutans* (MO BIO kit) (7) Extracted DNA of *Candida albicans* (Bacterial epicentre kit) (8) Extracted DNA of *Candida albicans* (Yeast epicentre kit). (9) Extracted DNA of *Streptococcus mutans* (Bacterial epicentre kit). Note that the image is a composite since different extracts were analysed at different times.

4.5.3 Primer design

A set of oligonucleotide primers and probe for qPCR targeting *ITS2* of *C. albicans* has previously been described (Guiver *et al.*, 2001). However, the genome of *C. albicans* VPSA1 has not been sequenced previously. Therefore, in order to assess whether the published TaqMan assay was appropriate for *C. albicans* VPSA1, it was necessary to amplify and sequence the *ITS2* region. Using Primer3Plus (Untergasser et al., 2007), primers *CaFP* and *CaRP* (Table 4.1) were designed to amplify an approximately 800 bp fragment including *ITS2* and the surrounding region. These primers were used to amplify the target region by PCR. After checking the product on an agarose gel (not shown), the amplified DNA fragment was sent to Eurofins for sequencing. The sequence obtained was submitted to GenBank (accession KJ39863). This sequence was aligned with the genome sequence of *Candida albicans* strain WO1 (available through NCBI Genbank Reference Sequence: L28817.1) using ClustalW2 (Larkin *et al.*, 2007) and a mismatch of one base pair was detected between the *C. albicans* VPSA1 DNA sequence and the published reverse primer (Figure 4.2).

On the basis of sequence obtained from Eurofins, a new primer (*CaR1*) was designed (Figure 4.2). This primer showed no significant potential for hairpin formation and self-complementarity when checked by Oligonucleotide Properties Calculator (Kibbe, 2007).

For *S. mutans* and *L. casei* primers, the whole genome sequence from NCBI was saved as FASTA file. It was confirmed that primers and probes named in this study, had 100% complementarity with the previously published primers and probes for *S. mutans* (Smut3423T, Ssob298T, Uni177T) (Yoshida *et al.*, 2003) , and (F_alllact_IS, R_alllact_IS, P_alllact_IS) for *L. casei* (Haarman and Knol, 2006).

C. albicans-R 5'TTGAAGATATACGTGGTGGACGTTA-3' (Guiver *et al.*, 2001)

CaR1 5'TGAAGATATACGTGGTAGACGTTAG-3' This study

Figure 4.2 Reverse primers of *C. albicans* (*CaRP*) compared with the previously published primer (*C .albicans*-R), a one pair difference was required to obtain complementarity with the *C. albicans* VPSA1 *ITS2* gene sequence (red line). In order to maintain the GC:AT base ratio and keep the melting temperature consistent with the forward primer, the *CaR1* primer was shifted one base pair to delete the 5' thiamine base (green colour) and add a 3' guanine residue (yellow colour).

4.5.4 Preparation of DNA standards for qPCR

To generate plasmids for use as standards for absolute quantification (qPCR), target regions for qPCR were PCR-amplified with CaF1, CaR1 for *C. albicans,* SmF1, SmR1 for *S. mutans,* and LcF1, LcR1 for *L. casei* using chromosomal DNA as described in Section 4.3.5. The PCR products were purified using the EZ-10 spin column PCR products purification kit (NBS) and the concentrations of DNA were estimated by NanoDrop. The amplified DNA fragments were cloned in plasmid vector pCR2.1-TOPO (Invitrogen), generating plasmids pCR2.1-Ca (4039 bp), pCR2.1-Sm (4045bp) and pCR2.1-Lc (4020bp). *E-coli* TOP10F´ was transformed with plasmids, and transformants were selected on LB agar (Difco) supplemented with 100 µg ampicillin/ml. The resultant plasmids were then extracted from the *E. coli* host strain for analysis.

4.5.5 Confirmation of plasmids

To confirm that the plasmids contained the correct inserts, restriction digests were carried out each plasmid using *Eco*RI (Biolabs). Fragments of approximately 125 bp, 132 bp, and 107 bp were released from pTOPO-*Ca*, pTOPO-*Sm* and pTOPO-*Lc*, respectively (Figure 4.3), indicating that these plasmids contained the expected inserts. To further confirm that inserts were as anticipated, the entire insert region was sequenced in both directions using M13 reverse and T7 forward primers (Table 4.1).

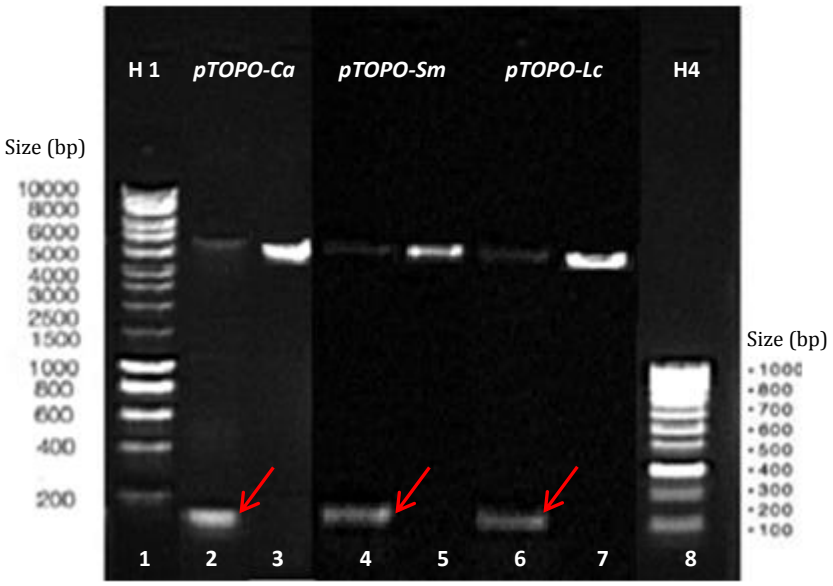


Figure 4.3 Agarose gel electrophoresis of plasmid DNA fragments digested by *Eco*RI. (1) Hyperladder one (2) Digested *pTOPO-Ca* (red arrow)(3) undigested *pTOPO-Ca* (4) Digested *pTOPO-Sm* (red arrow) (5) undigested *pTOPO-Sm* (6) Digested *pTOPO-Lc* (red arrow) (7) undigested *pTOPO-Lc* (8) Hyperladder four. Note that the image is a composite since different extracts were analysed at different times.

4.5.6 Plasmid Purification

The amount of each plasmid purified using the Qiagen midi prep kit was measured by NanoDrop and the concentrations were found to be: pTOPO-Ca, 64 ng μl^{-1}; pTOPO-Sm, 326 ng μl^{-1}, and pTOPO-Lc, 292 ng μl^{-1}. To further confirm the amount of plasmids obtained, they were investigated on agarose gel (Figure 4.4). Therefore, large quantities of plasmid DNA were prepared that could be used for standardising qPCR assays throughout the study.

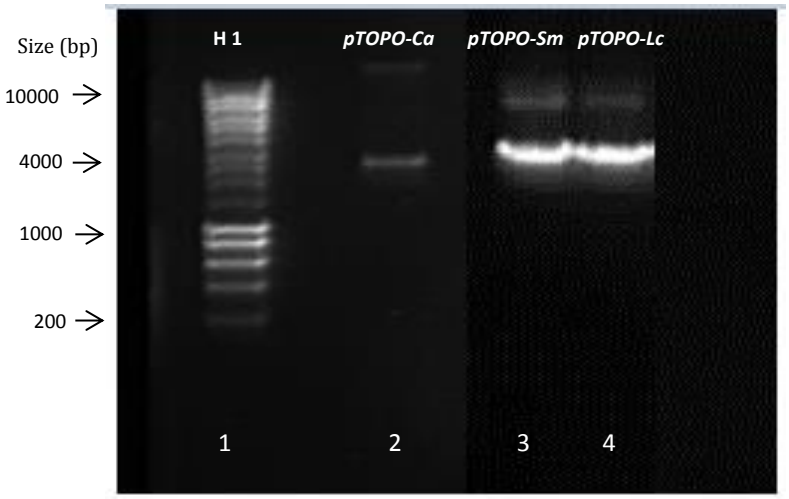


Figure 4.4 Agarose gel electrophoresis of plasmids purified using Qiagen midi prep kit (1) Hyperladder one (2) *pTOPO-Ca* (3) *pTOPO-Sm* (4) *pTOPO-Lc*. Note that the image is a composite since different extracts were analysed at different times.

4.5.7 Plasmid quantification

For absolute quantification using qPCR, it is necessary to determine the concentrations of DNA in the standards accurately. Therefore, PicoGreen dye was used for quantifying the DNA plasmid of the three microorganisms. Picogreen is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA) in solution. Using the Quant-iT™ PicoGreen kit, λ DNA standards were diluted to create a linear six-point standard curve (Figure 4.5). From these standard curves, stock concentrations of plasmids were found to be: pTOPO-Ca, 11.6 ng μl^{-1}; pTOPO-Sm, 581 ng μl^{-1}, and pTOPO-Lc, 349 ng μl^{-1}.

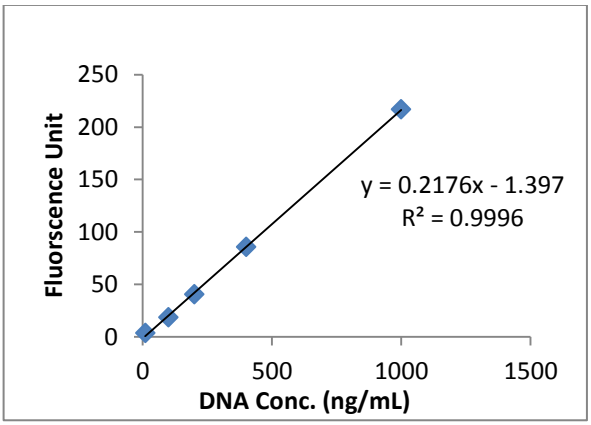


Figure 4.5 Linearity of PicoGreen assay. The total amount of DNA in ng ml^{-1} is plotted against fluorescence units recorded. The value of the TE blank was subtracted as background.

4.5.8 Real-time quantitative PCR assay

To develop qPCR assays that could be used for quantification of microorganisms in biofilms, it was necessary to assess the efficiency and specificity of amplification with the primer/probe combinations using purified template DNA. Initially, several different qPCR reaction mixes (DyNAmo flash qPCR kit, Sensimix NoRef, and Takara) were assessed by investigating the reaction efficiency of standard curves using *C. albicans* chromosomal DNA. Furthermore, a selection of PCR products from each run were analysed on agarose gels, and in each case the products were of the anticipated sizes (not shown). Two trials for each master mix were done and the mean of reaction efficiency for DyNAmo flash qPCR kit was (117.7%), Sensimix NoRef kit (114.7%) and Takara kit (101.7%). Reaction efficiencies of >100% may indicate non-specific amplification. Therefore, the Takara master mix was considered to be the best and this was used from here on.

4.5.9 Comparison of qPCR with total viable counts for enumeration of microbial cells

Enumeration of different microorganisms in complex mixtures by using qPCR and TVC's often gives slightly different results because both have inherent assumptions

and limitations. To compare qPCR enumeration with TVC's, *C. albicans, S. mutans*, and *L. casei* were cultured for 20 h at 37°C in 20 ml SDA, THYE, and MRS, respectively. Cultures were diluted 1:20 in pre-warmed fresh broth and incubated at 37°C. The optical densities of cultures at 600 nm (OD_{600}) were read in a UV/Vis Spectrometer (Unicam UV 2, ATI, USA) every hour for 5-7 h until mid-logarithmic phase ($OD_{600} \sim 0.5$). Cells of each species were harvested by centrifugation at 3500 x g, 4°C for 10 min and the pellet was resuspended in 1 ml PBS. Serial ten-fold dilutions of cell suspensions were prepared, and cells were quantified by TVC's and qPCR. As shown in Figure 4.6 which is showing a data of three independent experiments (a), the qPCR assay for *C. albicans* was found to correlate reasonably well with TVC's ($R^2 = 0.86$). Between 10^4 cells and 10^9 cells, cell counts were approximately 1 order of magnitude higher by qPCR than TVC's. There were also strong correlations between TVC's and qPCR quantification for *S. mutans* ($R^2 = 0.906$) and *L. casei* ($R^2 = 0.910$) [Figure 4.6 (b) and (C)]. Similar to *C. albicans*, for *S. mutans,* cell counts were <1 order of magnitude higher by qPCR than by TVC's. In the case of *L. casei*, cell counts were 2-3 orders of magnitude higher by qPCR than by TVC's. Overall, therefore, TVC's and qPCR appear to be relatively robust measures of cell counts, at least when compared against one another. The lower limit of *C. albicans* can be detected down to 10^3 cells, though the assay was a little unreliable at low concentrations. *S. mutans* can be detected down to 10^4 cells, and *L. casei* could be detected at $>10^3$ cells.

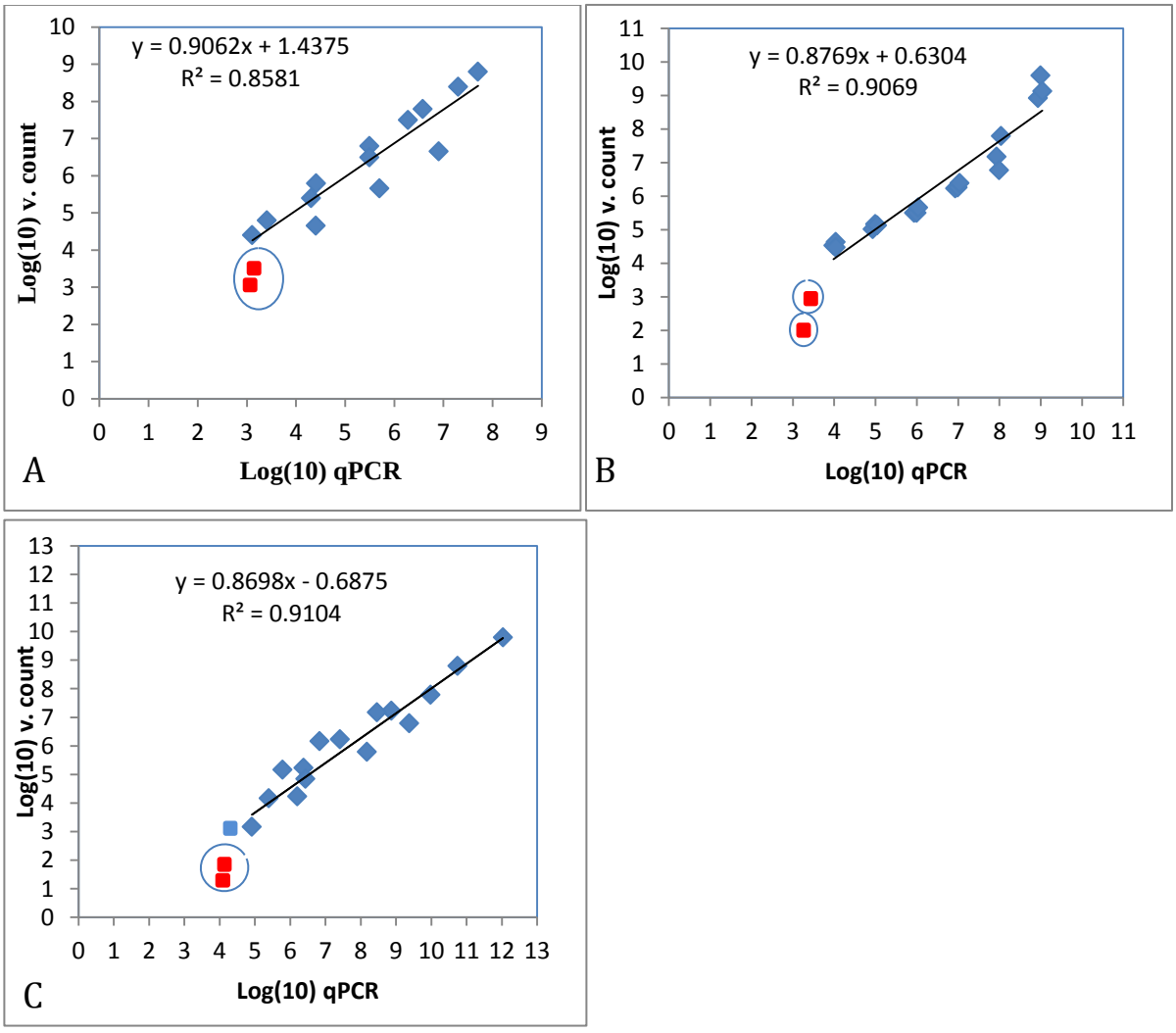


Figure 4.6 Correlations between the TVC'S and qPCR techniques. Dilutions of cells were prepared from mid-logarithmic cultures of (A) *C. albicans.* (B) *S. mutans.* (C) *L. casei*. Cell numbers quantified by TVC'S's were compared with numbers from qPCR assays. Data represented by the red points and within blue circles were considered unreliable and were not included for calculation of the trendline. Any data within and below these points will be omitted in the future work.

4.6 Discussion

Selection of microbial species that can be found in dentures and other related appliances was the first step in this part of study. Microorganisms which present orally interact with each other in different ways, such as using each other's metabolic end-products, or communicating more directly through signaling molecules (Pereira-Cenci *et al.*, 2008). The lifestyle of fungal and bacterial biofilms can be affected by environmental stress including nutrient supplies restrictions (Palková and Váchová, 2006). Furthermore, microorganisms can coaggregate with two or more genetically different strains interacting through specific cell to cell recognition. Such interaction has been seen between *C. albicans* and many other microorganisms like *S. mutans, S. mitis, L. casei* and *L. acidophilus* (Hsu *et al.*, 1990) and is considered an important factor in microbial colonization and progression of infections in the oral cavity. The study of fungal bacterial biofilms might provide insight into new strategies that can be exploited for the control of some oral diseases. Depending on the local conditions, bacteria can supply fungi with compounds that modulate the expression of virulence determinants of fungi (Wargo and Hogan, 2006; Peleg *et al.*, 2010). Fungal/bacterial biofilms are commonly visualized together in oral appliances (Branting *et al.*, 1989; Baena-Monroy *et al.*, 2005). Baena-Monroy *et al.* (2005) found *C. albicans and S. mutans* in dental prostheses of a large number of patients as this bacterial species can enhance *Candida* adherence in the oral epithelium and denture surfaces. In addition to its major role in caries induction, *S. mutans* is also considered an important pathogen in patients who wear removable partial dentures (Thein *et al.*, 2006). Furthermore, lactobacilli was also reported in patients with removable appliances (Tanaka *et al.*, 2009). In (2009), Bilhan and co-workers studied the association between denture stomatitis and denture age on 91 patients wearing maxillary and mandibular complete dentures. This study found a significant relationship between denture stomatitis and the presence of hyphae and *C. albicans*. A further interesting finding was the presence of higher *Lactobacillus* counts in the saliva and the palate of patients with denture stomatitis. Especially when *C. albicans* hyphae in the saliva, *Lactobacillus* growth was found significantly high (Bilhan *et al.*, 2009). This indicates *Lactobacillus* also play an important role in the initiation of this disease (Sakki *et al.*, 1997). *C.*

albicans, *L. casei* and *S. mutans* have the ability to produce acidic environment which increases the microorganisms' development and promote their adherence (Baena-Monroy *et al.*, 2005). As mentioned in the literature review, it is generally considered that *Candida albicans* is one of the main microorganisms in the induction of denture related diseases and particularly denture stomatitis. However, the incorporation of other two bacterial species in this study was important, firstly because they enhance *C. albicans* development and adherence, and secondly, as they have major roles in the initiation and development of caries and especially root caries with people who use oral appliances.

Microbial isolation for multispecies biofilm quantification by TVC's was achieved after selecting two specific media for *C. albicans, S. mutans* and *L. casei*. A considerable amount of literature has been published on the agar media that used to isolate these microorganisms (Rogosa *et al.*, 1951; Jarvis, 1973; Schaeken *et al.*, 1986; Kolaczkowska *et al.*, 2010). Choosing of TYCSB for *S. mutans*, was in agreement with Wan *et al.* (2002) who found that TYCSB is highly suitable for the isolation and enumeration of *S. mutans* compared to the other media including MSB. The frequently used isolation medium for *Candida* is SDA as it can suppress the growth of many species of oral bacteria due to its low pH (Odds, 1991). In this study, for unclear reason YPD showed a better growth and isolation of *C. albicans*. The growth of *L. casei* in Rogosa agar was better than MRS as Rogosa agar might be more selective for lactobacilli than MRS. The addition of antimicrobials and especially natamycin was found effective for *C. albicans* growth inhibition as shown previously by Pedersen (1992). Therefore, the isolation by using these selective media with antimicrobial addition was facilitative in cell isolation and quantification.

Two different methods were developed for the quantification of microorganisms in this study, namely TVC's and absolute qPCR. For qPCR, high quality DNA is required. So far there is no one common and simple procedure for genomic DNA extraction that can be used on a large scale for different organisms. Four different commercial DNA extraction kits were tried. The MasterPure DNA purification kit was commonly used for extracting bacterial DNA (Psoter *et al.*, 2011; Torlakovic *et al.*, 2012). In this study,

this kit was efficient in extracting *S. mutans* DNA but not *C. albicans*. Another kit investigated was The UltraClean Microbial DNA isolation kit. Many previous studies have shown this kit to be capable of fungal and bacterial DNA extraction (Karakousis *et al.*, 2006; Wise *et al.*, 2007; Boyanton *et al.*, 2008; Mahmoudi *et al.*, 2011). However, in this study, the kit was not efficient for extracting *C. albicans* DNA. At this point, The MasterPure yeast DNA purification kit was tried as a positive control to ensure that it is possible to extract the DNA of the *C. albicans* species used in this study. Finally, the ZR fungal/bacterial kit employed and was found to be effective for extracting the DNA of the three species, although the DNA yields were no higher than other kits. This also agrees with earlier observations that used the kit successfully to extract DNA from many fungal and bacterial species (Hyvärinen *et al.*, 2009; Vanneste *et al.*, 2011; Lee *et al.*, 2013), Therefore, the ZR kit was considered to be the best and this was used subsequently.

TVC's was the method of choice for viability determination. Cells that achieve sufficient growth on selective agar media can be measured within specified incubation times. However, this conventional method has many disadvantages, including the relatively long times needed for the growth of colonies, inaccuracies introduced by cells clumping (Jakubovics *et al.,* 2008) or, in the case of *C. albicans* forming hyphae, (Yano *et al.*, 2002), and rapidly growing bacterial cells usually contain more than one chromosome (Lick and Heller, 1998). This can lead to underestimation of cell quantification. In contrast, PCR techniques have several advantages over culture assays. qPCR is more specific than TVC's for microbial quantification, and consumes less time and effort than conventional methods (Kodkeaw *et al.*, 2010; Su *et al.*, 2010). However, quantification by PCR depends on the efficacy of the lysis and DNA extraction protocols (Yano *et al.*, 2002). In addition, using molecular methods requires a prior knowledge of the genome of the target species or strains (Douwes *et al.*, 2003). qPCR allows the detection of viable and nonviable microorganisms. This can cause the overestimation of cells which could be not harmful (neglected) if they are dead. This issue can be overcome by using propidium monoazide DNA-binding dye that detects only viable microorganisms by qPCR (Vesper *et al.*, 2008).

Despite molecular methods being more sensitive, conventional methods are still needed when it is necessary to quantify cells and identify culture contamination. The results in here further demonstrated that the detection limit of cell quantification by TVC's is lower than that by qPCR which can be advantageous in situations of small biofilms quantification.

TVC's and qPCR are complementary methods; they both have limitations, so it is important to apply both methods.

Despite the variation between these two techniques, the result of their correlation of the three species showed that cell counts were 1-2 orders of magnitude higher by qPCR than by TVC's. qPCR does not necessarily produce results comparable to culture based method because qPCR measures a genetic, rather than a growth of cells (Converse *et al.*, 2012). Furthermore, cells could be in viable but non culturable state (VNC) (Barer *et al.*, 1993) and that can cause underestimation of cell quantification by TVC. Therefore, it is not unusual to find the numbers of cells quantified by TVC's are lower than those by qPCR. The present findings are consistent with other research (Yano *et al.*, 2002; Haugland *et al.*, 2005; Morrison *et al.*, 2008) which found that qPCR and culture based methods generally correlate well, even though qPCR usually gives higher value.

Overall, the work in this chapter has established techniques for the selective quantification of *C. albicans*, *S. mutans* and *L. casei* in mixed cultures using TVC's and qPCR. These methods will be applied for analyzing biofilms in the next sections.

Chapter 5 . Effects of fluoride on single species biofilm formation

5.1 Introduction

There has been an increase over the last two decades in the use of antimicrobials for the treatment of oral diseases all over the world. Fluoride is considered one of the antimicrobials central to the prevention and treatment of dental caries through its properties of inhibiting bacterial acid production, in addition to its roles in strengthening the enamel structure (Hamilton, 1990; Brambilla, 2001; Marquis *et al.*, 2003). Fluoride may be introduced either locally or systemically, and in both cases it is important that an appropriate concentration is reached at the target site. Previous studies have demonstrated that fluoride concentrations as low as 0.024 ppm can be enough to protect tooth enamel from demineralization following exposure to pH 4.3 for 72 h (Ten Cate and Duijsters, 1983; Margolis *et al.*, 1986). However, it appears that the concentrations of fluoride required for antimicrobial activity are much higher. For example, a previous study by Maltz and Emilson (1982) showed that fluoride can kill oral streptococci *in vitro* at concentrations exceeding 4500-5000 ppm. One major effect of fluoride on oral bacteria is to modify their metabolic capability. A large and growing body of literature has investigated the fluoride effects on *S. mutans* growth. Liu and co-workers (2012) found that 1200 μg/ml of fluoride is the minimum concentration required to inhibit *S. mutans* biofilm formation on polystyrene blocks. In (1978), Hamilton and Ellwood showed that 2000 ppm fluoride has a significant inhibitory effect on carbohydrate metabolism by *S. mutans* grown in a chemostat system. In addition, Curran *et al.* (1994) demonstrated that fluoride concentrations in excess of 0.2 ppm were sufficient to act as a quasi-irreversible inhibitor of enolase in permeabilized *S. mutans* cells.

The effects of fluoride on *L. casei* growth have also been investigated by many researchers. *In vitro* experiments have shown a decrease in acid production in suspensions of oral lactobacilli and oral streptococci after cells come in contact with fluoridated enamel surfaces (Briner and Francis, 1962; Luoma and Luoma, 1982; Harper and Loesche, 1986; Marsh and Bradshaw, 1990). A higher fluoride concentration is required to inhibit carbohydrate metabolism in *L. casei* than *S.*

mutans (Hamilton *et al.*, 1985). Milnes *et al.* (1985) showed that ten-fold higher concentrations of fluoride are required to inhibit acid production by lactobacilli than by streptococci. It is possible that *Lactobacillus* sp. cells have fluoride-resistant glycolytic enzymes.

So far, there has been relatively little work on the effects of fluoride on fungal related diseases. In a 12-month open trial in elderly nursing home subjects who had high salivary fungal counts. A combination of amine fluoride and stannous fluoride (AmF/SnF_2) containing mouthwash and toothpaste, used twice daily, resulted in average decreases of fungal cell positive subjects from 26% at baseline to 9% at follow-up (Meurman *et al.*, 2006). An expanded *in vivo* study by the same group in (2009), studied the antimicrobial effects of a mouth rinse (Meridol) containing 250 ppm AmF/SnF_2 on 43 reference and clinical strains of *Candida albicans, C. dubliniensis, C. glabrata, C. guilliermondii, C. krusei, C. parapsilosis,* and *C. tropicalis*. *C. albicans* was highly sensitive to the agent and almost 90% of *C. albicans* strains were killed within 5 minutes. On the other hand the non-*albicans* strains, *C. glabrata*, *C. krusei* and *C. dubliniensis* were more resistant in ascending order. Overall, therefore, fluoride is clearly an important agent for the control of oral diseases with microbial aetiology (Bowden, 1990).

A better understanding of biofilm growth on different antimicrobial impregnated materials requires experimental models to produce *in vitro* biofilms on supports that are easy to manipulate in the laboratory (Chandra *et al.*, 2001a). Numerous model biofilm systems have been developed, from simple static models to open systems that include the flow of fresh medium to simulate physiological conditions and to prevent the decline of the culture by nutrient depletion. Such instrumentation is easy to assemble in a microbiology laboratory and requires only an incubator, peristaltic pump, fresh medium flasks, tubing, and chambers where the material of interest can be inserted and inoculated with microorganisms (Abuzar *et al.*, 2010). High throughput biofilm models have also been developed that employ static incubation and appropriate surfaces such as hydroxyapatite disks immersed in growth medium

with gentle shaking (Radford *et al.*, 1998). The material, nutrient medium, and oral bacteria selected for study vary significantly among these models (Lin *et al.*, 2010).

Several different devices have been developed for continuous flow biofilm studies. For example, the Modified Robbins Device (MRD) is commonly used to investigate the effect of antimicrobial incorporated materials (Nickel *et al.*, 1985; Rupf *et al.*, 2003; Sartori *et al.*, 2006; Coenye *et al.*, 2008; Kreth *et al.*, 2008). Unlike traditional testing approaches that study free-floating planktonic bacteria, the MRD permits the quantification of bacteria that are sessile and adherent to the surface (Machado *et al.*, 2009). This model is composed of a small channel shaped chamber with openings in which biomaterial discs can be inserted and mounted to form the channel wall. Parallel chambers allow independent samples to be produced under similar conditions (nutrients, flow rate, temperature, and incubation time). These devices are provided with a liquid circulator for low-pressure applications. Microorganisms introduced into the fluid stream can adhere to the plugs and generate a biofilm on a disc of substrates that is easy to remove for analysis (Coenye *et al.*, 2008).

A large number of studies have investigated the structure of biofilms grown using *in vitro* model systems (Wood *et al.*, 2000). One of the most common techniques used to investigate the microbial biofilm formation is scanning electron microscopy (Brecx *et al.*, 1994). Historically, scanning electron microscopy (SEM) has been the method of choice for investigating biofilm composition and structure because of the high resolution that can be obtained and the ability to capture the granular appearance of the biofilm matrix (Lawrence *et al.*, 2003; Mukherjee *et al.*, 2005). However, this approach requires the dehydration, fixing, embedding and gold coating of samples which may cause substantial damage or distortion to the biofilm structure (Wood *et al.*, 2000). Regardless of these limitations, SEM analyses can reveal surface topography of microbial biofilms at very high magnification (Mukherjee *et al.*, 2005).

During the last two decades, the optical sectioning ability of confocal microscopes has largely taken over from SEM as the method of choice for biofilm imaging (Lawrence *et al.*, 1991; Demandolx *et al.*, 1997). It has many advantages including the ability to employ lenses that can be immersed in oil or water, for imaging with or without a

coverslip, respectively. Minimal preparation of samples is required and dehydration is unnecessary. Additionally, CSLM microscopes overcome the out of focus haze associated with conventional light or fluorescence microscopes (Auschill *et al.*, 2002), and it is possible to undertake subsurface examinations and to optically section in a vertical or horizontal plane (Auschill *et al.*, 2002). Furthermore, CSLM can be employed in conjunction with image analysis software for quantitative fluorescence imaging in three dimensions (3D) (Demandolx *et al.*, 1997; Mukherjee *et al.*, 2005). Together, the high resolution of SEM and the non-disruptive nature of CLSM provide a powerful combination for the analysis of biofilm samples.

5.2 Aims

This chapter aimed to develop a model that enabled the formation of single species biofilms of *C. albicans, L. casei*, and *S. mutans* on the surface of the fluoridated and non-fluoridated copolymer materials. The specific objectives were as follows:

1- Establish a model system for culturing single species biofilms on the non-fluoridated and fluoridated copolymer material.
2- Investigate the effects of fluoride on biofilms by quantifying the extent of biofilm formation by each species on non-fluoridated and fluoridated copolymer materials using total viable counts and qPCR.
3- Investigate the structure and composition of the biofilms using scanning electron microscopy and confocal laser microscopy.

5.3 Material and methods

5.3.1 MRDs moulds and samples preparation.

Moulds were designed for the production of acrylic samples in an appropriate shape to fit within the MRD system (Tyler Research Co., Alberta, Canada) using small plastic containers (Figure 5.1) with a hole created at the bottom of each mould to fit a steel screw. From inside the containers, these screws were fastened to the steel studs that came with the MRD system (Figure 5.1). A silicone impression material (Silaplast Futur, Germany) was used to fill these containers. After the silicone had set, these studs were pulled out using the screws and removed to create moulds for preparation of samples to fit in the MRD biofilm model (Figure 5.1).

According to the material mixing formula in Chapter Three (Table 3.1), 50 mg of PMMA powder was weighed using a digital scale (Mettler-Toledo Ltd. AE 240, Switzerland) into a 1.5 ml microcentrifuge tube (StarLab GmBH, Ahrensburg, Germany) and 350 µl of MMA/HEMA liquid were added using a micropipette (Poulten and Graf Ltd, UK). Samples were mixed using a Rotomix (ESPE Seefeld, Germany) for 20 seconds (a technique that was adapted from Zahroon (2014)). A plastic spatula was used to pour the material into the moulds. While the material set, moulds carrying the samples were stabilized on dental die stone (GC FUJIROCK, Alsip, USA) prepared base that was coated with separating medium (Metrodent, Ltd. UK), with a pressure force of 10 Newtons for 20 minutes. After the acrylic was set, samples were removed from the moulds, cleaned of any excess material and stored in a humid container at 37°C.

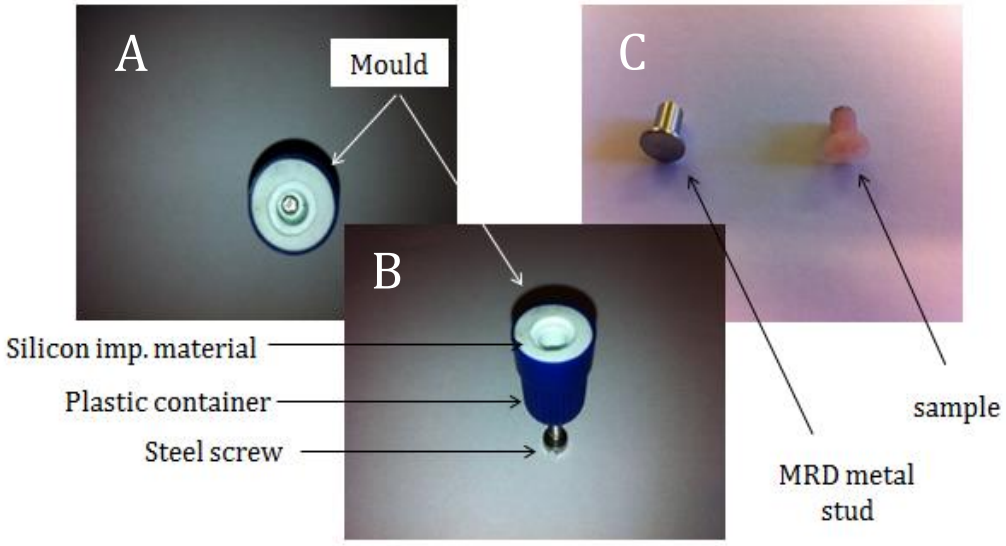


Figure 5.1 Mould design. A) topical view of the mould with the screw at the bottom that pushes the sample once it has set, B) Parts of mould used to construct MRD samples, C) MRD metal stud and the MRD copolymer sample of matching shape and dimensions.

5.3.2 Natural and artificial saliva preparation

Natural parafilm-stimulated saliva was collected from 6 volunteers and kept on ice (Palmer *et al.*, 2001). Dithiothreitol (Sigma-Aldrich, St. Louis, Mo.) was added from a 250 mM stock to a final concentration of 2.5 mM and stirred gently on ice for 10 min. Samples were centrifuged at 15,000 *g* for 30 min, 4°C and the clarified saliva supernatant was decanted. Saliva was sterilised by filtration through syringe filters of 0.2 µm pore size (PALL.Co, USA) and stored at -20°C until use.

Artificial saliva was prepared according to the method of Pratten *et al.* (1998) by combining: Lab-lemco (OXOID Ltd, UK) 1 g/L, yeast extract (Melford Ltd, UK) 2 g/L , proteose peptone (Sigma) 5 g/L, type III hog gastric mucin (Sigma) 2 g/L, sodium chloride (AnalaR, UK) 0.35 g/L, potassium chloride (AnalaR, UK) 0.2 g/L, calcium chloride (Sigma) 0.2 g/L. After autoclaving, filter sterilized urea (Sigma) was added at a concentration of 500 mg/L to the saliva and stored at 4°C.

5.3.3 Preparation of the MRD system and Biofilm quantification

A 20 ml aliquot of sterilized natural saliva was thawed and used to coat the surface of samples prior to inoculation with microorganisms. Stock cultures of *C. albicans*, *L. casei* and *S. mutans* were prepared as described in Section 4.3.4. In addition, 4 L of sterilized artificial saliva was prepared in a 5 L Duran bottle and 2 L of 10% sucrose in distilled water.

A flow system for biofilm formation was developed based on two Polysulfone MRDs (Tyler Research, Alberta, Canada) that were run in parallel (Figure 5.2). Each MRD contained 12 individual ports in a linear array along a channel of rectangular cross-section. Each port accepts a press-fit sample with a surface area of approximately 47.8 mm^2. Six samples from each group (fluoridated or non-fluoridated) were fitted along each of the two MRDs and sterilized by autoclaving at 121°C for 15 minutes. The tubing was connected to a peristaltic pump (401U/DM3, Watson-Marlow, UK) set at a flow rate of 40 ml/h according to (Honraet and Nelis, 2006). The entire system was assembled in an air flow cabinet to minimise the risk of contamination, and then transferred to a laboratory incubator to maintain a constant temperature of 37°C. Metal clamps were used at various points to control the flow of the solutions.

The acrylic samples were coated with sterile natural saliva for 2 h. Each MRD was inoculated with 20 ml artificial saliva containing 5 x 10^5 CFU/ml *C. albicans*, 5 x 10^7 CFU/ml *L. casei* or 5 x 10^7 CFU/ml *S. mutans*, as appropriate, and incubated at 37°C for 2 h. Biofilms were developed in artificial saliva with occasional (three times per day) pulsing of 10% sucrose for 30 min. After 48 h, the microbial biofilms of five samples from each MRD were harvested by scraping in 1 ml PBS. The scraped biofilm from each sample was transferred into a microcentrifuge tube. Samples were diluted and CFU were determined by the total viable counts technique (as described in Section 4.3.3). Agar media SDA, THYE and MRS (as described in Section 4.3.1) were employed for growth of *C. albicans*, *S. mutans* and *L. casei* respectively.

In addition, chromosomal DNA from each species was extracted using the ZR Fungal/Bacterial DNA MiniPrep (as described in Section 4.3.5) and the number of copies of each genome was quantified using an Opticon 2 qPCR System (MJ Research, UK). The data were analysed using Opticon 3 software and transferred to Microsoft Excel 2010 for further analysis. The remaining sample from each group was prepared for either SEM or CLSM imaging.

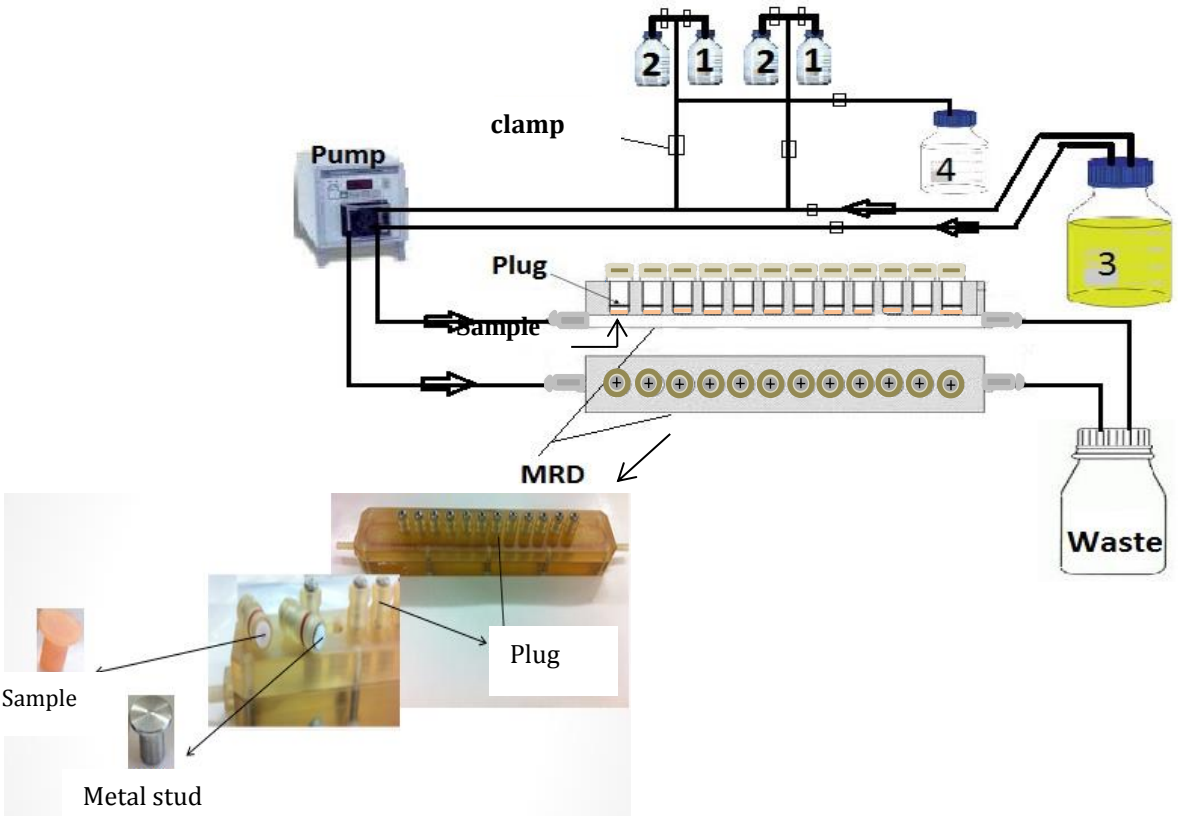


Figure 5.2 Schematic diagram of the MRD model system, 1) Natural Saliva, 2) Inoculum, 3) Artificial saliva, 4) Sucrose 10% (wt/vol). Sucrose was pumped over the biofilms for 30 min, three times daily (9 a.m., 1 p.m., and 5 p.m.), roughly modelling the total mean daily intake of sucrose of an adult.

5.3.4 Biofilm imaging by Scanning Electron Microscopy (SEM)

Samples were fixed for at least 20 h in 2% Gluteraldehyde at 4°C. Samples were then rinsed in Sorensons Phosphate Buffer for 2 x 15 min and dehydrated in a series of ethanol washes (25% for 30 min, 50% for 30 min, 75% for 30 min, and two washes in

100% for 1 h). The final dehydration of samples was carried out using carbon dioxide in a Baltec critical point dryer. Aluminum stubs were used to mount the samples using Achesons silver dag (Agar Scientific, Essex, UK) and dried overnight. Finally, samples were coated with gold (standard 15 nm) using a Polaron SEM coating unit. Samples were visualized in a Stereoscan S40 SEM.

5.3.5 Biofilm imaging by using Confocal Laser Microscopy (CLSM)

Live/Dead BacLight Bacterial Viability Kit (Life technologies Ltd, Paisley, UK) was used to stain samples for visualisation by CLSM. Syto9 was used to stain live cells while propidium iodide (PI) was used for dead cells. A stock solution was prepared containing a concentration 5 μM of Syto9 and 30 μM of PI and kept in total darkness at 23°C. The bases of samples were fixed in 6-well plate using sticky wax. Samples were submerged in the BacLight stain solution and incubated at room temperature in the dark on a rocking plate with a speed of 10 rpm. After 15 minutes, excess stain was washed off with PBS for 2-3 times carefully to avoid biofilm disruption. A circular cover slip (1 cm^2 in diameter) with a 0.5 mm rubber O ring (W&H Ltd, Bürmoos, Austria) was fitted over the sample surface using sticky wax (Figure 5.3) in order to keep the biofilm more stable during imaging. PBS was added to the wells until samples were fully immersed in liquid. Samples were visualised in a Leica SP2 CLSM microscope [Leica, Microsystems, Heidelberg, GMBH] using an excitation at 488 nm and emission at 520-540 nm for Syto9, and excitation at 530 nm and emission at 630-650nm for propidium iodide.

In some cases, biofilms formed by *L. casei* were also stained by Calcofluor White (CW) stain (Life technologies Ltd, Paisley, UK) in combination with PI to investigate polysaccharide production by *L. casei*. A stock solution of CW at a final concentration of 5 μM was added alongside the PI and applied for 15 min. CW stain excitation was at a wavelength of 360 nm and emission at 450-500 nm. Biofilms imaging was performed with CLSM. The objective used was a water immersion lens (x40 and x63, Zeiss, Carl Zeiss Ltd., Surrey, UK). Images were processed using LCS 2.61 software (Leica Microsystems, Heidelberg, GMBH).

The whole three-dimensional structure (XYZ direction) of the biofilm was rebuilt by combining each of the sectional analyses (XY direction) of 1.5-μm thickness (XZ direction). The rebuilding was carried out with Imaris software (Bitplane, Zurich, Switzerland).

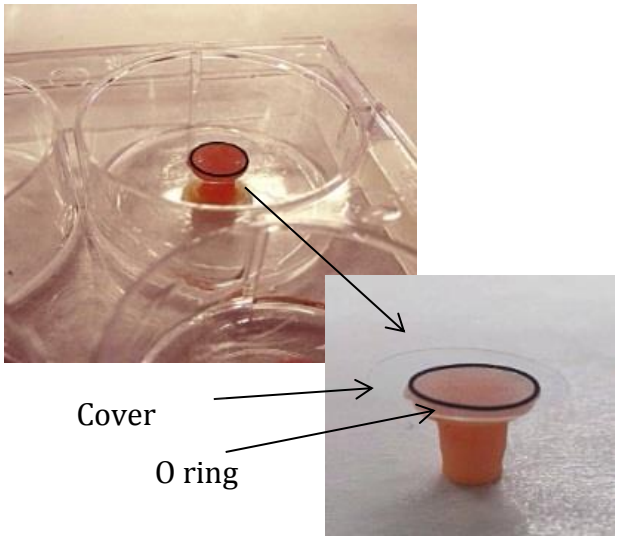


Figure 5.3 Stained sample with O ring and cover slip covering the surface to be imaged by CLSM.

5.4. Results

5.4.1 Establishing the MRD system

The MRD was set up using samples of copolymer with and without fluoride, as described in section 5.3.3. Initial experiments focused on single species biofilms, and *C. albicans* was the first organism tested. Preliminary experiments aimed at developing single-species *C. albicans* biofilms in unsupplemented artificial saliva resulted in only low levels of growth. Sucrose has previously been shown to have an important role in enhancing virulence, adhesion and growth of *C. albicans* (Samaranayake *et al.*, 1980; McCourtie and Douglas, 1981; McCourtie and Douglas, 1985). Therefore, to enhance *C. albicans* biofilm growth and to more closely mimic the dietary sugar exposure that occurs in the natural human oral environment, 10% sucrose was added three times daily for 30 min. Initial data by TVC's revealed that *C. albicans* growth on both fluoridated and non-fluoridated copolymer material was increased approximately 30-fold by the addition of sucrose (Table 5.1).

Table 5.1 Growth of *C. albicans* on non-fluoridated or fluoridated copolymer in the MRD system under artificial saliva with or without added sucrose (n=1).

	Group[A]	
	Non fluoridated Mean	Fluoridated Mean
Without sucrose	$7x10^3$	$8.9x10^3$
With sucrose	$2.2x10^5$	$2.9x10^5$

[A]Biofilms were gently scraped, and TVC's were enumerated on SDA agar. Values represent CFU/mm^2 of copolymer from two independent experiments.

5.4.2 C. albicans quantification by TVC's and qPCR

Having established conditions that permitted growth of *C. albicans* in the MRD system, the effects of incorporation of fluoride into the copolymer substratum were assessed. For these experiments the growth of *C. albicans* was monitored both by the 'gold standard' TVC's method, and by qPCR, which is potentially more appropriate for enumerating hyphae, and more easily adaptable to mixed-species cultures. Using TVC's to determine cell numbers, there was no significant difference in the density of surface colonization by *C. albicans* after 48 h on non-fluoridated or fluoridated surfaces (independent sample T test, $p>0.05$;Table 5.2). This result was confirmed by qPCR quantification, which also showed no significant differences in surface colonization with or without fluoride (independent sample T test, $p>0.05$; Table 5.2). Therefore, the incorporation of fluoride into the copolymer appears to have no major effects on *C. albicans* growth after 48 h in MRD system. It is noteworthy that similar counts were obtained between the two different methods that were used for quantification.

Table 5.2 Growth of *C. albicans* on non-fluoridated and fluoridated copolymer in the MRD system with 10% sucrose pulsed three time in a day for 48 h.

	[A]Group	
	Non fluoridated Mean (SD)	Fluoridated Mean (SD)
TVC's (CFU/cm^2)	$2.7x10^5$ ($1.2x10^5$)	$3.5x10^5$ ($1.3x10^5$)
qPCR (cell/ cm^2)	$1.2x10^5$ ($4x10^4$)	$2.3x10^5$ ($1.2 x10^5$)

[A]Biofilms were gently scraped, and TVC's were enumerated on SDA agar while the remaining of the biofilm was quantified by qPCR. The values represented were from three independent experiments. In all quantification experiments, the number of cells quantified was divided by the surface area of the sample.

5.4.3 Biofilm imaging of Candida albicans by SEM

To assess the structure of *C. albicans* biofilms, samples were visualised by SEM (Figure 5.4). *C. albicans* biofilms were present on both non-fluoridated and fluoridated samples, and were similar in appearance on the different surfaces. In both cases, clusters of cells and hyphae were spread in small patches over the samples. The surface of the sample was apparently affected by the chemical process of sample preparation for SEM imaging. Cracks, large and small holes can be seen along the surface of the sample.

5.4.4 Biofilm imaging of Candida albicans by using CLSM

In the first trial of biofilm imaging by CLSM using a dipping (water immersion) lens, blurred images were obtained due to movement of the hyphae and the biofilm in the PBS during image capture (Figure 5.5). To overcome this problem, a new technique was developed by covering the disc with a circular slide cover slip with 0.5mm rubber O ring as explained in Section 5.3.5. This kept the biofilm more stable during the imaging process, whilst avoiding compression of the biofilm with the coverslip.

Using the new technique, clear biofilm images were visualized using Live/Dead stain. These stains are optimized for assessment of bacterial cell viability and do not necessarily distinguish between the live and dead cells of fungi, but they can still be used to stain all the cells present and give a good overview of the biofilm structure. The CLSM images showed the spread of *C. albicans* cells and hyphae with no structural differences between biofilms on the non-fluoridated and fluoridated groups (not shown). Three dimensional images gave more details about the structure, consistency and the shape of the biofilms as shown in Figure 5.6.

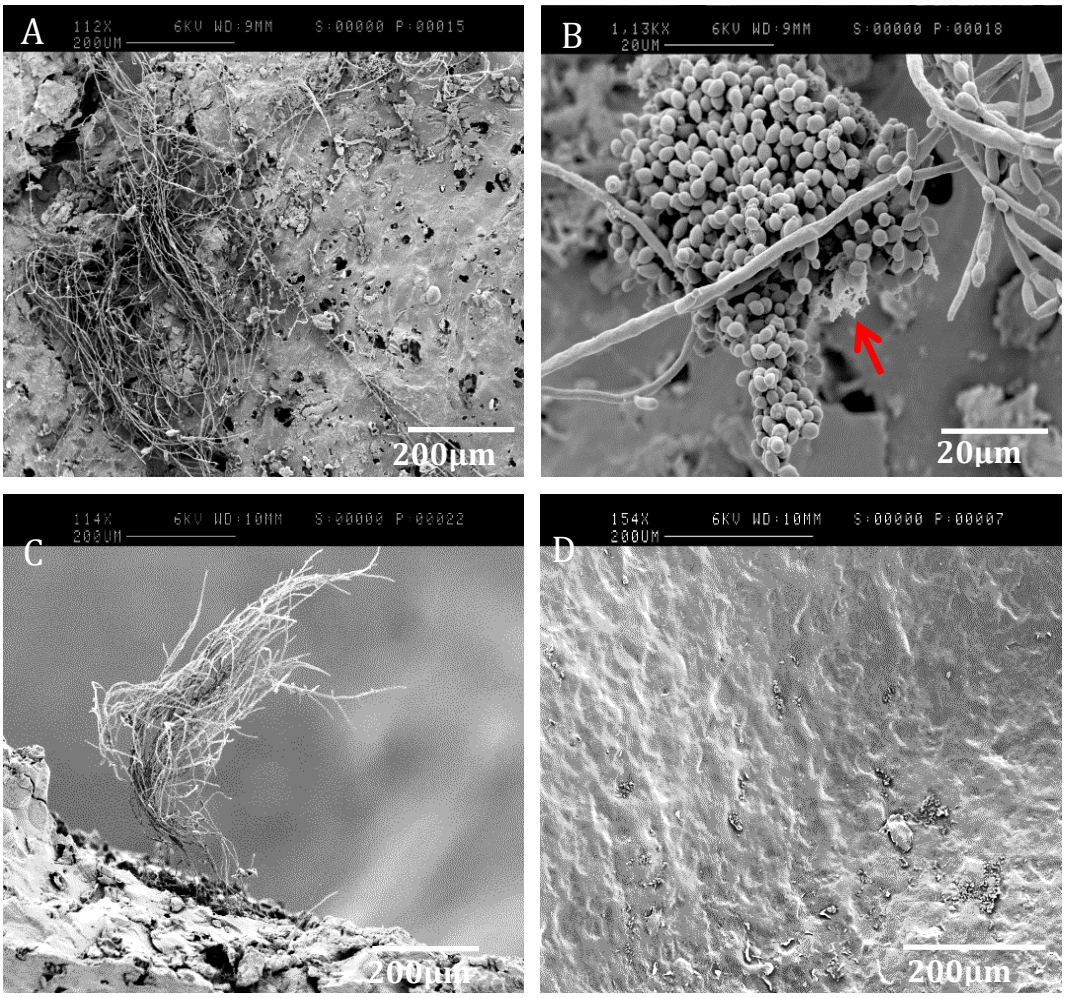


Figure 5.4 *C. albicans* biofilm imaging by SEM. A) An overview of the hyphae and biofilm spread over the sample, B) *C. albicans* yeast cells were observed aggregated with the hyphae. The arrow indicates extracellular material, likely remnants of EPS, C) Hyphae protruding from the edge of the sample, and D) remnants of biofilm cells that were still attached to the surface after scraping the sample. Scraping the surface of samples removed the vast majority of cells. Only very occasional small clu**s**ters remained.

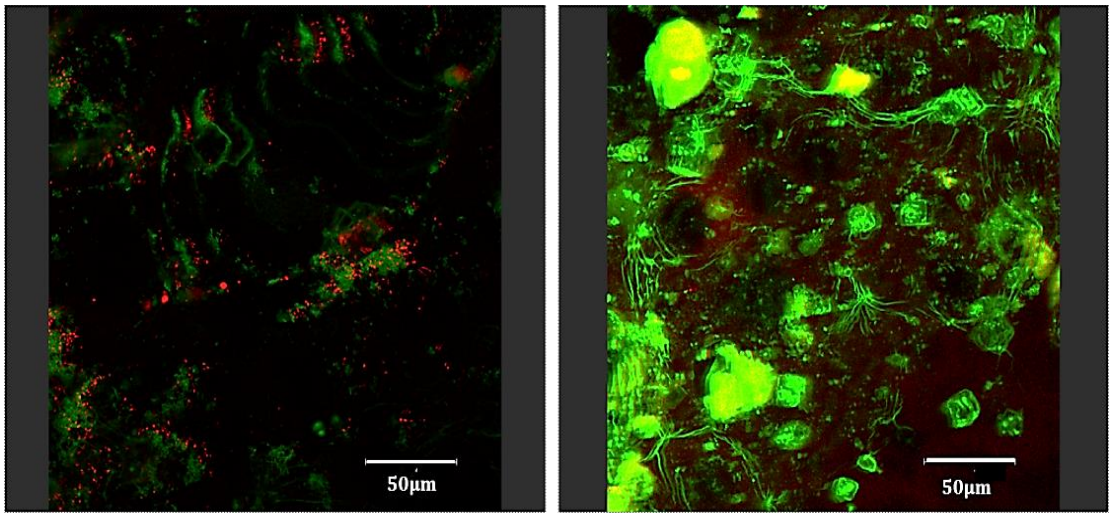


Figure 5.5 Blurred images with unclear biofilm structure by CLSM with a dipping lens.

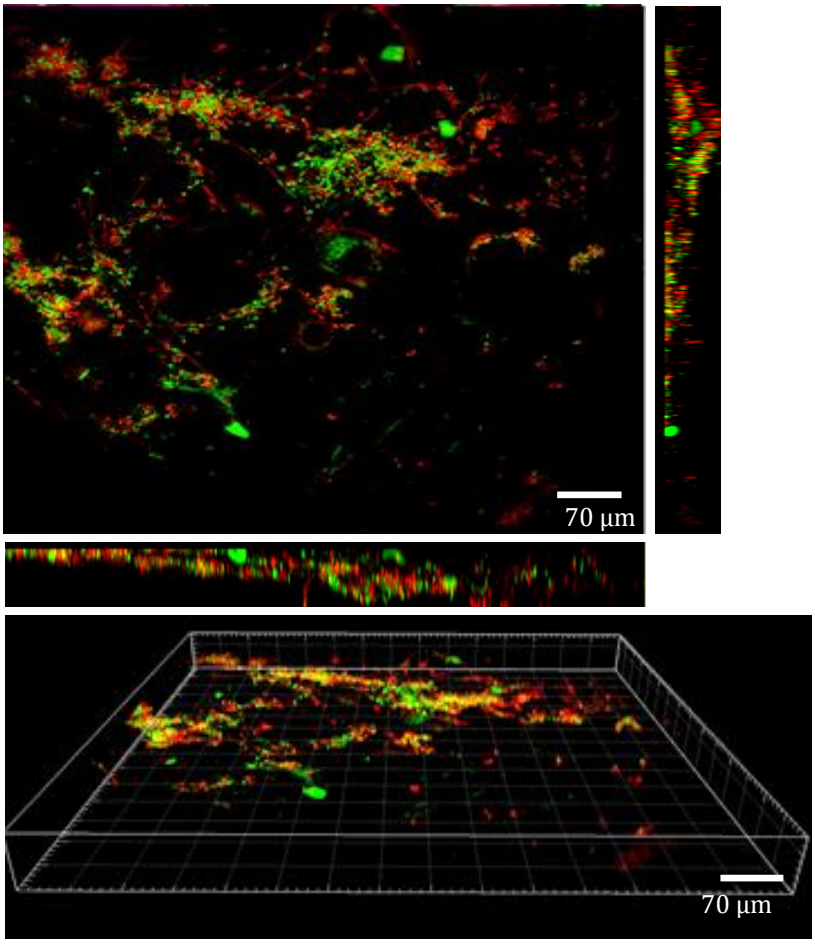


Figure 5.6 *C. albicans* biofilm cultured in the MRD for 48 h and visualised by CLSM. Samples were stained with Syto9 and PI, and 2D and 3D images are shown.

5.4.5 L. casei quantification by TVC's and qPCR

After quantification of *C. albicans* biofilms, the effects of incorporation of fluoride into the copolymer substratum on *L. casei* growth were assessed. The results of cells quantification by TVC's or qPCR showed no significant difference in the density of surface colonization by *L. casei* after 48 h on non-fluoridated or fluoridated surfaces (independent sample T test, $p>0.05$;Table 5.3). However, there was a small decrease in numbers of *L. casei* on the fluoridated surfaces even though it was not statistically significant. Higher numbers of cells were obtained by qPCR than by TVC's, as was previously observed for planktonic cells (Section 4.5.9).

Table 5.3 Growth of *L. casei* on non-fluoridated and fluoridated copolymer in the MRD system with 10% sucrose pulsed three time in a day for 48 h.

	[A]Group	
	Non fluoridated Mean (SD)	Fluoridated Mean (SD)
TVC's (CFU/cm^2)	$1.6x10^7$ ($5.6x10^6$)	$9.2x10^6$ ($4.6x10^5$)
qPCR (cell/ cm^2)	$3.1x10^9$ ($2.1x10^9$)	$1.3x10^9$ ($6.1x10^8$)

[A]Biofilms were gently scraped, and TVC's were enumerated on MRS agar while the remaining of the biofilm was quantified by qPCR. The values represented were from three independent experiments. In all quantification experiments, the number of cells quantified was divided by the surface area of the sample.

5.4.6 Biofilm imaging of L. casei by using SEM

By SEM, *L. casei* cells were spread over the surface of the samples in long chains and aggregates (Figure 5.7). As with *C. albicans*, there were no clear differences in the structure of biofilms on fluoridated or non-fluoridated samples (not shown). In some images, exopolymeric material was observed surrounding *L. casei* cells (for example, see Figure 5.7 B below).

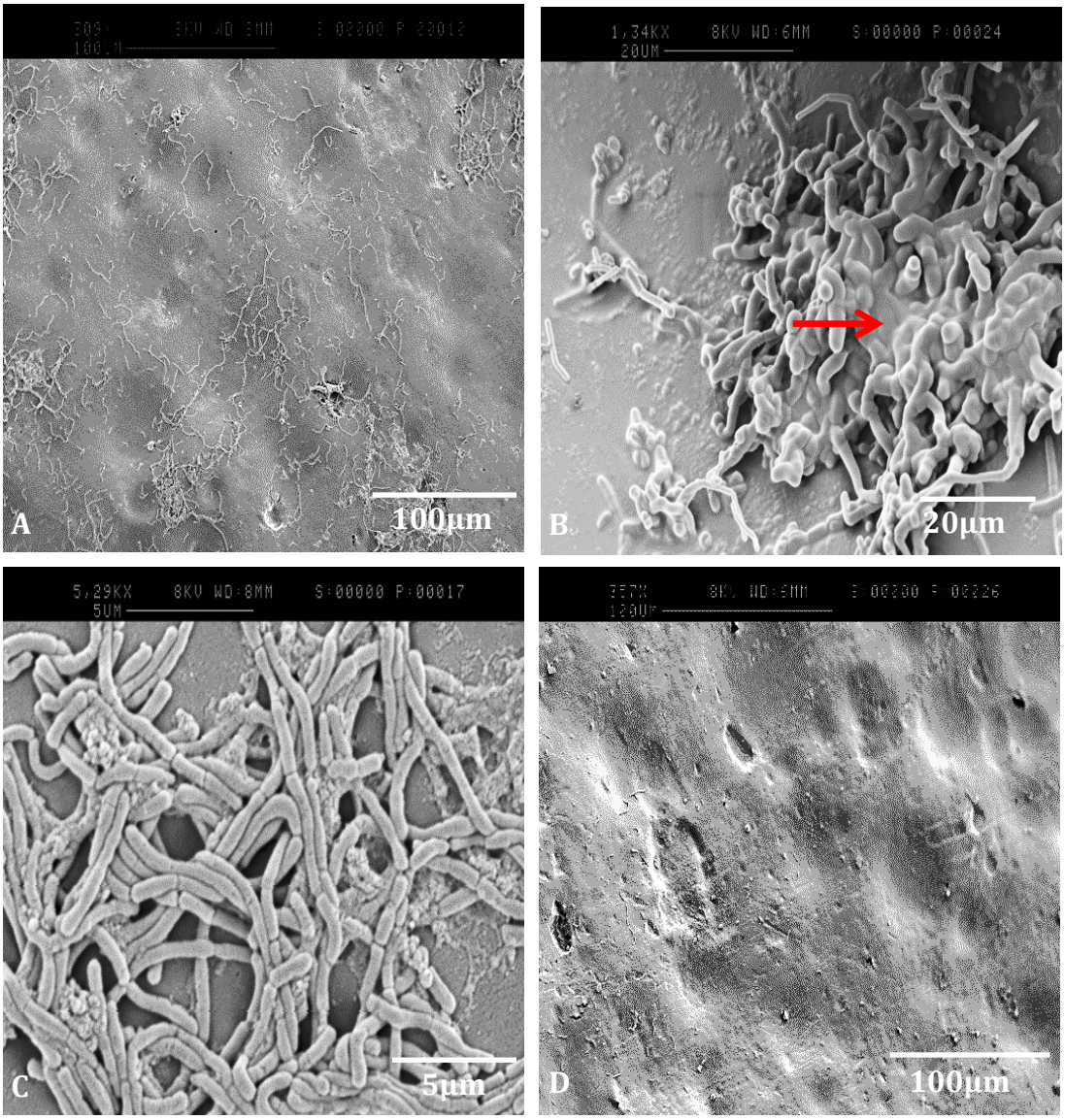


Figure 5.7 *L. casei* biofilms formed on non-fluoridated surfaces in the MRD for 48 h and imaged by SEM. A) Wide field view of the cells spread on sample surface, B) An aggregate of cells covered by exopolymeric material is shown by the arrow, C) A higher magnification of *L. casei* biofilm, and D) Very few cells were left after scraping the sample to recover cells for quantification.

5.4.7 Biofilm imaging of L. casei by using CLSM

Live/Dead stain should distinguish between viable and nonviable cells of bacteria as a function of the membrane integrity of the cell. The majority of *L. casei* cells in the biofilm appeared to be viable (green staining). In line with low-power SEM images (for example, Figure 5.7 A), biofilms appeared very thin and spread diffusely over the surface (Figure 5.8). Across the images, there was a background of red staining that

was apparent on the screen but was difficult to render in printed images. This possibly could have arisen from interaction of the PI stain with exopolysaccharides. To investigate this further, CW stain was used to monitor polysaccharides (Figure 5.9). Although there appeared to be some polysaccharides, CW staining occurred only in relatively large clumps and therefore it is unlikely that the diffuse PI fluorescence had arisen from exopolysaccharide material. Instead, perhaps the PI stain picked up extracellular DNA in the biofilm matrix.

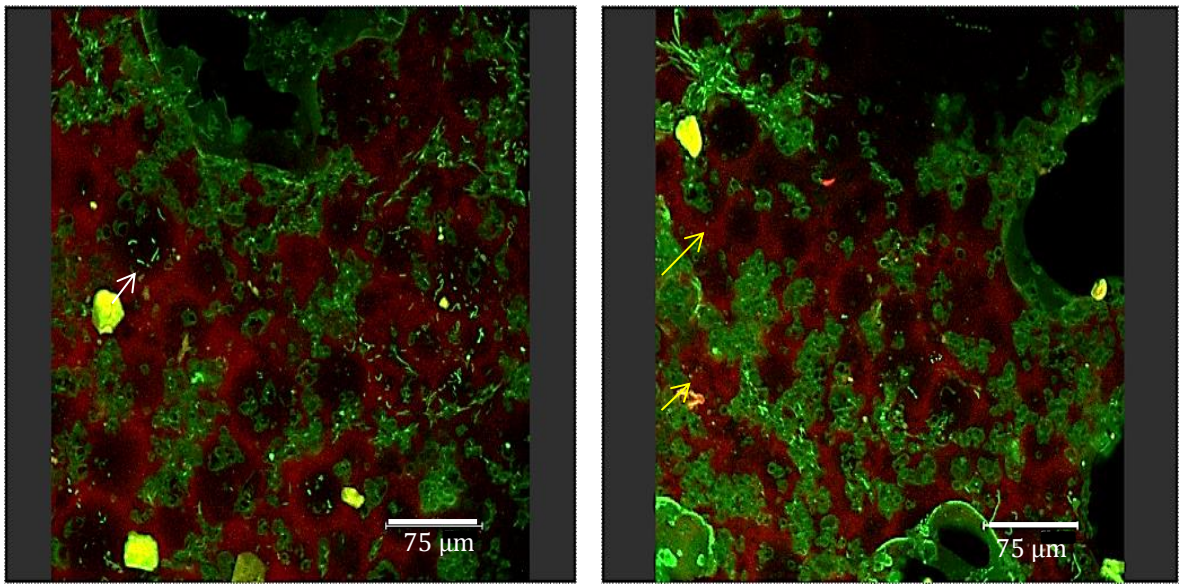


Figure 5.8 *L. casei* biofilm imaging by CLSM using LIVE/DEAD stain. The live cells stained green while the dead cells stained red. Single and small aggregates of *L. casei* cells were spread all over the sample (white arrows). Diffuse stain was apparent in the background (yellow arrows).

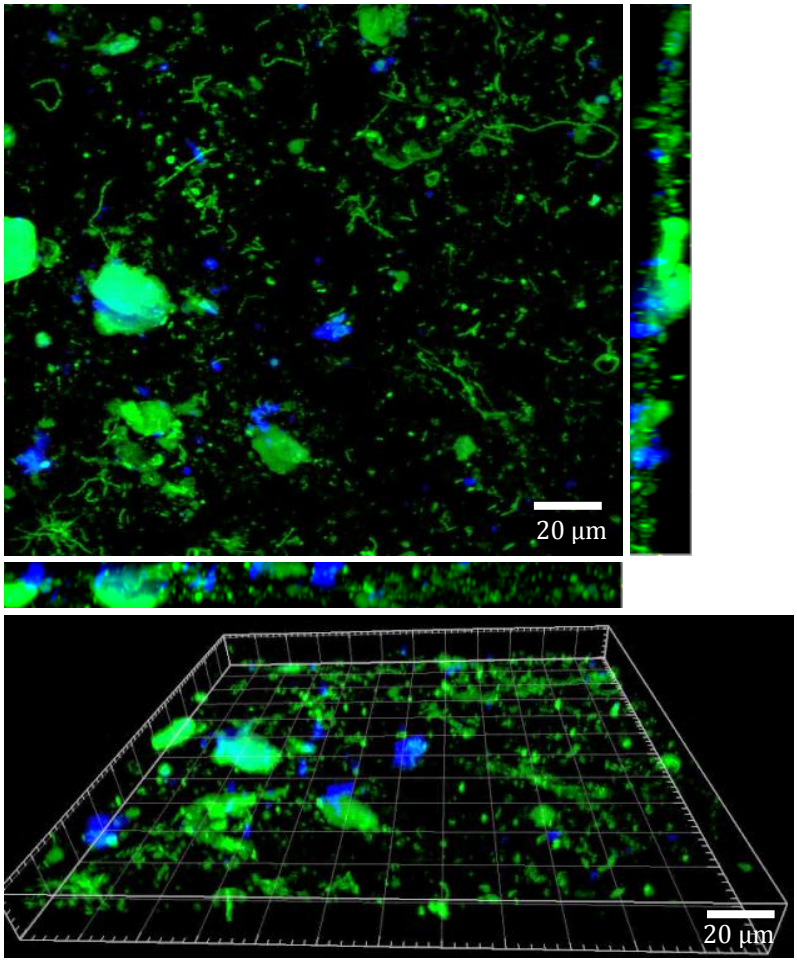


Figure 5.9 *L. casei* staining with Syto9 (green) and CW (blue). The blue staining was generally restricted to relatively large areas, and was not spread across the surface. Long chains and small aggregates of *L. casei* can be seen stained by Syto9.

5.4.8 S. mutans quantification by TVC's and qPCR

The final organism that was intended to be included in a three-species acidogenic biofilm community model was *S. mutans* UA159. The MRD system was set up as previously, using artificial saliva pulsed with sucrose three times per day and inoculated with *S. mutans*. However, despite several attempts, it was not possible to obtain strong and reproducible growth of *S. mutans* under these conditions. Therefore, the system was modified by introducing 1% sucrose continuously in place

of the three daily pulses with 10% sucrose. Under these conditions, strong biofilm growth of *S. mutans* was obtained (Table 5.4).

There was a slight decrease in the number of cells on fluoridated samples by both TVC's and qPCR, although differences were not statistically significant (Independent sample T test, $p>0.05$). As with *L. casei*, the total counts obtained by qPCR were consistently higher than by TVC's, as was previously observed for planktonic cells (Section 4.5.9).

Table 5.4 Growth of *S. mutans* on non-fluoridated and fluoridated copolymer in the MRD system with 1% sucrose pulsed continuously for 48 h.

	[A]Group	
	Non fluoridated Mean (SD)	Fluoridated Mean (SD)
TVC's (CFU/cm^2)	$8.1x10^5$ ($1x10^3$)	$7.6x10^5$ ($1.1x10^5$)
qPCR (cell/ cm^2)	$6.3x10^8$ ($2.8x10^8$)	$3.7x10^8$ ($7.4x10^7$)

[A]Biofilms were gently scraped, and TVC's were enumerated on THYE agar while the remainder of the biofilm was quantified by qPCR. The values represented were from three independent experiments. In all quantification experiments, the number of cells quantified was divided by the surface area of the sample.

5.4.9 Biofilm imaging of S. mutans by SEM

Using SEM for *S. mutans* biofilm visualization demonstrated large numbers of clumps of *S. mutans* cells covering the surfaces of non-fluoridated and fluoridated samples (Figure 5.10). With higher magnifications, images showed extracellular material that was presumably the collapsed exopolysaccharide matrix of the biofilm (Figure 5.10, C). No clear differences were noticed in the shape or the structure of the biofilms that formed on fluoridated or non-fluoridated copolymer (not shown).

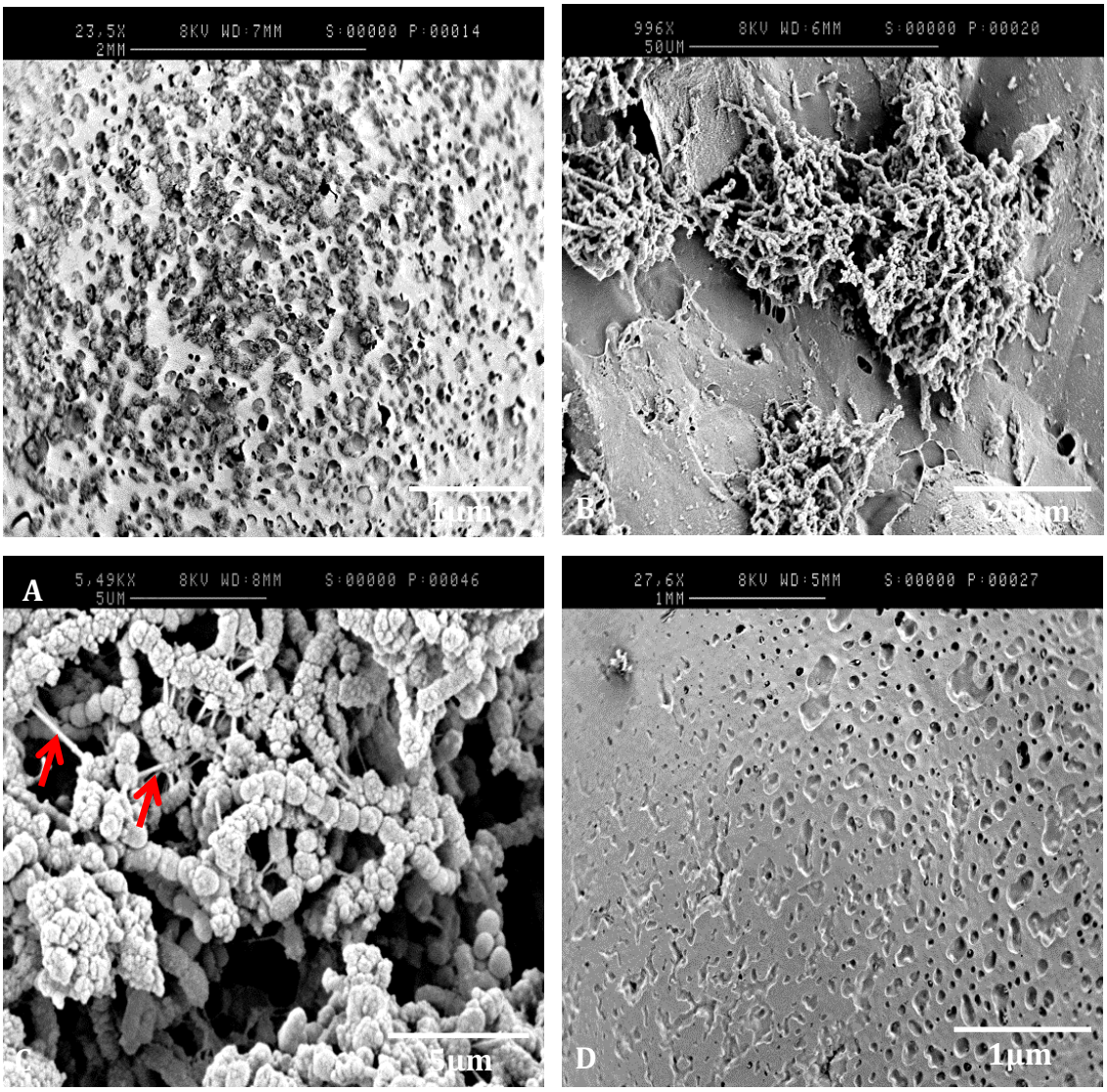


Figure 5.10 *S. mutans* biofilm imaging by SEM. A) At low magnification clusters of *S. mutans* aggregates can be seen on the sample surface, B) At higher magnification the patches of *S. mutans* can be seen, C) In a close-up view the matrix of water-insoluble glucans and extracellular polysaccharide is seen as indicated by arrows, and D) At low magnification after sample scraping few cells can be seen on the surface.

5.4.10 Biofilm imaging of S. mutans by using CLSM

With Live/Dead stain, Patches of *S. mutans* were stained green and red representing the live and dead cells respectively. The majority of *S. mutans* cells in the biofilm

appeared to be viable with no structural differences between biofilms on the non-fluoridated and fluoridated groups (not shown) (Figure 5.11)

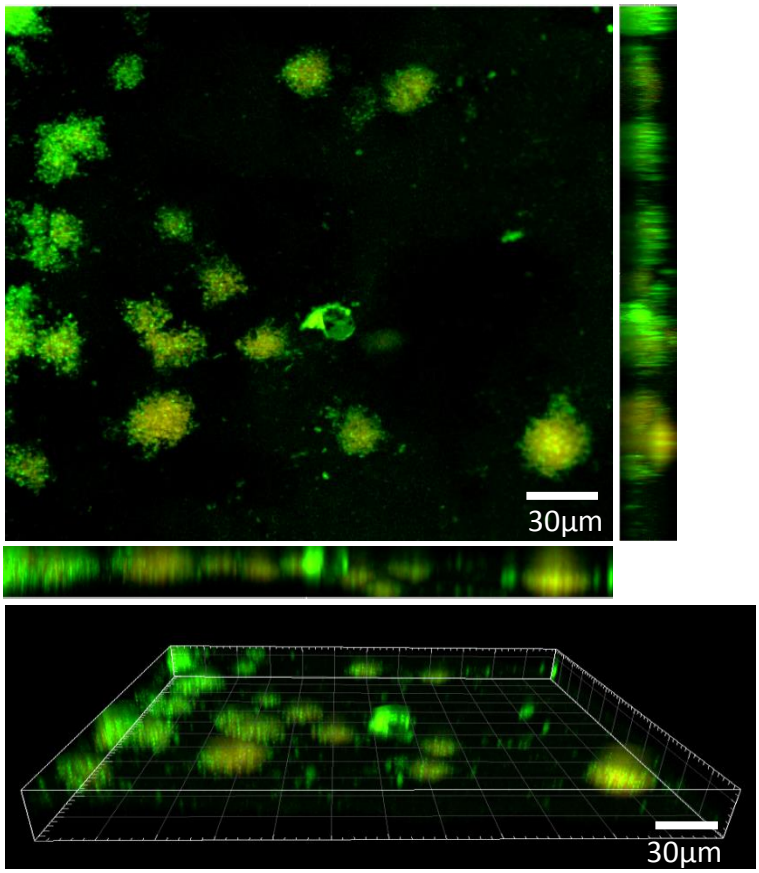


Figure 5.11 Small and large biofilm clumps of *S. mutans* stained by Live/Dead stain.

5.1 Discussion

In this chapter, an MRD system was established for biofilm formation by *C. albicans, L. casei* and *S. mutans* on non-fluoridated and fluoridated copolymer material. Unlike static biofilm models, the MRD system is a flow device that allows the growth of sessile microorganisms under continually replenished nutrient and enables the study of different types of substratum materials on microbial growth. Though the MRD system has many advantages over other models, it had some limitations that affected this study. For example, within a single flow chamber the same liquid runs over all the samples, and therefore the number of different treatments is limited by the number of chambers that can be assembled in parallel. In addition, any contamination in the system will spread on all over the samples. Here, we found that biofilms were unevenly distributed across the chamber and it was necessary to ensure that quantitative analyses utilized five discs that were spread evenly along the model. Similar problems have previously been noted when using the MRD (Araujo *et al.*, 2000).

Artificial saliva was used as the growth medium as it was difficult to collect the quantities of natural saliva that would be needed for the MRD system. However, natural saliva was used first to condition the surfaces with salivary components such as mucin, amylase, proline-rich glycoproteins and proline-rich peptides which are part of the natural acquired pellicle (Lendenmann *et al.*, 2000; Helmerhorst and Oppenheim, 2007). These components can promote microbial adhesion (Whittaker *et al.*, 1996; Lenander-Lumikari and Loimaranta, 2000; Okahashi *et al.*, 2011; Heo *et al.*, 2013). Edgerton and Levine (1992) studied the composition of acquired denture pellicle which has many components (i.e. amylase, albumin and mucin) similar to that of natural enamel pellicle. This study found these components have an important role in modulating microbial colonization on dentures. In this study, samples were coated for 2 hours with natural saliva, and this was based on previous studies which have employed periods of between 1-4 h for conditioning samples (Honraet and Nelis, 2006; Ramage *et al.*, 2008).

Sucrose was used in this study to mimic the normal meals that adult takes every day as it has beneficial effects on microbial growth and adhesion (Samaranayake *et al.*, 1980; Colby and Russell, 1997; Almståhl *et al.*, 2013). However, periodic introduction of sucrose to mimic normal intake of sucrose was sufficient for biofilm formation by *C. albicans* and *L. casei* but not *S. mutans.* The *S. mutans* is known to produce three different GTFs that are necessary for sucrose-dependent adherence (Ooshima *et al.*, 2001). Failure of *S. mutans* adherence and biofilm formation might relate to its inability to synthesize insoluble glucans (Branting *et al.*, 1989) and it is also not a primary coloniser like *S. sanguinis*. This result was in contrast with previous studies by Embleton *et al.* (1998) that showed *S. sanguinis* ability to grow when 10% sucrose pumped three times daily in the presence of amine fluoride. Furthermore, Pratten and Wilson (1999) who found a successful growth of streptococci biofilm on bovine enamel when 10% sucrose pulsed 3 times /day using CDFF. Successful use of a constant 1% sucrose infusion was in agreement with Liu *et al.* (2012) who used this ratio to grow *S. mutans* biofilm on polystyrene blocks.

Once the system was established, it was employed to assess the effects of incorporating fluoride into a copolymer on biofilm formation by *C. albicans*, *L. casei* and *S. mutans*. With *C. albicans,* fluoride demonstrated no inhibitory effect on *C. albicans* growth and the findings of the current study are consistent with those of Flisfisch *et al.* (2008) who found NaF has no inhibitory effect on *Candida* species that were grown planktonically. There was some indication that *C. albicans* colonization was slightly higher on fluoridated copolymer than non-fluoridated material, although differences were not statistically significant. This phenomenon might have been caused by the higher roughness of fluoridated samples as shown in Chapter Three. Surface roughness is known to be a factor that can lead to entrapment of microorganisms on the surfaces and protect them from shear forces. For example, higher numbers of fungal cells were retained on rough denture surfaces than smooth denture surfaces in a previous investigation by Verran and Maryan (1997).

Biofilm formation by *L. casei* on fluoridated and non-fluoridated samples revealed no significant difference between the two groups. *Lactobacillus casei* is well-known

acidogenic and aciduric microorganism that can tolerate different environmental pressures. Once it is attached to the surface, it can grow and metabolize in acidic environments in the presence of fluoride to a greater extent than other acidogenic species, which give *L. casei* a competitive advantage in the ecosystem (Hamilton *et al.*, 1985). There are similarities between the behaviour expressed by *L. casei* in this study and those described by Milnes *et al.* (1985) and Maltz and Emilson (1982) with several possible explanations for this result. The amount of fluoride released from samples might not be enough to inhibit their growth. Fluoride might reduce or inhibit the growth of *L. casei* at the start of the experiment but since there was a decrease in the amount of fluoride released from the material with time; these cells may have multiplied and increased in numbers after the fluoride release diminished. Therefore, a higher fluoride concentration is required to inhibit their growth. This was previously shown by Maltz and Emilson (1982) who found that lactobacilli are the most resistant microorganism to different fluoride salts when compared with streptococci and *Actinomyces* species. In addition, it is possible that fluoride is effective only against certain strains. This could be anticipated from the results of Milnes *et al.* (1985), who studied the effect of NaF on the metabolism of glucose by twenty-eight fresh and seven type strains of *Lactobacillus* species. This study found that freshly isolated strains were generally more fluoride-tolerant than the type strains, indicating that there is apparently variation in the degree of resistance between and within *Lactobacillus* species. In conclusion, it appears that *L. casei* are relatively unaffected by fluoride at low pH (Milnes *et al.*, 1984; Marquis, 1995).

Many previous studies have shown a positive inhibitory effect of fluoride against *S. mutans* (Brown *et al.*, 1980; Caufield and Wannemuehler, 1982; Van Loveren, 1990; Friedl *et al.*, 1997; Pandit *et al.*, 2011a; Pandit *et al.*, 2011b; Erdem *et al.*, 2012; Liu *et al.*, 2012). However, in this study *S. mutans* did not demonstrate any differences in growth on the surface of non-fluoridated and fluoridated copolymer. These results are consistent with previous work demonstrating that fluoride cannot affect *S. mutans* biofilm formation when frequent sucrose rinses were used (Duggal *et al.*, 2001; Ccahuana-Vásquez *et al.*, 2006). However, these findings need to be interpreted carefully because all previous studies were undertaken in conditions different from

this study. Another possible explanation for the lack of *S. mutans* inhibition by fluoride might relate to the development of bacterial resistance to fluoride over the course of growth. Oral streptococci can adapt to inhibitory concentrations of fluoride *in vitro* (Brown *et al.*, 1980). It is possible that fluoride tolerance is related to changes in expression of two glucose transport systems, one of which is fluoride insensitive (Hamilton and Ellwood, 1978). The amount of fluoride that is released from the material is also one of the important factors that affect biofilm growth on the surface. A previous study by Brown *et al.* (1980) found 75 ppm was not enough to stop *S. mutans* growth in an *in vitro* study. Therefore, the amount of fluoride released from the material might not be sufficient to inhibit or reduce *S. mutans* growth. Another possible explanation is that fluoride might have an influence during the first 24 hours but with time as the fluoride release decreases there may be a point at which microbial cells can start to grow. This explanation has also been proposed by Erdem *et al.* (2012) who studied the effect of fluoride containing varnish on *S. mutans* biofilm formation *in vitro*. Moreover, fluoride bacterial inhibition might not be direct but through the development of a more aciduric environment as identified previously by Giertsen *et al.* (2000). Therefore, it can be stated that many factors can play a role in fluoride antimicrobial effect such as fluoride concentration, time of exposure and the pH of the environment.

Two methods of biofilm quantification were used here: total viable counts (TVC's), which has historically been employed as the 'gold standard' for enumeration of bacteria, and qPCR, which is a highly sensitive approach that can easily enumerate different species with a mixed consortium. In comparing the data from TVC's with those from qPCR, it is important to note that there are many differences in the underlying assumptions, as well as the sources of error between viable counts and qPCR, as discussed previously in chapter four. For *C. albicans*, the data obtained from both methods showed a close match in the number of cells quantified by TVC's or qPCR. It might be expected that qPCR would give higher counts since it will include live and dead cells, as well as multiple copies of the *C. albicans* genome within individual hyphae (Yano *et al.*, 2002; Rawashdeh *et al.*, 2008). However, PCR quantification depends on the efficacy of the DNA extraction procedure and it is likely

that not all cells were fully disrupted. Yeast cells in general have a thick and rigid cell wall that is not easily lysed (Cryer *et al.*, 1976). In addition, the efficacy of DNA extraction can diminish with the aging of the fungal biofilms due to an increased deposition of melanin in the cell walls of fungi. This leads to the formation of complexes with proteins and carbohydrates that increase the rigidity of fungal cell walls (Karakousis *et al.*, 2006). Therefore, much DNA maybe lost during the procedure of DNA extraction. For *L. casei* and *S. mutans,* results from TVC's and qPCR were close to the standardization experiments (section 4.5.9) indicating that under biofilm growth, as with planktonic cells, qPCR tends to give higher cell counts of these organisms than TVC's.

The architecture of the microbial biofilms is one of the factors investigated in this study. Two methods were used to visualize biofilms on samples: SEM which is the conventional method for studying biofilm composition and structure, and CLSM which demonstrates the biofilm in more natural hydrated state (Kania *et al.*, 2010). *C. albicans* biofilms consisted of complex networks of fungal cells and hyphae which were frequently embedded in cracks, holes, and imperfections on the material surface that were particularly apparent after fluoride had leached out. These images confirm the importance of the morphogenetic conversions in the formation of biofilms (Baillie and Douglas, 1999; Ramage *et al.*, 2012). The dehydration procedure that is required for SEM sample preparation has disadvantages due to the artifacts that are created on the surface of the substratum which creates porosities and irregularities that change the morphology of the surface (Pereira-Cenci *et al.*, 2008). In addition it can reduce the total volume of the EPS material and alter its structure (Kania *et al.*, 2010; Weber *et al.*, 2014). Therefore, a very small amount of EPS is seen attached to the biofilm surface. *L. casei* cells were visible as long chains and aggregates that were sometimes associated with EPS material. *S. mutans* formed a large number of patches all over the samples with clump shaped structures that could be related to the sucrose supplement resulting in a higher amount of EPS material produced by cells. These clumpy shaped biofilms were observed previously by Yoshida *et al.* (2005). Neither the number of cells nor the consistency of EPS material were found to be different

between the fluoridated or non-fluoridated copolymer samples, even when higher magnifications were used for investigation.

Non-invasive CLSM was used to visualize the intact non disturbed biofilms. Covering sample surface with the cover slip was found to be a beneficial way to overcome the blurred images that were created initially. One of the drawbacks of using this technique was the possibility of water bubble entrapment between the sample surface and the cover slip, although this was not found to be a major problem here. Staining of *C. albicans* using Syto9 and PI has been used previously by Zhang and Fang (2004) who used these stains to quantify fungal biofilms. However, for future experiments, a specific Live/Dead stain for yeast is recommended. For *L. casei,* the background appeared to stain with PI which may be due to the presence of EPS or extracellular DNA, or could simply be related to the improper washing out of stains by PBS during sample preparation. Similar to the SEM images, CLSM images of *S. mutans* biofilm showed clumpy patches distributed across the sample surface. This characteristic distribution of *S. mutans* biofilms has also been recently identified by Chau *et al.* (2014).

In summary, a model system has been developed for culturing single species biofilms on dental materials. The concentration of fluoride used in this experiment might not be high enough to inhibit single species biofilm formation. Further research is required to investigate the amount of fluoride that is released from the material under conditions found in the MRD model. In addition, the antimicrobial mechanism of fluoride is strongly influenced by the pH as previously reported by many researchers (Hamilton, 1990; Marquis, 1990; Marsh and Bradshaw, 1990). Therefore, pH monitoring should be undertaken in future experiments. Studying the effect of fluoride on single species biofilm does not reflect the biofilm formation in the oral environment, where bacteria are usually present in multispecies communities that can spread and cause different diseases inside the oral cavity. The sensitivity of microorganisms to antimicrobials may be different in mixed species biofilms compared with monospecies cultures (Kara *et al.*, 2007). Resistance of biofilm cells to antimicrobials is well documented and may depend on many factors such as the

biofilm thickness, the number of Live/Dead cells present, gene expression, biofilm structure and the life cycle of bacteria which in turn will be influenced by metabolic interactions. Studying the effect of fluoride on single species biofilm is not enough as these species might behave completely different when they live together. Therefore, fluoride interaction with single species biofilm is the first step along the way to growing multiple species biofilms in order to optimize the effects of fluoride on their growth.

Chapter 6 . Effects of fluoride on multiple species biofilm formation

6.1 Introduction

Microorganisms do not usually live in single cell cultures but instead they are most commonly seen in polymicrobial communities as films or biofilms (Flemming and Wingender, 2010). The construction of a multispecies biofilm is influenced by the surface nature of the material, the nature of the conditioning layer that coats the surface and the microbial composition of the colonizing community (Teles *et al.*, 2012). In addition, mixed biofilms enhance opportunities for interactions such as horizontal gene transfer and co-metabolism (Burmølle *et al.*, 2006). The interactions between microorganisms in the mixed biofilms may be synergistic or competitive. Competition arises from factors such as bacteriocins or hydrogen peroxide that are produced by some members of the community and inhibit other species. In synergistic interactions one microorganism can generate a niche for other species, for example to facilitate their retention by a process known by coaggregation (Metwalli *et al.*, 2013). The formation of multi-species biofilms increases the chance of survival for many microorganisms (Pereira-Cenci *et al.*, 2008) and enhances their resistance to antimicrobial attack (Burmølle *et al.*, 2006).

Fluoride is generally considered to be the most effective anticaries agent, in part due to its effects against cariogenic microorganisms. The antimicrobial effects of fluoride against multispecies communities have been investigated previously. In (2002), Kamotsay and co-workers reported that 500 mg/L of NaF reduced the generation time of *C. albicans, L. acidophilus*, and *S. mutans* when co-cultured in milk. Another study by Bradshaw *et al.* (1990) investigated the effect of low sodium fluoride concentration (20 ppm) on nine bacterial species (including *L. casei*, and *S. mutans) in vitro* using a Chemostat system. The antibacterial activity of fluoride has been reported to involve interference with bacterial acid production and metabolic activity (Phan *et al.*, 2002; Neilands *et al.*, 2014). Bacterial cells that grow in dental plaque have different sensitivities to fluoride (Mandell, 1983). Potentially, fluoride may modulate inter-bacterial interactions and could influence the proportions of different

plaque bacteria during sugar metabolism (Marsh and Bradshaw, 1990). The interaction between *S. mutans* and *L. casei* grown together in Chemostat continuous culture with glucose and a fluoride concentration of 400 ppm, was studied by Bowden and Hamilton (1989). The growth of *S. mutans* was suppressed when the pH dropped to 5.5, but after cessation of fluoride, *S. mutans* re-established and was able to competite with *L. casei*. This demonstrates the ability of *S. mutans* to adapt and survive in these types of ecological system.

It is well-known that fluoride can inhibit the metabolism of many microorganisms *in vitro* (Hamilton and Bowden, 1988; Bradshaw *et al.*, 2002; Marquis *et al.*, 2003). Fluoride has been shown to decrease glycolytic activity, sugar transport and acid tolerance of many Gram-positive bacteria and these effects may be increased at low environmental pH (Marsh and Bradshaw, 1990; van Loveren, 2001; Marsh, 2006).

After studying the impact of fluoride with single species biofilms of *C. albicans, L. casei*, and *S. mutans* in chapter five, it was considered important to investigate the effects of fluoride on multiple species biofilms composed of the three microorganisms. In addition, pH and the amount of fluoride released into the medium were monitored to give insight into the interactions occurring during biofilm growth on fluoridated and non-fluoridated substrates.

6.2 Aims

The aim of this section was to investigate the formation of multiple species biofilms of *C. albicans, L. casei*, and *S. mutans* on the surface of the non- fluoridated and fluoridated copolymer materials.

The objectives were as follows:

1- Investigate the effects of fluoride by quantification of the biofilm cells for each species on non-fluoridated and fluoridated samples using total viable counts and qPCR.
2- Investigate the structure and composition of the biofilms by scanning electron microscopy and confocal laser scanning microscopy.
3- Measure the pH of the growth medium following biofilm formation on non-fluoridated and fluoridated copolymer.
4- Measure the amount of fluoride released from copolymer samples over the course of 48 h in the MRD biofilm system.

6.3 Materials and Methods

6.3.1 Preparation of MRD system and biofilm quantification

The MRD biofilm device was prepared as previously described (section 5.3.3). Inoculae of each microorganism were prepared and the microorganisms were flushed through the system in turn, in the order: *C. albicans* followed by *L. casei* and then *S. mutans*. Two hours were allowed between the introduction of each species to maximize the potential for microbial attachment and biofilm formation.

Biofilms were scraped from the surfaces for quantification by TVC's and qPCR after 48 h as previously described (section 5.3.3). In addition to the 48 h time point, mixed-species growth in the MRD was also monitored after 6 h and 24 h to assess the rate of biofilm accumulation over time.

6.3.2 Biofilm imaging using confocal laser scanning microscopy (CLSM)

CLSM was used to visualize the mixed species biofilms after 48 h. In this technique, stocks of three types of stains were prepared. a) 1 mg ml^{-1} (1.5 mM) Propidium iodide (PI) (Sigma-Aldrich, Inc) was prepared and stored at 4°C in darkness for 1 h, b) 5 mg ml^{-1} (18 mM) 4',6-diamidino-2-phenylindole (DAPI) (Life technologies Ltd, Paisley, UK) was prepared and stored at -20°C for 1 h, c) 1 mg ml^{-1} (9.6 µM) Concanavalin A Alexa Fluor 488 (ConA) (Life Technologies Ltd, Paisley, UK) was prepared in 0.1 M sodium bicarbonate (pH 8.3) and stored at -20°C for 1 h.

Samples were gently washed with PBS three times, and immersed in 2 ml PBS. To each sample, 10 µl (7.5 µM final concentration) of PI stain was added to stain the total DNA. The sample was then incubated in darkness at 23°C on a moving plate. After 5 min, the stain was washed off with 2 ml of PBS, and the sample was immersed in 2 ml fresh PBS. From the stock solution of ConA, 100 µl (0.48 µM final concentration) was added to stain the *Candida* cell wall for five minutes. The stain was washed off with 2 ml of PBS, and the sample was immersed in 2 ml fresh PBS. From the stock solution of DAPI, 0.8 µl (7.2 µM final concentration) was used additionally to highlight the DNA in all cells. The sample was incubated in darkness at 23°C and washed after 15 min three

times using PBS solution. Each sample was then mounted in a six well plate (Corning Life Sciences DL, Tewksbury, USA), and prepared for imaging as previously discussed in section 5.3.5. ConA stain excitation was at a wavelength of 485 nm and emission at 519 nm. PI excitation was at 530 nm and emission at 630nm. DAPI excitation was 358 nm and emission at 461 nm.

For SEM, the same procedure as that used in chapter five (Section 5.3.4) was applied to visualize mixed species biofilms.

6.3.3 pH and fluoride release measurement

A pH meter (Seven Easy S20, Mettler Toledo Co., Ltd., UK) was used to measure the pH of artificial saliva flowing from the outlet of each independent MRD after biofilm formation for 6 h, 24 h, and 48 h, respectively. A total of 10 ml was collected in a glass universal bottle. The pH meter was calibrated before each experiment using two standard buffer solutions of pH 7.00 and 4.01 for neutral and acidic solutions, respectively.

For fluoride analysis, 5 ml of spent medium was collected from the MRD outlet port and fluoride was measured using an ion selective electrode as previously described in chapter three. In addition, an extra MRD system was run separately at 23°C with deionised water and no microorganisms to investigate whether there is a difference in the amount of fluoride released in the presence or absence of biofilm and growth medium.

6.4 Results

6.4.1 Preliminary experiment for multispecies biofilm development

In the first trial of multispecies biofilm formation, the microorganisms were cultured in artificial saliva supplemented with 1 % sucrose. However, it was observed a large amount of EPS material was produced and the outlet tubing became blocked as a consequence. These three microorganisms have previously been shown to produce EPS material, when sucrose is available (McCourtie and Douglas, 1985; Gamar *et al.*, 1997; Bowen and Koo, 2011; Falsetta *et al.*, 2014). Therefore, to minimise problems due to excessive EPS production and blockage of the tubes, the concentration of sucrose in the artificial saliva was decreased from 1% to 0.1%.

6.4.2 Multiple species biofilm quantification by TVC's and qPCR

The aim of this part of study was to investigate the effects of fluoride on the growth of the three species on the surface of non-fluoridated and fluoridated copolymer material. In the first part of this experiment the impact of fluoride release from the material on the microbial species present on sample surface were investigated at three different time points, to assess the accumulation of biofilms over time. For all three organisms, colonization was detected after 6 h. Cell numbers increased at 24 h, and increased further after 48 h, indicating that each species was growing in the mixed-species biofilm model (Figure 6.1,Figure 6.2). *C. albicans* cell numbers were consistently lower than *S. mutans* and *L. casei*, perhaps partly due to the lower inoculum that was employed for *C. albicans*. It should be noted that the qPCR for *C. albicans* gave readings that were at or close to the limit of detection for this organism (see Section 4.5.9). However, *C. albicans* TVC's are also likely to be inaccurate, since hyphae will not be counted appropriately in a TVC's assay. Nevertheless, it is clear that *C. albicans* grew in the biofilm and there were sufficient cells for qPCR analysis by 48 h.

The results of *C. albicans* quantification by TVC's showed a higher number of cells on the fluoridated group in the first 6 h (Table 6.1). In addition, biofilm quantification by

TVC's and qPCR (Table 6.2; Table 6.3) for *S. mutans* and *L. casei* showed higher cell growth for both species on fluoridated samples at 6 h. After 24 h, the situation was changed. Quantification of *C. albicans* (depending on TVC only, Table 6.1), *S. mutans* and *L. casei* (depending on TVC and qPCR, Table 6.2; Table 6.3) showed a reduction in cell growth on fluoridated samples.

Using TVC and qPCR to determine cell numbers after 48 h, there was significant reduction in the density of surface colonization by *C. albicans, S. mutans and L. casei* on fluoridated surfaces (independent sample T test, $p<0.05$; Table 6.1; Table 6.2; Table 6.3). These results indicate that the presence of fluoride in the material showed a significant effect in the reduction of microbial growth.

After 48 h, in comparing between the two techniques of quantification, there were no significant differences in the numbers of *C. albicans* cells quantified by TVC or qPCR. On the other hand, for *S. mutans* and *L. casei*, the total counts obtained by qPCR were consistently higher than by TVC's, as was previously observed for planktonic cells (Section 4.5.9).

Table 6.1 Quantification of *C. albicans* cells in mixed species biofilm after 6 h, 24 h and 48 h with 0.1% sucrose added continuously in the MRD.

C. albicans		Group[A]	
		Non-fluoridated Mean (SD)	Fluoridated Mean (SD)
6 h	TVC's (CFU/cm^2)	1.1×10^3 (1.4×10^2)	1.9×10^3 (1.1×10^2)
	qPCR ($cell/cm^2$)	2.2×10^2 (5×10^1)	3.9×10^2 (4×10^1)
24 h	TVC's (CFU/cm^2)	4.2×10^4 (3.3×10^4)	3.2×10^3 (2.8×10^2)
	qPCR ($cell/cm^2$)	2.4×10^3 (1.1×10^3)	1.2×10^3 (2.2×10^2)
48 h	TVC's (CFU/cm^2)	5.6×10^4 (2.8×10^3)	4.5×10^3 (4.9×10^2)*
	qPCR ($cell/cm^2$)	2.8×10^4 (2×10^3)	5.6×10^3 (7×10^2)*

[A]Biofilms were gently scraped, and TVC's were enumerated on YPD agar. The remainder of the sample was used for *C. albicans* quantification by qPCR. The values of biofilms quantified after 6 h and 24 h were from two independent experiments and those quantified after 48 h were from three independent experiments.

Table 6.2 Quantification of *S. mutans* cells in mixed species biofilm after 6, 24 and 48 h with 0.1% sucrose pulsed continuously in the MRD

S. mutans		Group[A]	
		Non-fluoridated Mean (SD)	Fluoridated Mean (SD)
6 h	TVC's (CFU/cm^2)	4.6 x 10^3 (6.4x10^2)	9 x 10^3 (1.1x10^2)
	qPCR (cell/cm^2)	2 x 10^5 (9.7x10^3)	3.2 x 10^5 (1.6x10^3)
24 h	TVC's (CFU/cm^2)	5.4 x 10^4 (2.8x10^3)	2 x 10^4 (3.5x10^3)
	qPCR (cell/cm^2)	6.4 x 10^6 (2x10^4)	3.7 x 10^5 (9.4x10^4)
48 h	TVC's (CFU/cm^2)	5.7 x 10^5 (1.7x10^4)	1.8 x 10^5 (2.9x10^4)*
	qPCR (cell/cm^2)	1.3 x 10^8 (5.8x10^7)	1.7 x 10^7 (8.7x10^6)*

[A]Biofilms were gently scraped, and TVC's were enumerated on TYCS agar. The remainder of the sample was used for *C. albicans* quantification by qPCR. The values of biofilms quantified after 6 h and 24 h were from two independent experiments and those quantified after 48 h were from three independent experiments.

Table 6.3 Quantification of *L. casei* cells in mixed species biofilm after 6, 24 and 48 h with 0.1% sucrose pulsed continuously in the MRD.

L. casei		Group [A]	
		Non-fluoridated Mean (SD)	Fluoridated Mean (SD)
6 h	TVC's (CFU/cm^2)	4.1×10^3 (1.3×10^3)	1.0×10^4 (8.1×10^2)
	qPCR ($cell/cm^2$)	1.8×10^7 (1.2×10^7)	4.9×10^7 (9×10^6)
24 h	TVC's (CFU/cm^2)	1.5×10^5 (1.1×10^4)	5.7×10^4 (6.4×10^3)
	qPCR ($cell/cm^2$)	1.6×10^8 (2.9×10^7)	8.8×10^7 (3.5×10^6)
48 h	TVC's (CFU/cm^2)	5×10^5 (1×10^5)	1×10^5 (1.3×10^4)*
	qPCR ($cell/cm^2$)	3.2×10^8 (7.5×10^7)	1.1×10^8 (1.8×10^7)*

[A]Biofilms were gently scraped, and TVC's were enumerated on Rogosa agar. The remainder of the sample was used for *C. albicans* quantification by qPCR. The values of biofilms quantified after 6 h and 24 h were from two independent experiments and those quantified after 48 h were from three independent experiments.

6.4.3 Biofilm imaging using scanning electron microscopy (SEM)

The relationships and interactions between the three species were investigated by SEM (Figure 6.1). In low power images, clusters of microorganisms were seen to be spread over large areas of the non-fluoridated sample surfaces, while there were few aggregates found on the surface of the fluoridated material. Tight coadherence was seen between *C. albicans*, *S. mutans* and *L. casei* in higher power fields of view (Figure 6.1, A_3).

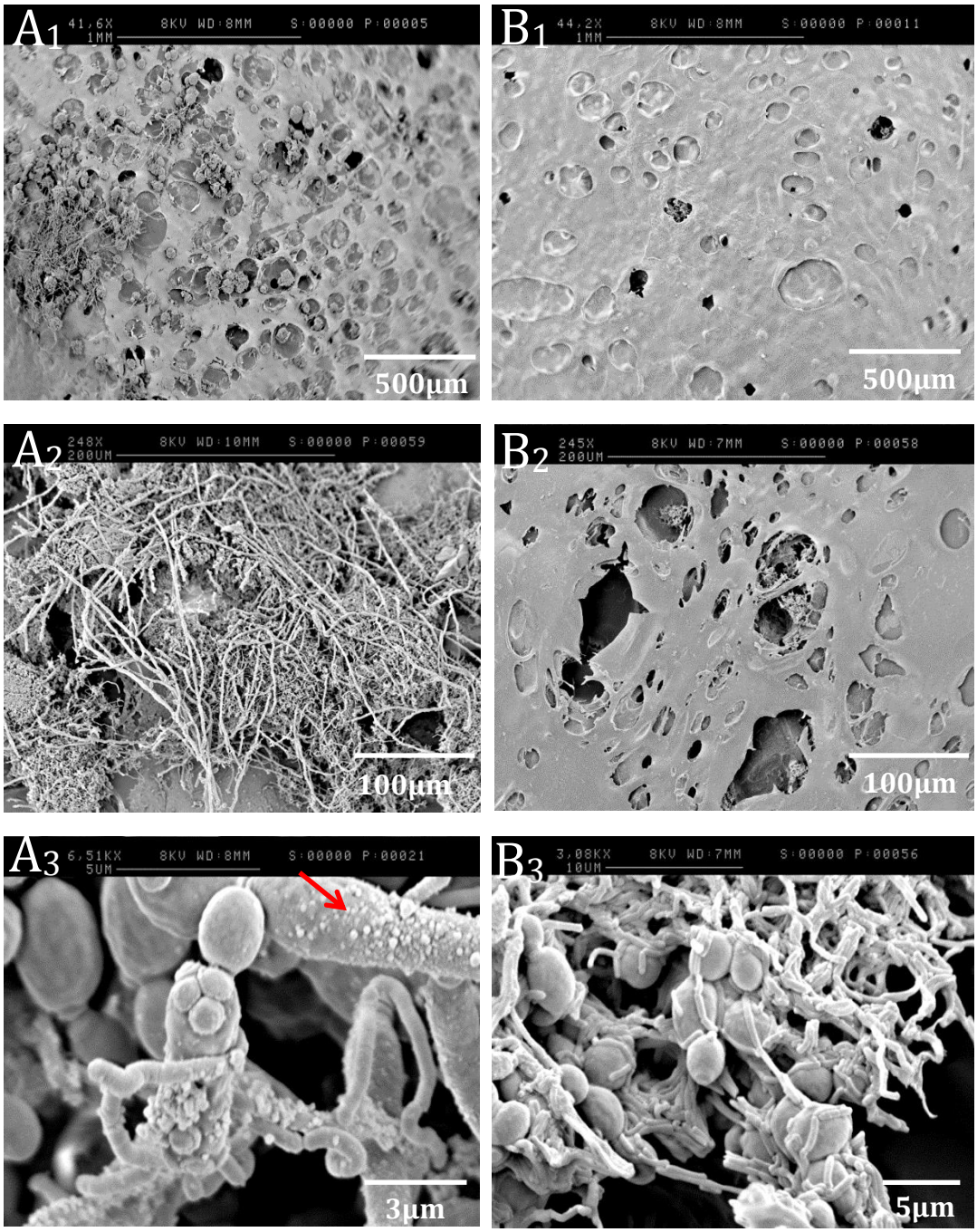


Figure 6.1 Mixed species biofilm imaging by SEM. $A_{1\text{-}3}$) biofilms formed on non-fluoridated samples, $B_{1\text{-}3}$) biofilms formed on fluoridated samples. Interactions between these three microorganisms can be observed in these images. Images from $A_{1\text{-}3}$ and $B_{1\text{-}3}$ represent the gradual increase in magnification. In A_3 and B_3 two different magnifications are shown to highlight the microbial interactions between the 3 species. Remnants of EPS material were still can be seen after biofilm dehydration (red arrow).

6.4.4 Biofilm imaging using Confocal Laser Microscopy (CLSM)

CLSM was used to visualize the mixed species biofilm of *C. albicans, S. mutans* and *L. casei*. The images showed a clear difference in the number of cells on fluoridated versus non-fluoridated surfaces (Figure 6.2). Thick biofilms of microbial cells were visualized on non-fluoridated samples with a high number of *C. albicans* cells, hyphae and psuodohyphae that interacted with other bacteria to produce large microbial aggregates. The structure and composition of the biofilms formed on fluoridated samples showed small clumpy biofilms spread irregularly over the surface. The interaction between the species was apparently decreased by the effect of fluoride and *Candida* hyphae appeared shorter when compared with non-fluoridated samples.

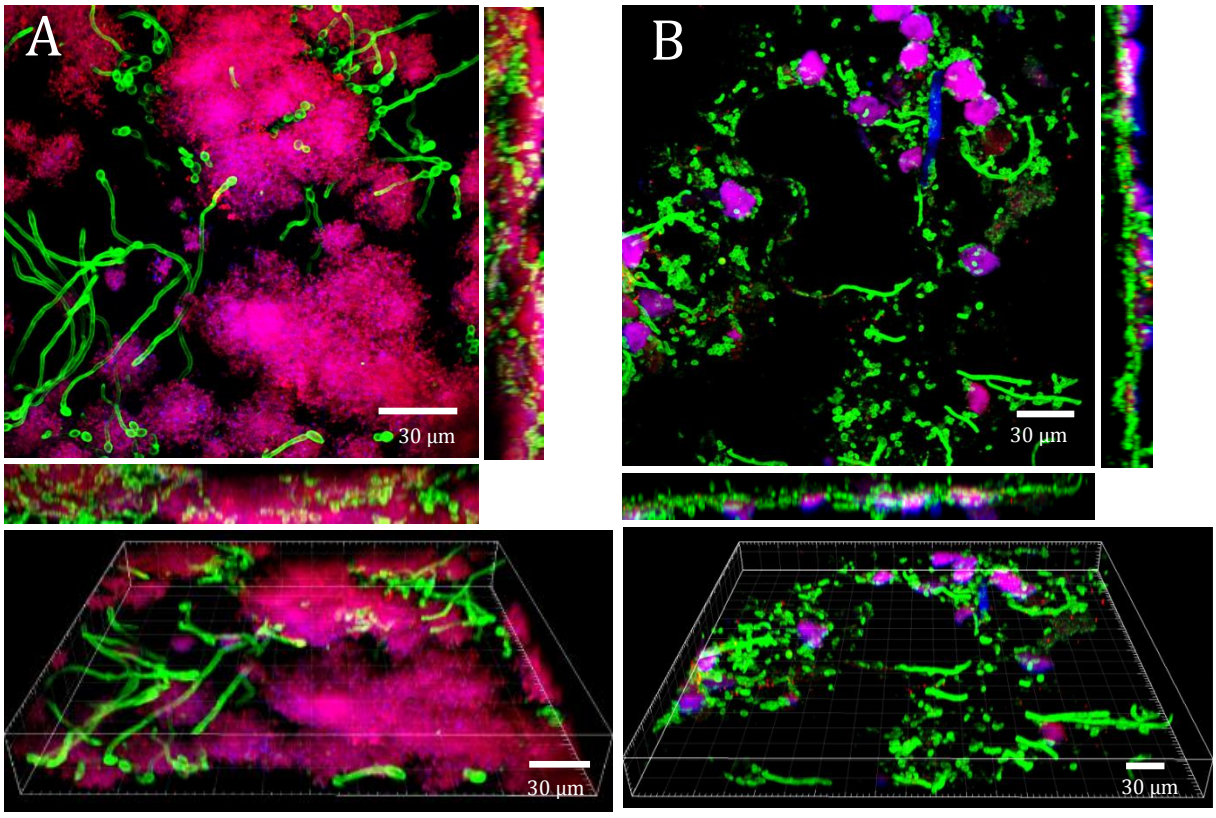


Figure 6.2 Mixed species biofilms formed on non-fluoridated surfaces (A) and on fluoridated surfaces (B). The green cells stained by ConA represent *Candida* cells in yeast, hyphal, and pseudohyphal morphologies. Total DNA was stained red/purple/blue using the combination of PI and DAPI.

6.4.5 pH measurement

The pH of saliva collected from the MRD was measured to investigate the effects of fluoride on microbial acid production. The pH of the artificial saliva was (6.99 ±0.03) at the start of the experiment. A significant difference (Independent sample T test, $p<0.05$) was observed between the pH of artificial saliva that had flowed over biofilms on fluoridated versus non-fluoridated surfaces after 6 h and 24 h (Table 6.4). The fluoridated samples tended to maintain a higher pH value than non-fluoridated samples over the 48 h duration of the experiment. After 48 h, the pH values did not show a significant difference (Independent sample T test, $p>0.05$, Table 6.4) between groups. This indicates that fluoride suppressed the acidogenicity of biofilms for up to 24 h, but this effect was not sustained over a 48 h period.

Table 6.4 pH of artificial saliva that measured from the MRDs output after 6 h, 24 h and 48 h.

	Non-fluoridated group	Fluoridated group
	Mean (SD)[A]	Mean (SD)[A]
6 h	4.88 (0.03)	5.04 (0.01)
24 h	4.38 (0.02)	4.47 (0.04)
48 h	4.32 (0.01)	4.35 (0.05)

[A]pH of the spent medium from the MRD. Data were collected from three independent experiments

6.4.6 Fluoride release measurement

In Chapter 3, the release of fluoride from copolymer samples was investigated under static conditions. However, it was considered important to measure fluoride release in the MRD system, since it was possible that continual removal of fluoride might increase the rate of release from surfaces. Therefore the release of fluoride from copolymer samples was measured both in the presence of growth medium and microgorganisms, and in sterile deionized water (Table 6.5). There was a significant

decrease (Independent sample T test, $p<0.05$, Table 6.5) in the amount of fluoride released in the presence of artificial saliva and biofilms compared with release in deionized water alone during 6, 24, and 48 h. The amount of fluoride released from the material decreased throughout the experiment in the presence or absence of biofilm and artificial saliva.

Table 6.5 Comparison of fluoride release from the fluoridated samples in two different conditions after 6 h, 24 h and 48 h.

	Fluoride release (μg/cm^2.h) in artificial saliva after mixed sp. biofilm formation	Fluoride release (μg/cm^2.h) in deionized water
	Mean (SD)[A]	Mean (SD)[A]
After 6 hours	343 (14.9)	433 (63.7)
After 24 hours	68.1 (9.2)	143 (27.5)
After 48 hours	28.7 (5.8)	93 (22.4)

[A]5ml of deionized water or artificial saliva were collected from the outlet of MRDs using plastic vial and fluoride measured as shown previously in section 3.3.1. Data were collected from three independent experiments. The fluoride concentration for the deionized water and fresh artificial saliva was below the minimum level of the detection by the ISE.

6.5 Discussion

Studying the effect of fluoride on multispecies biofilms was the main goal of this project. The results of experiments to investigate the impact of fluoride on a single species microbial biofilms did not reveal reduction in their growth. However, it is known that the behaviour displayed by microbial organisms grown separately is different from those of similar microorganisms grown in communities such as oral biofilms (Costerton *et al.*, 1999). Further investigation of these variations through assessing gene expression and protein expression, has shown that some properties of oral bacteria grown within biofilms are different from those of single cultures (Burne *et al.*, 1997; Black *et al.*, 2004). It is logical to assume that the interactions between the oral microbial inhabitants may influence the behaviour of the whole community. These interactions could include competition for nutrients, synergistic interactions, antagonist production from one habitant that can prevent the growth of others, and virulence factor neutralization by other residents (Kuramitsu *et al.*, 2007).

To the best of our knowledge, there are no previous published studies that investigate biofilm formation by mixed cultures of *C. albicans*, *L. casei* and *S. mutans*, including the relationship between the species and the impact of fluoride on their growth. In this study, SEM images demonstrated the affinity of *S. mutans* to the yeast and hyphal elements of *C. albicans*. This interaction has been previously visualized by Metwalli *et al.* (2013). An ultrastructure study on mixed biofilm of two of these organisms showed the positive interaction of both morphological forms of *Candida* with *S. mutans* cells (Jenkinson and Douglas, 2002). A mutual growth stimulation and co-aggregation between *S. mutans* and *C. albicans* can enhance the adhesion process but at the same time, *S. mutans* has the capability to suppress hyphae formation of *C. albicans* (Pereira-Cenci et al., 2008). On the other hand, Thein and co-workers (2006) reported contradictory evidence, showing no significant effect of *S. mutans* on hyphal formation in a biofilm co-cultured with *C. albicans*. Recently, Falsetta *et al.* (2014) found that there was a mutualistic relationship between *S. mutans* and *C. albicans* in the presence of sucrose and EPS which worked as a key mediator of a cospecies biofilm between these two species. This relationship was confirmed and visualised using SEM images.

A slightly different interaction was observed between *L. casei* and *C. albicans* by SEM. Here, the cells of *L. casei* were twisted around the hyphae and budding cells of *C. albicans*. There is a reason to believe that *Candida* and lactobacilli can be compatible to some extent, especially because yeasts may improve the growth of lactobacilli by supplying them with necessary vitamins (Koser *et al.*, 1960; Wilson and Goaz, 1960). This interaction was also visualised by Bilhan *et al.* (2009) when they found a high percentage of *L. casei* and *C. albicans* on the dentures of patients with denture induced stomatitis.

The coaggregation between *L. casei* and *S. mutans* is well-known through their interaction and biofilm formation in dental plaque (Carlsson *et al.*, 1975; Babaahmady *et al.*, 1997; Twetman *et al.*, 2009; Huang *et al.*, 2011). This positive synergism was demonstrated by Wen *et al.* (2010) where real time PCR quantification showed an increase in the number of cells quantified when they grow in co-species biofilm in comparing with mono-species biofilm. This enhancement was shown to involve *S. mutans* glucosyltransferase enzymes, which provide *L. casei* with glucans that enhance bacterial adhesion and coaggregation. Therefore, our results of biofilm assessment by SEM are in agreement with other researchers who have confirmed a positive relationship between these species.

The results of biofilm growth on non-fluoridated and fluoridated samples during the first 6 hours of the experiment revealed slightly higher initial colonisation of fluoridated samples by each microorganism. One plausible explanation for this is that the rougher surface of fluoridated samples might lead to increased attachment by cells (section 3.4.3). Preferential retention of microbial cells on rough surfaces occurs due to increased protection from shear forces, with even a small increase in roughness being shown to have a significant effect (Bollen *et al.*, 1997). Many previous studies have demonstrated the relationship between surface roughness and microbial colonization on denture surface, confirming that cracked and rough surfaces facilitate microorganism attachment and biofilm development (Quirynen *et al.*, 1990; Verran *et al.*, 1991; Moura *et al.*, 2006; Von Fraunhofer and Loewy, 2008).

After 24 hours, inhibitory effects of fluoride on bacterial growth were apparent. It is possible that these results are due to the amount of fluoride released from the material which might be enough to interrupt the biofilm formation by these species. It was shown previously (section 3.4.1) that a high amount of fluoride is released from the material during the first day. This finding is in agreement with Erdem *et al.* (2012) who showed fluoride that released from varnish was high enough to suppress biofilm formation by *S. mutans* and *S. sobrinus* in the first 24 h. It is not clear exactly how fluoride inhibits biofilm growth, but the low pH that was generated over the first 24 h may have contributed to antimicrobial activity. This point will be discussed in greater detail below.

With the continuing release of fluoride after 48 h, fluoride appeared to still be effective in suppressing microbial growth of the three species, despite the fact that the number of cells started to increase by this point. A possible explanation for these results might be that the interaction between the microorganisms in the presence of sucrose caused a further reduction in the pH. This phenomenon enhances fluoride entrance inside the cells as HF, whereupon it dissociates to fluoride ions and inhibits sugar uptake by reducing the glycolysis (Hamilton, 1977; Mayanagi *et al.*, 2014). If the extracellular environment acidity continued to increase (pH~4.5), then the intracellular fluoride uptake was highly sufficient that tiny amount of fluoride of 5-10 ppm could be inhibitory (Harper and Loesche, 1986). The inhibitory effects of fluoride in this case may be attributed to the inhibition of glycolytic enzymes such as enolase that in turn disrupts the phosphoenolpyruvate-energized phosphotransferase transport systems of the microorganisms (Hamilton, 1977; Nouri and Titley, 2003; van Loveren *et al.*, 2008). In addition, fluoride would further impair bacterial metabolism and the aciduric capability of microorganisms through the inhibition of ATPase enzyme that plays a main role in the maintenance of the intracellular pH by pumping out protons (Sutton *et al.*, 1987; Hayacibara *et al.*, 2003).

A reduction of *C. albicans* growth was seen on the surfaces of non-fluoridated and fluoridated samples when compared with their growth in single species biofilms. This may have resulted from the lower sucrose concentration that was used in the mixed-

species model (0.1% sucrose, compared with 1% sucrose for single species biofilms). The interspecies interactions between the three species might also have had a role in the reduction of *C. albicans* growth. *S. mutans* has the capability to release one or more diffusible molecules that impair germ tube formation of *C. albicans*. For example, competence-stimulating peptide has been shown to inhibit *C. albicans* hypha formation (Jarosz *et al.*, 2009). On the other hand, lactobacilli are also capable of suppressing *C. albicans* growth (Thein *et al.*, 2006) in spite of interaction that visualised here between the two organisms using the SEM . Lactic acid production by *L. casei* might have the role in this suppression as growth of *C. albicans* in a biofilm with *L. casei* did not attain as high counts as when growing in the same medium in pure culture (Koser *et al.*, 1960; Wagner *et al.*, 1997).

The structure, design and interactions within the multispecies biofilm were studied using SEM. Fluoride released appears to play a role in suppressing biofilm formation and only small amounts of aggregates were found hiding in the holes and cracks that formed on the surface of fluoridated samples. These findings were confirmed by CLSM when three different stains were used to visualize the biofilm that formed by the three species. In these images, it was clear that biofilm formation was reduced on samples containing fluoride compared with non-fluoridated copolymer. Regarding the role of *S. mutans* in inhibiting hyphal formation of *C. albicans,* images by SEM and CLSM did not reveal this suppression and hyphae were found in both groups of the material. This confirms the recent findings of Falsetta *et al.* (2014) in *in vivo*, who showed that the bacterium-fungus relationship between *S. mutans* and *C. albicans* was mutualistic at a clinically relevant site, and could amplify the severity of carious lesions.

The measurement of pH was helpful to estimate the influence of fluoride on reducing microbial acid production. The method used to measure the pH might underestimate the impact on acidity close to or within the biofilm surface. Different electrodes or microelectrodes have been described for measuring the pH of biofilm as shown previously by Liermann *et al.* (2000). Owing to technical difficulties with sampling biofilms in the MRD model and concerns regarding potential contamination, the waste medium collected from the outlet of each MRD was used to give an indication of the

pH difference between the groups. The results indicated that fluoride was able to moderate the reduction in pH after 24 h. The present findings are consistent with other research in *in vitro* by Bibby and Fu (1986) who found 10 ppm of NaF was enough to prevent pH drop of dental plaque from 4.51 to 4.31 of that group without fluoride. In addition Jenkins *et al.* (1969) reported that 2.5 ppm fluoride significantly moderated the pH drop by about 0.06 units in *in vitro* plaque bacteria growth. This also agrees with a later observation by Bradshaw *et al.* (2002), who showed that fluoride played a role in preventing pH drop of a biofilm fed by glucose in a CDFF model system. In this case, the final pH was 4.83 in the presence of 20 ppm fluoride in comparison to 4.41 of control group. This result may be explained by the fact that fluoride can act directly on glycolysis to reduce the acidification of the biofilm or through interfering with carbohydrate degradation (Hamilton and Ellwood, 1978; Van Loveren, 1990; Marquis *et al.*, 2003). Recently, Mayanagi *et al.* (2014) found that four fluoride releasing restorative materials, glass-ionomer cement (GIC), resin-modified glass-ionomer cement (RMGIC), resin composite (RC) and flowable resin composite have the ability to maintain higher level of pH values in comparing with PMMA material used as control group in *in vitro* dental plaque model of *S. mutans*. Even with low concentrations of fluoride can reduce (but not eliminate) the production of acid by microorganisms and the decrease in acid may negate the ecological benefit afforded to aciduric species (Marsh and Bradshaw, 1990). In my experiment, pH was 6.99 at the start of the experiment and it dropped to the range of 4.3 - 4.4 after 24 h. Therefore, this drop was beneficial in enhancing antimicrobial role of fluoride in the depletion of microbial growth as shown previously. It can be suggested now that a small change in pH generated by these cells in acidic environment resulted in small uptake of fluoride inside the cells which can cause a smaller inhibitory effect.

Another important factor that affects the amount of biofilms formed on fluoridated samples is the amount of fluoride released from the material. Higher amounts of fluoride were released from samples in the MRD during the first six hours (343 $\mu g/cm^2$. h = 33 ppm. h) than after 24 h or 48 h. This is usual with most fluoride releasing materials (Kamijo *et al.*, 2009; Su *et al.*, 2010; Erdem *et al.*, 2012). Despite the high concentration of fluoride released during the first hours, it was found that

slightly higher numbers of cells were attached to the surface of fluoridated samples. This was possibly related to the rough surface of the material, and to the fact that higher number of cells adhered to the surface of the material at the start of the experiment. It can be considered that fluoride had no direct effect in the reduction of microbial attachment at the first 6 hours of the experiment. This result seems to be consistent with *in vivo* study by Kilian *et al.* (1979), who found that for the first five hours after rinsing with 0.2% NaF, the numbers of bacteria adhered onto enamel surfaces were not significantly reduced. Fluoride might have less effect at the start of the experiment due to the relatively high pH environment (Hamilton and Ellwood, 1978). As the pH decreases, this can increase the dissolution of the material leading to a higher fluoride level in the medium (Wiegand et al., 2007), and by the same time enhancing uptake into the microbial cell.

Most previous studies tried to measure the amount of fluoride released from the material by using distilled water which does not represent the oral fluid as other ingredients of saliva such as mucin and oral flora are missing (Okada *et al.*, 2001). In this study, the amount of fluoride released in distilled deionized water was compared with the amount released during biofilm formation. A significant decrease in amount of fluoride released from the group covered with biofilm was shown at all measurement points. There are several possible explanations for this. Fluoride release is greatly influenced by the medium the material is stored in (Kosior and Kaczmarek, 2005). For instance, the amount of fluoride released into natural or artificial saliva is lower than that released into distilled water. This could be related to the organic compounds that are present which interfere with the release of fluoride ions (Preston *et al.*, 2003). Furthermore, the biofilm and plaque layer formed on the surface of the restoration may reduce fluoride release and part of fluoride released could be accumulating within these layers. In addition, there is a possibility of an uptake of part of the fluoride that released back into the material (Tam *et al.*, 1996). Therefore, for future experiments, fluoride releasing restorations should be measured in conditions that closely mimic conditions encountered in the oral cavity.

In summary, the interaction between the three microorganisms was clearly visualised using SEM and CLSM. The observed coaggregation closely simulates the nature of microbial communication which occurs inside the oral cavity. The EPS material produced by these three species might play an important role in the coadherence between these microorganisms. Two techniques of biofilm quantification were successfully used to represent the number of cells that grew as biofilms on the material surface. The inclusion of fluoride within the material resulted in a reduction in multispecies biofilm formation, and this copolymer material is potentially beneficial for microbial control in the oral cavity. This study highlights the need to study the impact of this material on biofilms formed by the natural microbiota of human denture plaque.

Chapter 7 . General discussion

A wide range of techniques and materials are used for cleaning oral appliances and keeping them in a hygienic condition. The microporous surfaces of acrylic appliances offer a wide range of environments to support microorganisms that can threaten the health of many types of patients. The maintenance of a prostheses is important for the health of patients and to maintain an aesthetic, odour-free appliance (Shay, 2000).

Past research has attempted to decrease diseases associated with biofilm formation on these appliances using mechanical and chemical cleansing techniques or by incorporating materials within PMMA to minimize biofilm formation on the acrylic prosthesis (Edgerton *et al.*, 1995; Sheen and Harrison, 2000; Guggenheim *et al.*, 2004; Devine *et al.*, 2007). Fluoride is particularly effective against cariogenic microorganisms, since it inhibits acid production as well as limiting microbial growth. In attempts to control denture plaque, fluoride has been incorporated into a denture-coating material (Kodkeaw *et al.*, 2010), and denture lining material (Hayakawa *et al.*, 2006). However, none of these materials have been tested for their ability to control the growth of acidogenic microorganisms. Here, a new model was developed to monitor mixed-species acidogenic biofilm formation, and tested the biofilm inhibitory properties of a novel fluoride-releasing denture lining material consisting of a copolymer of methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA). This material was previously developed with potential to be used as an orthodontic bonding material. The composition of this resin has been refined to make it suitable for use as a liner in dental acrylic appliances such as complete and partial dentures, orthodontic removable appliances, surgical obturators and splints, overdentures, or for a hard relining material.

7.1 Discussion of the key findings.

7.1.1 Properties of the copolymer

This project began with the selection of the appropriate formula of the copolymer material. PMMA is a linear polymer with low density chains which forms a mixture with MMA that is easy to handle and use with many positive properties encouraging its use as a denture or denture liner such as high tensile and compressive strength, dimensional accuracy and aesthetic (Ferracane, 2006; McCabe and Walls, 2008). HEMA was added to the material as it is a hydrophilic material which enhances the absorption and diffusion of water that is necessary to facilitate fluoride release (McCabe and Rusby, 2004). The source of fluoride was NaF, as selected in the previous studies by Zahroon (2014) and Su *et al.* (2010). This salt is highly soluble and less sensitive to environmental pH changes than other fluoride sources (Shen *et al.*, 2007). In addition, it is able to immediately yield free ions once placed in solution (Nakajo *et al.*, 2009). The release ratio of NaF from materials is relatively stable across a range of pH values which is important for a sustained long-term release of NaF from the materials (Shen *et al.*, 2007). A relatively high concentration of fluoride was selected to investigate the impact of fluoride on the biofilms that were formed on the material surface.

Prior to laboratory work investigating biofilm growth, it was necessary to select a sterilization method which had minimal effects on fluoride release properties (Chapter 3). Four methods beside the control (unsterile) were chosen to sterilize fluoridated discs, three based on autoclaving (Chang and Merritt, 1992) and the last group sterilized by dry heat. A high rate of variation in the amount of fluoride released was noticed during the first week. This difference in the amount of fluoride released with relatively high standard deviations between samples within the same treatment group, is most probably related to the uneven distribution of fluoride inside the material (Thevadass *et al.*, 1996) as a manual method was used to mix the ingredients of these specimens. Fluoride release continued at a low rate for six months. These results were in line with previous studies (Kodkeaw *et al.*, 2010). Under static conditions, fluoride was released at a relatively high rate initially,

followed by a gradually decreasing rate of release. Even after six months, fluoride release was in the range of 12-15 μg/cm^2.day. Although we did not measure the fluoride concentration in the immediate vicinity of the disc, it is likely that this sustained release of fluoride will have significant effects on dental plaque bacteria in the base of a biofilm formed on the resin. Furthermore, it has been demonstrated that similar copolymer material can be recharged by soaking in an NaF solution (Zahroon, 2014).

The chemical composition and average roughness (R_a) were thought to be other factors that could be affected by autoclaving. Using FTIR, it was found that autoclaving has no impact on the chemical composition of the material. Roughness values, measured using stylus profilometry showed no difference between fluoridated and non-fluoridated groups before autoclaving with an increase in R_a was noticed in fluoridated group after autoclaving. This change was attributed to the breakdown of the material surface layer and craze formation due to autoclaving, or leaching out of residual monomer or fluoride that left small holes on the material surface.

7.1.2 Microbial preparation

C. albicans, S. mutans, and *L. casei* were selected for investigation, as these acidogenic microorganisms are commonly implicated in oral appliance related diseases (Branting *et al.*, 1989; Baena-Monroy *et al.*, 2005; Thein *et al.*, 2006; Li *et al.*, 2010; Mantzourani *et al.*, 2010; Preshaw *et al.*, 2011). Optimizing DNA extraction from the three species was a challenge in this part of the study. Different DNA extraction kits were tried, but following further research and depending on previous study (Seyran *et al.*, 2010; Douglas and Klaenhammer, 2011), ZR kit was found efficient in the DNA extraction from the fungal-bacterial cells that used in this study.

Two methods were chosen for biofilm quantification, the conventional culture method, also known as total viable count (TVC's) and absolute qPCR. Quantification by qPCR and TVC's are difficult to compare because both have inherent assumptions and limitations. Viable counts depend on being able to recover cells from biofilms, and to disperse aggregated microorganisms and this can result in an underestimation of cell

numbers by TVC's. It is also likely that qPCR gives higher counts due to the inclusion of DNA from non-viable cells. In this section of study, a comparison between the two techniques was undertaken. The results demonstrated correlation coefficients greater than 0.87 for all the cases evaluated between both quantitative methods, indicating that the qPCR method developed allows reliable determination of the total number of cells.

7.1.3 The impact of fluoridated copolymer on the formation of biofilms

This part of the study aimed to develop a biofilm model system to monitor microbial cell growth. In the MRD biofilm model, the amount of fluoride released from the material under flowing condition was measured. High levels of fluoride release (28.7 $\mu g/cm^2.h$ = 2.8 ppm) were sustained for at least 48 h. This rate of fluoride release was sufficient in comparison with static conditions, because samples were exposed to a continuous removal of dissolved fluoride from the system. The mouth is essentially an open system, and fluoride will be removed by swallowing. In future, it will be important to determine the rate of fluoride release over longer time periods. Nevertheless, the rate of fluoride release was sufficient to reduce mixed-species biofilm formation by oral acidogenic microorganisms in the model system. Interestingly, when biofilms were developed with single species of microorganisms, there were no significant differences between biofilm development of *C. albicans*, *L. casei* or *S. mutans* on fluoridated or non-fluoridated copolymer (Chapter five). It is now well-documented that oral bacteria communicate with one another and with oral *Candida* spp., and that these interactions are critical for biofilm development (Thein *et al.*, 2006; Jarosz *et al.*, 2009; Falsetta *et al.*, 2014). Therefore, it is possible that fluoride interferes with some aspect of cell-cell communication, and that this destabilizes the biofilm. In addition, it is likely that fluoride inhibits carbohydrate metabolism and acid generation with mixed-species biofilms since fluoride is well-known to inhibit sugar uptake and glycolysis (Hamilton and Ellwood, 1978; Van Loveren, 1990; Nakajo *et al.*, 2009).

Although there was a significant reduction in acid production by biofilms on non-fluoridated versus fluoridated copolymer after 24 h, no significant differences

between the pH in the effluents from biofilms on the different surfaces were seen at 48 h. This was presumably related to the sufficient growth on both types of material to create a low pH. However, the data indicated that the fluoride was effective in controlling microbial growth and acidogenicity during the early stages of biofilm formation. Within more complex microbial communities, fluoride can shift the ecological balance and prevent the overgrowth of strongly acidogenic microorganisms such as *S. mutans* (Marsh and Bradshaw, 1990). An interesting future goal would be to assess whether the fluoridated copolymer can shift the microbial balance in more complex communities by selectively inhibiting the acidogenic species.

The mixed-species acidogenic denture plaque model developed here can be used for future studies on antimicrobial properties of different acrylic resin materials. Approaches were developed to quantify both the viable cells on the surface and the total DNA from each species, which is derived from live and dead cells. For *C. albicans* the two techniques gave similar cell counts. It is likely that this is in part due to coincidence, as both methods have limitations. Various hyphal and budding forms of *C. albicans* were observed by SEM and CSLM that were not well-separated, and would have resulted in an underestimation of cell numbers by TVC's. The qPCR method depends on disruption of thick fungal cell walls that are not easily lysed (Cryer *et al.*, 1976). Therefore, qPCR may also have underestimated *C. albicans* cell numbers. For *L. casei* and *S. mutans,* higher cell counts were obtained by qPCR than by TVC's. A previous study also found greater sensitivity of qPCR for enumeration of *S. mutans*, and >10-fold higher counts estimated by qPCR than by TVC's (Childers et al., 2011). It is likely that qPCR gives higher counts due to the inclusion of DNA from non-viable cells.

7.1.4 Research techniques

Many experimental techniques have been used and developed during the course of the study. For example, to overcome the blurred images obtained from CLSM, a cover slip with O ring was adapted over biofilm surface and enhanced biofilm visualization. Custom made plastic moulds were designed in dental material laboratory to produce stubs of the correct dimension to fit the MRDs. A homogenous mixing procedure using

Rotomix (adapting (Zahroon, 2014) technique) has been modified and applied successfully to prepare MRD stubs. This technique was an important development because the rate of fluoride release was more constant per time measurement than with hand mixing. The biofilm model system was developed by adjusting the sucrose ratio to facilitate microbial growth. Initially, biofilms were cultured on non-fluoridated copolymer in artificial saliva medium, supplemented with sucrose three times daily to mimic meals. In monoculture, *C. albicans* and *L. casei* grew well in the model and formed diffuse biofilms on saliva-conditioned copolymer (Chapter five). However, it was not possible to develop reproducible *S. mutans* biofilms under these conditions. To enhance the growth of *S. mutans*, 1% (w/v) sucrose (Sigma-Aldrich) was introduced continuously with the artificial saliva. Here, *S. mutans* grew well and formed biofilms consisting of aggregated microcolonies. Although experiments with single species had indicated that supplementation of artificial saliva with 1% (w/v) sucrose was required for *S. mutans* growth, in mixed-species cultures this concentration of sucrose led to persistent blockage of the tubing by extracellular polymers. Therefore, the concentration of sucrose was reduced to 0.1% for mixed-species cultures.

Having started the PhD as a prosthodontist many microbiological, biomaterials and molecular biological research techniques have been acquired during this study.

Chapter 8 . Conclusions and Suggestions

8.1 Conclusions

The research in this thesis has made novel contributions to the development of a fluoride releasing copolymer material that has impact on the oral biofilms related diseases. The aims of this study were to investigate the developed copolymer properties (fluoride release, chemical composition and roughness) and develop a model system to quantify single and mixed species biofilm formation on the material surface). The output of this study displayed the following results:

-Chemically cured copolymer material was developed with a sustained amount of fluoride release that continued for at least six months and offers the possibility of recharging.

-Autoclaving of the material has no effect on the chemical composition of the material, but a small increase in roughness was noticed in the fluoridated copolymer after autoclaving. It could be caused by the breakdown of the surface layer, vaporization of the residual monomer or fluoride leaching out from the material due to the high temperature reached during sterilization. SEM images showed small holes on the sample surface of fluoridated material after autoclaving.

-A model system has been developed for culturing single- and multiple-species biofilms which can be used for future studies on antimicrobial properties of different acrylic resin materials.

-During model development, the sucrose concentration was adapted along the experiments to optimise microorganisms growth for mixed species biofilms. Therefore, this study is not in a position to compare between single and mixed species biofilms as the conditions applied were not similar.

- Quantification of *S. mutans and L. casei* cells was 2-3 orders of magnitude higher by qPCR than by TVC's while no difference was found with *C. albicans*.

-Biofilm inhibition was apparent for mixed-species cultures but not for single-species biofilms, indicating that fluoride may interfere with microbial cell-cell interactions. This work demonstrates the potential for the fluoride-releasing material to control complex oral biofilms and provides the foundations for future clinical investigations.

8.2 Suggestions

Based on the results of this research, an experimental fluoridated acrylic resin was developed for the use of lining material. This material offers potential for clinical applications where control of acidogenic biofilms is important, for example denture liners, obturators and surgical coverplates. However further investigations are still required, as follows:

-Studying other mechanical and physical properties of the material with a chance of adding other cross linking agents to improve material properties.

-Studying the incorporation of other fluoride containing filler particles (CaF_2, SnF_2, Al_2F_3, pre-reacted glass ionomer fillers…ets) on material properties and its impact on biofilm growth.

-Measuring the amount of residual monomer in the material and trying to use other types of polymers such as heat cured or light cured PMMA to increase the degree of polymerisation.

- Measuring the roughness of the MRD samples after 6, 24 and 48 h to investigate the relation of the biofilm formation with the roughness.

-Find an alternative method of samples sterilization that has no effect on material properties.

-Studying material capability of fluoride release for an extended period of time with an attempt to studying material property of recharging.

-Reduce the flow conditions to a rate that mimics the nature of the oral cavity which can also decrease the leaching out rate of fluoride from the material

-Storage media such as natural or artificial saliva could be utilized to study the fluoride release behaviour in conditions similar or closer of the oral environment.

-Studying a lower concentration of NaF incorporated in the material on biofilms growth, considering lower amount of NaF can enhance material properties.

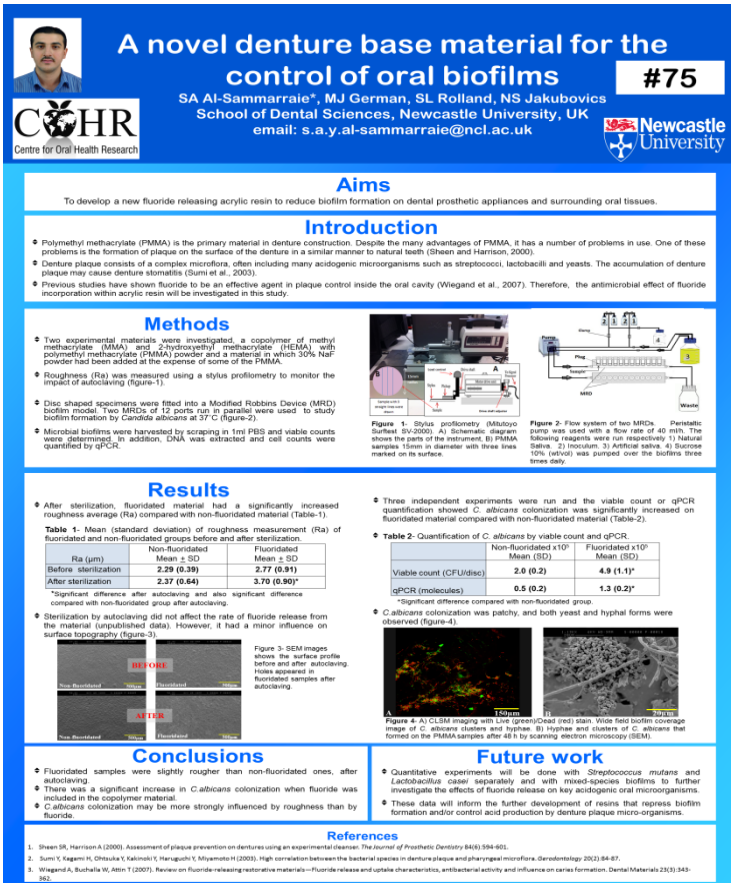
- Studying NaF effects on other microbial species that are frequently found in oral appliances.

-Standardise the sucrose concentration to be applied on single and mixed species biofilms.

-Using the model to study fluoride biofilm interaction for a period longer than 48 h with a possibility of flushing microbial cells continuously but in lower amount to more closely mimic the nature of the oral environment.

Appendix: Conference Attendance

9 September 2013: British Society for Oral and Dental Research 2013, bath (Poster presentation)



Title: A novel denture base material for the control of oral biofilms.

Objectives: Denture plaque consists of a complex microflora, often including many acidogenic microorganisms such as streptococci, lactobacilli and yeasts. The accumulation of denture plaque may cause denture stomatitis and caries in the adjacent teeth. This study aims to assess the efficacy in reducing biofilm formation of a new fluoride-releasing acrylic resin for potential use as a denture base.

Methods: Two experimental materials were investigated, a copolymer of methyl methacrylate (MMA) and 2-hydroxyethyl methacrylate (HEMA) with polymethyl

methacrylate (PMMA) powder and a material in which 30% NaF powder had been added at the expense of some of the PMMA. Disc shaped specimens were then fitted into a Modified Robbins Device (MRD) biofilm model. Two Polysulfone MRDs run in parallel were used to study monospecies biofilm formation by *Candida albicans*. After 48 h, biofilms were harvested and total viable counts were determined. In addition cell counts were quantified by qPCR. Roughness (R_a) was measured using a stylus profilometer.

Results: The fluoride-containing specimens exhibited a significant increase in colonisation by *C. albicans* ((P<0.05), table below). These specimens were found to be singificantly rougher (R_a) than the non-fluoride containing specimens ((P<0.05), table below).

Method	Groups	
	Non fluoridated Mean + SD	Fluoridated Mean + SD
v.quant (CFU/disc)	$2.7x10^5$ ($1.2x10^5$)	$6.8x10^5$ ($3.6x10^5$)*
qPCR (molecules/disc)	$1.2x10^5$ ($1.3x10^5$)	$2.6x10^5$ ($1.7 x10^5$)*
($R_{a)}$ µm	2.37 (0.64)	3.699 (0.9)*

* Significantly difference compared with non fluoridated group

Conclusion: This work has established a model for testing the propagation of oral biofilms on acrylic materials. There was an increase in colonization of *C. albicans* when fluoride was included. While this increase may be due to the increased roughness of these specimens, the inclusion of fluoride may not be directly inhibitory to all denture plaque micro-organisms.

Appendix

18 March2014: American Society for Oral and Dental Research 2014, Charlotte (Oral presentation)

Title: Oral biofilm growth on fluoridated denture material

Objectives: Denture plaque is an oral biofilm that can lead to caries on adjacent teeth or oral candiasis. These conditions are caused by aciduric microorganisms within the biofilm, including mutans streptococci, lactobacilli, and *Candida albicans*. Here, we assessed the efficacy of a new fluoride-releasing acrylic resin for reducing biofilm formation on oral appliances.

Methods: Discs of a copolymer of methyl methacrylate and 2-hydroxyethyl methacrylate with polymethyl methacrylate powder were produced. For one group, 30% NaF powder was added at the expense of some of the PMMA. Samples were fitted into two polysulfone Modified Robbins Devices. Single species biofilm of *C. albicans, S. mutans* and *L. casei* were cultured (48h), harvested, and microorganisms were quantified by total viable counts (TVC's) and quantitative PCR (qPCR).

Results: There were no signficant differences between *C. albicans, S. mutans* and *L. casei* levels on non-fluoridated or fluoridated surfaces (Mann-Whitney U Test $p>0.05$, table below). In general, much higher cell counts were obtained by qPCR than TVC's, possibly due to the quantification of non-viable microorganisms. Patchy and clumpy thick biofilms were formed by these microorganisms.

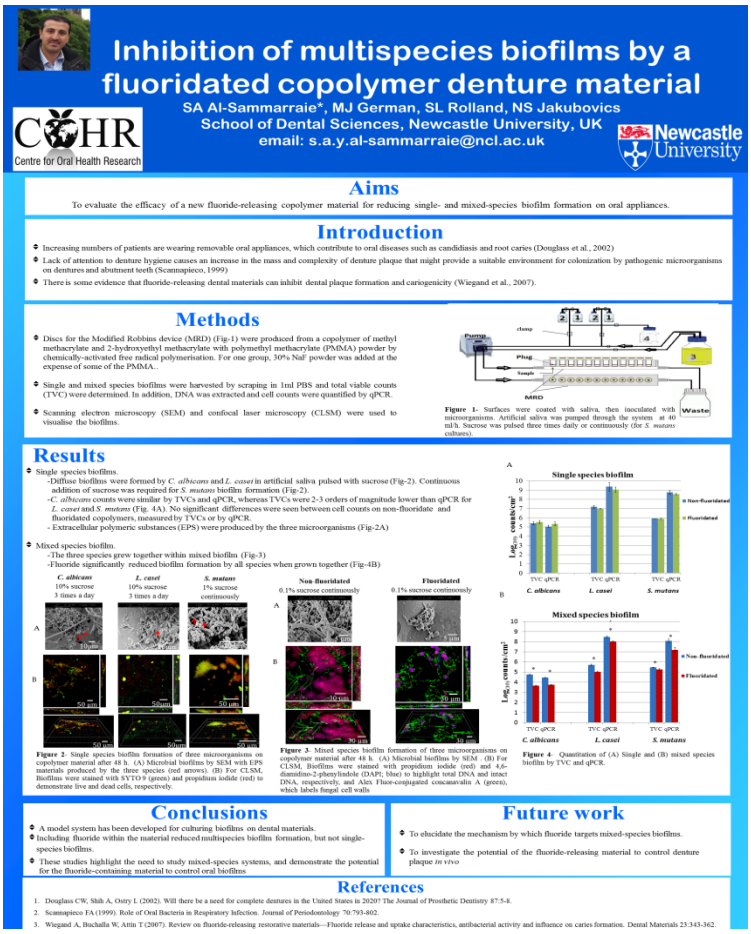
Appendix

		Non-fluoridated Median (Interq. Range)	Fluoridated Median (Interq. Range)
C. albicans	TVC's (CFU/disc)	$3.7x10^5$ ($5.7x10^4$)	$4.1x10^5$ ($1.3x10^5$)
	qPCR (cell/disc)	$1.8x10^5$ ($2.2x10^4$)	$1.9x10^5$ ($7.4x10^4$)
S. mutans	TVC's (CFU/disc)	$7.5x10^5$ ($3x10^5$)	$6.8x10^5$ ($9x10^4$)
	qPCR (cell /disc)	$3.7x10^8$ ($1x10^8$)	$3.4x10^8$ ($2.3x10^8$)
L. casei	TVC's (CFU/disc)	$4.5x10^5$ ($4x10^4$)	$2.9x10^5$ ($2.5x10^5$)
	qPCR (cell /disc)	$9x10^7$ ($3.3x10^7$)	$7.6x10^7$ ($2.9x10^7$)

Conclusions: A model system has been developed for culturing single- and multiple-species biofilms on dental materials. Our initial studies did not reveal an impact of incorporating fluoride into acrylic resin on growth of monospecies biofilms. The model will now be exploited to study the effects of including fluoride in the substratum on acid production by mixed-species oral biofilms.

Appendix

11 May2014: Biofilm 6 conference 2014, Vienna (Poster presentation)



Title: Inhibition of multispecies biofilms by a fluoridated copolymer denture material

Objectives: Increasing numbers of patients are wearing removable oral appliances, which contribute to increase of oral diseases such as candidiasis and root caries. Fluoride is one of the most effective agents for caries control. In this study, we evaluated the efficacy of a new fluoride-releasing copolymer material for reducing single- and mixed-species biofilm formation on oral appliances.

Methods: Discs of a copolymer of methyl methacrylate and 2-hydroxyethyl methacrylate with polymethyl methacrylate powder were produced by chemically-activated free radical polymerisation. For one group, 30% NaF powder was added at the expense of some of the PMMA. Samples were fitted into two polysulfone Modified

Robbins Devices. Single- and mixed-species biofilms of *Candida albicans, Streptococcus mutans* and *Lactobacillus casei* were cultured for 48h, harvested, and microorganisms were quantified by total viable counts (TVC's) and quantitative PCR (qPCR). Scanning electron microscopy and confocal laser microscopy were used to visualise the biofilms.

Results: The inclusion of fluoride within the copolymer resulted in significantly reduced colonisation by *C. albicans, S. mutans* and *L. casei* in mixed-species biofilms (T Test, $p<0.05$). However, when microorganisms were grown in single-species biofilms, all the three organisms were not significantly (T Test, $p>0.05$) reduced by fluoride. In mixed species colonization, thick biofilms were formed by these microorganisms on non-fluoridated samples while small patchy biofilms were found on fluoridated samples.

Conclusions: A model system has been developed for culturing single- and multiple-species biofilms on dental materials. The inclusion of fluoride within the material resulted in a reduction in multispecies biofilm formation, but not in single-species biofilms. These studies highlight the need to study mixed-species systems, and demonstrate the potential for the fluoride-containing material against complex oral biofilms. Further work will aim to elucidate the mechanism by which fluoride specifically targets mixed-species biofilms.

10 September 2014: International Society for Oral and Dental Research/ PER congress 2014, Dubrovnik (Oral presentation)

Title: Effect of fluoride containing copolymer on multispecies biofilm reduction

Objectives: Polymethyl methacrylate (PMMA) is a widely used denture base material. This material continues to be used as it has good working and esthetic characteristics. Despite its clinical success, it has a number of problems in use such as plaque formation on the surface of the denture. In this study, we evaluated the efficacy of a

Appendix

new fluoride-releasing copolymer material for reducing mixed-species biofilm formation on oral appliances.

Methods: Discs of a copolymer of methyl methacrylate and 2-hydroxyethyl methacrylate with PMMA powder were produced by chemically-activated free radical polymerisation. For one group, 30% NaF powder was added at the expense of some of the PMMA. Samples were fitted into two polysulfone Modified Robbins Devices. Mixed-species biofilms of *Candida albicans, Streptococcus mutans* and *Lactobacillus casei* were cultured for 48h, harvested, and microorganisms were quantified by total viable counts (TVC's) and quantitative PCR (qPCR). The pH of both groups was monitored during biofilm formation and fluoride release from the material was measured for six months.

Results: The inclusion of fluoride within the copolymer significantly reduced colonisation by *C. albicans, S. mutans* and *L. casei* in mixed-species biofilms (T Test, $p<0.05$). Fluoride suppressed the acidogenicity of biofilms for up to 24 h. High rates of fluoride release (>300 μg/cm2/day) were sustained for 6 days, after which low levels of fluoride were released for 6 months.

Conclusions: A model system has been developed for multiple-species biofilms on dental materials. The inclusion of fluoride within a copolymer resulted in a reduction in multispecies biofilm formation. This model will now be exploited to look at the effects of the fluoride-releasing material on biofilms formed by the natural microbiota of human denture plaque.

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