DENTAL BIOFILM FORMATION IN YOUNG CHILDREN AND THE LONG TERM EFFECTS OF FLUORIDE VARNISH.

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approximately pH 5.5, the average enamel demineralisation threshold.

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List of Abbreviations

2PG.	2-phosphoglycerate.
3PG.	3-phosphoglycerate.
ADP.	Adenosine diphosphate.
ADS.	Arginine deiminase system.
ANOVA.	Analysis of variance.
ATP.	Adenosine triphosphate.
BEH.	Behavioural intervention group.
BL.	Baseline.
BS.	Bright smiles.
CDC.	Centers for disease control and prevention.
CEJ.	Cemento enamel junction.
CFU.	Colony forming units.

CON.	Control intervention group.
CRB.	Criminal records bureau.
CRN.	Clinical research network.
CST.	College of Science and Technology (University of Salford).
CV.	Curriculum vitae.
DBS.	Disclosure and barring service.
DEJ.	Dento-enamel junction.
Demin.	Demineralisation.
DNA.	Deoxyribonucleic acid.
ECC.	Early childhood caries.
EPS.	Exopolysaccharide.
ES.	Excess saliva.
ESEM.	Environmental scanning electron microscopy.
F.	Fluoride.
FDA.	Food and Drug Administration.
FLU.	Fluoride intervention group.
FP.	Fluor protector.
FTF.	Fructosyltransferases.
GCP.	Good clinical practice.
GLM.	General linear model.
GM.	Gingival margin.
GSK.	Glaxosmithkline.
GTF.	Glucosyltransferases.
HA.	Hydroxyapatite.
HF.	Hydrogen fluoride.
HI.	High pH.
HOMIM.	Human oral microbe identification microarray.
IC.	Informed consent.
IPA.	Intercellular polysaccharide adhesin.
IRAS.	Integrated research application system.
KWT.	Kruskal Wallis test.
Low.	Low pH.
LS.	Isohelix DNA isolation Reagent (unconfirmed composition).
MED.	Median pH.
MRDe.	Modified Robbins device.
MRD.	Maximum recovery diluent.
MS.	Mutans streptococci.
MWU.	Mann Whitney U test.
NCIMB.	National Collection of Industrial, Marine and Food Bacteria.
NHS.	National health service.
NIHR.	National institute for health research and clinical research network.
NRES.	National research ethics service.
OD.	Optical density.
OH.	Hydroxyl.

PBS.   Phosphate buffered saline.
PCR.   Polymerase chain reaction.
PEP.   Phosphoenolpyruvate.
PGRM.   Plaque Glycolysis regrowth Method.
PIL.   Patient information leaflet.
PK.   Isohelix DNA isolation reagent (unconfirmed composition).
PRP.   Prolein rich proteins.
PVC.   Polysulfone, polyvinyl chloride.
Remin.   Remineralisation.
RNA.   Ribonucleic acid.
SA.   *Staphylococcus aureus*.
SAD.   Surface attachment defective.
SEM.   Scanning electron microscopy.
TE.   Tris Ethylenediaminetetraacetic acid.
TEM.   Transmission electron microscopy.
UK.   United Kingdom.
UK.   Unknown.
US.   United States.
USA.   United States of America.
VS.   Viridans streptococci.

Abstract

Introduction: Fluoride is widely used as a method of reducing caries and while much is known about the processes involved in enamel remineralisation the effects of fluoride on indigenous biofilms *in vivo* are unclear. **Aims:** The aim of this study was to combine data specifying the distribution of oral bacteria derived from clinical samples with *in vitro* observations in order to define the effects of improved behaviour and the use of fluoride varnish on the development and metabolic activity of oral biofilms. **Methods:** 30 children (aged approximately 12 months) were divided into three groups. The Behavioural group were instructed in improved dental hygiene (e.g. brushing and reduced sugar intake), the Fluoride group received biannual applications of fluoride varnish (Fluor Protector, 'FP', 0.1% fluoride) and the Control group received the standard NHS dental service. Over two years the bacterial composition of clinical samples (produced by the Human Oral Microbe Identification microarray, 'HOMIM') was compared to similar data generated from an *in vitro* study (utilising the Plaque Glycolysis Regrowth Method, 'PGRM') in order to describe the effects of FP on the composition and metabolic activity of oral biofilms. **Results:** *In vitro* the PGRM study indicated no difference in the acidogenicity between FP treated and untreated biofilms ($P = 0.96$) however, a significant increase in richness was observed in the presence of FP ($P = 0.048$). Similarly, samples collected from children, which were highly comparable at Baseline ($P > 0.05$), indicated a significant drop in the number of species identified in the Control group when compared to the Fluoride and the Behavioural groups ($P < 0.05$) by age 3. **Conclusions:** Results indicate that fluoride provides a homeostatic effect which promotes increased diversity in oral biofilms, a feature which has been associated with oral health.

1.   Introduction.

1.1.   The properties of teeth and enamel.

Typically, humans will experience the eruption of two sets of teeth during a lifetime. The initial shedding teeth are known as the deciduous (primary, baby or milk) set while the second, permanent dentition are known as the succedaneous (adult) set. Approximately twenty deciduous teeth will erupt between the ages of six to twenty-four months; ten in the maxillary (upper) jaw and ten in the mandibular (lower) jaw (Nelson, 2009). These teeth will remain in place until they are gradually replaced by the eruption of the succedaneous set, between the ages of six and twelve years; 32 teeth in total, 16 in each jaw (Nelson, 2009).

The human tooth is a multifaceted structure, composed of two visibly indistinct, yet characteristically different sections that exist above and below the gingival margin (GM). The crown is the visible portion which erupts through the GM, into the oral cavity and is separated from the root at the cemento-enamel junction (CEJ; Figure 1). The root (unless exposed by gingival recession) remains below the GM, via the CEJ and secures the tooth into the jaw.
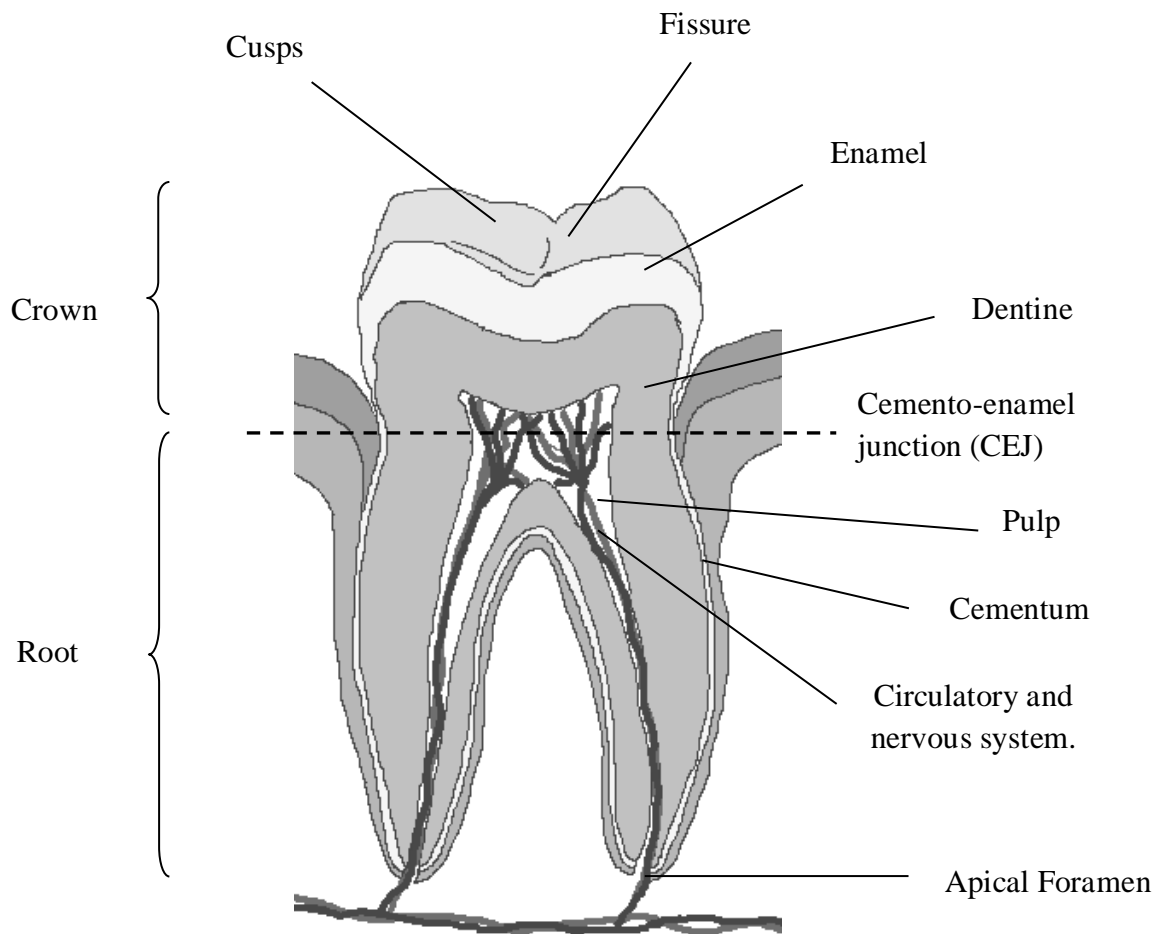
Figure 1. Schematic of a human molar and the basic tooth structures, adapted from Grassi and DeAngelo (2012) and Levison (2004).

The crown of the succedaneous molar extends into the oral cavity from the CEJ, to a ridged cusp which surrounds an aperture, also known as the 'fissure'. Below the CEJ, the root is encased in a bone-like protective coating known as cementum. Beneath the cementum, the dentine surrounds the core of the tooth, the pulp, which is a soft tissue through which the circulatory and nervous systems flow, via the pulp canals at the apical foramen (Garg & Garg, 2010; Grassi & DeAngelo, 2012; Levison, 2004).

Approximately 85% of enamel is comprised of highly organised, tightly packed crystalline rods of 'hydroxyapatite' (HA; $Ca_{10}(PO_4)_6(OH)_2$). These rods are enveloped in a mixture of water and organic matter which make up to 12% and 3% respectively of the remainder (de Sant'Anna *et al.*, 2009; Simmer & Hu, 2001).

Enamel formation (amelogenesis) is a highly complex process that occurs simultaneously with dentine production, along the border that will become the dentino-enamel junction (DEJ); an interface between the dentine, which surrounds the root, and the enamel coated crown (Simmer & Hu, 2001). The cells responsible for enamel formation, 'ameloblasts', coat the mineralisation front and express and secrete a number of matrix proteins and enzymes (e.g. amelogenins) which cluster together like scaffolding, promoting the structural integrity of longitudinal crystalline growth during mineral deposition. This prevents the thickening and fusion of adjacent rods while promoting longitudinal growth (Avery *et al.*, 2002; Mihu *et al.*, 2011; Simmer & Hu, 2001; Wakida *et al.*, 1999). The ameloblast 'membrane' will coat the enamel surface until the tooth erupts into the oral cavity where it is shed and lost. This renders the host insensitive to minor enamel damage but also prevents enamel regeneration in the event of decay (Mihu *et al.*, 2011; Nanci, 2008; Simmer & Hu, 2001).

At a genetically predetermined point ameloblasts begin to reduce the secretion of matrix proteins in favour of proteinases which degrade the structural amelogenins, causing the arrest of crystal rod elongation while subsequently accelerating their thickening; this is referred to as the enamel maturation stage. Ameloblasts then begin transporting phosphate, calcium and bicarbonate ions into the matrix, causing the gradual thickening and hardening of the enamel (Simmer & Hu, 2001; Wakida *et al.*, 1999).

Enamel is the hardest tissue in the vertebrate body; its strength and capacity to withstand repeated masticatory forces is due to the combination of tough, crystalline HA rods, and the dampening properties of water combined with an organic matrix (Simmer & Hu, 2001). The

HA rods branch out in a similar general but dissimilar local orientation which is thought to reduce the potential for fracture (Mihu *et al.*, 2011). This is reinforced by the presence of water and the organic components which act to cushion against impact (Baldassarri *et al.*, 2008; Maas & Dumont, 1999; Spears, 1997). However, organic inclusions are also much more susceptible to erosion and loss and therefore, exposing the internal HA structure to the surrounding oral environment promotes a greater potential for cavity formation (Lussi, 2006).

Tooth enamel is subject to numerous forms of damage including erosion, abrasion, attrition and dental caries (Dawes, 2008). Erosion is the loss of enamel via extrinsic processes, i.e. processes not linked to bacterial activity (Cheng *et al.*, 2009). These processes include the consumption of acidic foods (e.g. citrus fruit), medications (e.g. aspirin) or the effects of stomach acids during emesis (vomiting) or regurgitation (Dawes, 2008; Scheutzel, 1996; Zero, 1996). Abrasion is the physical loss of enamel by hard materials introduced with, though generally not associated with, food type (Dawes, 2008). Enamel abrasion is generally linked to habits such as aggressive brushing (Habsha, 1999; Wiegand *et al.*, 2008). Attrition is the wear of enamel by contact with opposing or adjacent teeth and is generally associated with 'bruxism', the grinding of teeth (Dawes, 2008; Taji & Seow, 2010). Finally, caries is the loss of enamel due to repeated and prolonged acidification of oral bacterial biofilms (Arora *et al.*, 2011); this will be discussed in greater detail later.

In each of these cases, prolonged damage to the enamel can lead to discomfort, pain and loss of teeth. However, the presence of saliva provides a protective action, slowing the dissolution and loss of dental enamel (Dawes, 2008).

1.2.   Saliva and the salivary pellicle.

The salivary system is comprised of hundreds of secretary organs embedded within the tissues of the oral cavity which are collectively referred to as the major and minor salivary systems (Holsinger & Bui, 2011; Schenkels *et al.*, 1995). The production of saliva is controlled by the autonomic nervous system which typically functions at the subconscious level; other physiological systems regulated in this way include respiration, blood pressure, metabolism and body temperature (Brown *et al.*, 2006; Holsinger & Bui, 2011; Lamont & Jenkinson, 2010; Sherwood, 2008).

The host will produce between approximately 500 ml and 1500 ml of saliva per day, depending upon diet and health. Un-stimulated flow ranges between $0.3 - 0.4$ ml/min^{-1} whereas stimulated (i.e. stimulated by food consumption or hunger) will have a much greater variation in flow rates depending on the type of food being chewed. An example of flow rates as stimulated by specific food types (as described in the literature) include eating rice or rhubarb pie which induce approximately $3 - 5$ ml/min^{-1} of flow whereas up to 7 ml/min^{-1} flow may be observed after consuming citrus fruit (Dawes, 2008; Marsh, 2003; Watanabe & Dawes, 1988).

Approximately 99.5% of saliva is water which is combined with approximately 0.2% inorganic materials such as bicarbonate, phosphate, calcium, sodium and potassium and 0.3% organic materials including mucins, α-amylase, amino acids and host and bacterially derived cellular fragments, including proteins, RNA and DNA (Lima *et al.*, 2010; Palanisamy & Wong, 2010; Park *et al.*, 2006; Samaranayake, 2006). Like many mucosal fluids, saliva acts to coat and protect vulnerable surface tissues within the oral cavity. Here, saliva is a major homeostatic component and associated with lubrication, digestion and immunity (Holsinger & Bui, 2011; Samaranayake, 2006). Highly abundant salivary mucins promote viscosity, aiding prolonged surface coverage, lubrication and prevention from desiccation (Dawes, 2008;

Tabak *et al.*, 1982; Talwar & Srivastava, 2004). Additionally, saliva aids in the protection of surface tissues via mechanisms which include the buffering of food and drink temperatures, the clearance of non-adherent cells from the mucosal surface and the delivery and distribution of immunological components to the surface (Dawes, 2008; Schenkels *et al.*, 1995).

In the oral cavity, the continuous turn-over of fresh saliva coats the exposed surfaces of the mouth and throat with a transient layer of water and organic materials forming a conditioning film (also known as the salivary pellicle) that ranges in thickness from between 100 and 1000nm, depending upon the volumes of saliva available and the shear-stresses which are unique to the location in the mouth (Lamont & Jenkinson, 2010; Lendenmann *et al.*, 2000; Reddy, 2008). The pellicle was first examined in 1839 by Alexander Nasmyth during a detailed analysis of teeth from numerous animal species, including those of humans. Nasmyth witnessed granular patterns across the enamel surface which he deduced as being an embryonic capsule or membrane (Nasmyth, 1839). He referred to this membrane as the 'persistent dental capsule' noting that it was likely to be present on the surfaces of all teeth from birth, gradually receding and lost due to physical wear. Muller, another researcher of the day hypothesised that the pellicle was not a membrane but instead, precipitated from the saliva (Muller, 1838). This observation was confirmed in 1849 when it was noted that 'Nasmyths membrane' could also be observed on abraded teeth, suggesting that the membrane could not be embryonic in nature (Frank, 1849; Lendenmann *et al.*, 2000). In 1963 the connection between these observations was made and it was concluded that Nasmyth's membrane was derived from the host saliva and has since been known as the acquired enamel pellicle (Dawes *et al.*, 1963; Dawes, 1968; Lendenmann *et al.*, 2000).

At the tooth surface, the salivary pellicle plays a major role in protecting the enamel from demineralisation, particularly by the dilution and neutralisation (buffering) of corrosive substances, while also providing a reservoir of elementary HA-derived particles that are

essential for enamel remineralisation. These include ionic calcium, phosphates and fluoride (Dodds *et al.*, 2005; Järvinen *et al.*, 1991; Lussi & Schaffner, 2000; Lussi & Jaeggi, 2008; Zero & Lussi, 2005). However, as previously noted, the pellicle is not evenly distributed throughout the dentition and so the protection provided can be highly variable.

Variations in pellicle distribution and accumulation across the entire dentition is associated with the availability and flow rate of saliva which can vary significantly with age (Amaechi *et al.*, 1999; Järvinen *et al.*, 1991; Navazesh *et al.*, 1992; Percival *et al.*, 1994; Rytömaa *et al.*, 1998). Amaechi *et al.* observed that the accumulation of the saliva at the tooth surface is largely a local phenomenon and is often dictated by mechanical abrasions from the adjacent tissues (such as the tongue) or friction encountered during mastication (Amaechi *et al.*, 1999). Amaechi's work indicated that the greatest accumulation of conditioning saliva on enamel was observed throughout the lingual surfaces (mandibular jaw, surrounding the tongue) which are constantly bathed in fresh saliva and are in semi-permanent contact with the non-keratinised, under-surface of the tongue. In contrast, the thinnest accumulations of saliva were observed on the palatal surfaces of the maxillary jaw, where surfaces do not receive the same volumes of fresh saliva. Furthermore, the saliva which does accumulate on the palatal surfaces is in constant contact with (and rapidly removed by) the abrasive actions of the keratinised, dorsal surface of the tongue. The same is true for the buccal (opposite the cheek) and labial surfaces (behind the lips) which are subjected to constant mechanical shearing from the surrounding tissues (Amaechi *et al.*, 1999).

Saliva-borne macromolecules undergo rapid adsorption onto the saliva-coated enamel surface in a process which begins with ionic interactions between the negatively charged phosphates in the HA and the positively charged proteinaceous molecules. All subsequent accumulation is discriminatory and based on protein-protein interactions (Demuth & Lamont, 2006;

Kolenbrander *et al.*, 2002; Marsh, 2003; Reddy, 2008; Samaranayake, 2006; Schüpbach *et al.*, 1996).

The flow and accretion of saliva to the enamel is of major significance, not only for the reasons described above, but also because it acts as the primary mechanism for the delivery of bacteria to the tooth. Bacteria are often highly receptor-specific and so early colonisation and accumulation is generally associated with specific interactions between colonising species. During pellicle colonisation species will bind to specific receptors (including, but not limited to, highly abundant α-amylases and mucins) within the pellicle (and often, to the adjacent cell) in a process known as aggregation and co-aggregation (respectively). This provides further substrate for subsequent binding of additional species (mid and late colonisers) during biofilm development (Demuth & Lamont, 2006; Kolenbrander *et al.*, 2002).

The succedaneous tooth is the only non-shedding part of the body which is freely colonised by bacteria. The particulate organic components of saliva provide a highly variable substrate for the localised bacterial colonisation; the foundations of biofilm development (Anon., 1999; Bos *et al.*, 1996; Frandsen *et al.*, 1991; Grassi & DeAngelo, 2012; Kolenbrander, 1988; Kolenbrander *et al.*, 2002; Marsh, 2003; Samaranayake, 2006).

1.3. Biofilms.

Biofilms are aggregations of micro-colonies of similar and/or dissimilar bacterial species, irreversibly associated to a molecularly-conditioned surface or interface, including between water and air, or existing planktonically in raft-like aggregates and enclosed in a polysaccharide matrix (Costerton *et al.*, 1995; Donlan & Costerton, 2002; O'Toole *et al.*, 2000a; Palmer & White, 1997). Single species biofilms are thought to exist only under strictly controlled laboratory conditions. In nature, biofilms are complex arrangements of different

species, derived from the immediate environment and existing as a mutually-beneficial collective (Palmer & White, 1997). In addition to these highly complex and interactive bacterial communities, the biofilm is also home to viruses and eukaryotic organisms such as fungi and protists (e.g. algae) and when the biofilm exists in association with another organism (e.g. the human gut or oral cavity), additional components will include host-derived cells, proteins and DNA fragments and food products (Palmer & White, 1997).

One of the earliest acknowledgments of the existence of biofilms was by Arthur Henrici (1933), who observed that fresh water bacterial life existed predominantly, not in a planktonic suspension but instead, as surface-associated colonies encapsulated in a 'slime' matrix.

Later ZoBell and Anderson (1936) observed that the hard surface areas of a given container did not increase in parallel with the volume of water it stored and that relatively fewer cells were obtained from increasingly larger units of stored seawater. This led to the conclusion that harvested bacteria came, predominantly, from the inner surfaces of the storage container rather than from planktonic suspension. It is now generally acknowledged that sessile, surface-associated biofilms over planktonic existence is the predominant form of bacterial life in nature (Palmer & White, 1997; Pratt & Kolter, 1998; Trémoulet *et al.*, 2002).

Biofilm 'development' is a continuum (Palmer & White, 1997). To use the word 'development' may generate the impression that before biofilm initiation the colonised substrate was free of bacterial inhabitants. In nature, this is highly unlikely. Any surface suitable for bacterial colonisation in the presence of moisture, nutrients and suitable shelter from the immediate environment will be subject to continuous periods of colonisation, partial clearance and recolonisation. This means that the observed niche will also undergo continuous physiochemical changes that initiate colonisation signals within differing organisms that prefer that temporal, physiochemical niche (Renner & Weibel, 2011).

The only environments which are not subject to this rule are those which are physically cleansed by natural events occurring within the environment (e.g. short term exposure to high temperatures), and those subject to chemical cleansing by human activity. These would result in a 'new start' for the surface, which, providing the physiochemical conditions of its immediate environment are not drastically changed, will be subject to repeated colonisation by similar organisms interacting with repeated exposure to a molecularly homologous conditioning film.

Although initiation signals for biofilm formation are known to vary among species they are generally considered to begin when changes in environmental conditions trigger the abandonment of planktonic life in favour of a nutritionally beneficial sessile existence (O'Toole *et al.*, 2000a; O'Toole & Kolter, 1998; O'Toole *et al.*, 2000b; Pratt & Kolter, 1998; Stoodley *et al.*, 1998; Wimpenny & Colasanti, 1997). Biofilm development and maturation is highly complex, such that it is no longer regarded simply as colonies but more like multicellular organisms (Costerton *et al.*, 1995).

1.3.1. Biofilm initiation signals.

Numerous reviews (Palmer & White, 1997; Stoodley *et al.*, 2002; Trulear & Characklis, 1982) have attempted to outline a universal developmental process however the complexity of biofilm formation is dictated by a potentially limitless combination of parameters making an all-encompassing model somewhat unachievable. The parameters which dictate biofilm formation not only include the microbial composition and rate of cellular replenishment from the immediate environment but also the cumulative effect of all physiochemical properties including the local hydrodynamics, the level of moisture and osmolality, nutrient availability, pH and temperature (Lawrence *et al.*, 1991; Percival *et al.*, 2011; Pringle & Fletcher, 1986; Robinson *et al.*, 1984; Stoodley *et al.*, 1998; Wimpenny & Colasanti, 1997).

1.3.1.1. Hydrodynamics.

The local hydrodynamics describes the dynamic nature (current, speed and direction) of the bulk, aquatic phase to which a biofilm may be exposed. Within an aquatic environment, such as saliva, river water or a marine environment, biofilms are subject to natural hydrodynamics that not only impose 'drag' and 'shear' but also the benefits of colony transport, relocation and nutrient delivery (Stoodley *et al.*, 1998). Within a prevailing current, the colonising cell needs to be capable of surface attachment in the first instance but also remain attached against the hydrodynamic forces. This is particularly true for species associated with colonisation of the urinary tract which is regularly exposed to rapid bursts hydrodynamic forces. An example of bacterial traits which have overcome hydrodynamic forces include some *Escherichia coli*, known to be responsible for up to 80% of urinary infections which utilise fimbriae to adhere to the urinary epithelia (Schembri & Klemm, 2001). Fimbriae are hair-like projections which extend from the surface of, mostly, Gram negative bacteria and provide an adhesive property permitting anchorage to mannose molecules exposed at the epithelial surface against the temporary passage of urine (Krogfelt *et al.*, 1990; Ofek *et al.*, 1977; Seltmann & Holst, 2013).

1.3.1.2. Osmolality.

Osmolality, the measure of solutes dissolved into a solvent (Koay & Walmsley, 1996), is vital for the formation of the conditioning film and a major factor in biofilm initiation (Donlan, 2002). In nature, abiotic surfaces are rarely sterile. Once exposed to moisture a surface is rapidly contaminated by molecular debris, derived from the immediate environment which rapidly adsorbs onto the surface, masking its original properties (Percival *et al.*, 2011; Pringle & Fletcher, 1986). However, surface conditioning is a highly complex process and is dependant, not only on the conditions of the immediate environment, but also on the characteristics of the surface including factors such as electrostatic charge (Krasowska &

Sigler, 2014). Reports have indicated that hydrophobic surfaces are more resistant to colonisation, due to a reduced interaction with the surrounding bulk phase solvent and therefore, the suspended micro and macro molecular particles which would condition that surface (Baier, 1981, Pringle & Fletcher, 1986). Conversely, hydrophilic surfaces were found to have no greater benefit to the bacteria during the attachment process, exceeding the primary condition that they are easier to hydrate (Pringle & Fletcher, 1986). However, over-hydration of a surface can also lead to reduced biofouling by early surface colonisers. In a review by Chen *et al*. (2010), it was shown that when a hydrophilic surface comes into contact with bulk water, the water molecules penetrate into the surfaces ultrastructure, forming hydrogen bonds with the surface polymers and preventing non-specific protein adsorption. This, it was suggested, can lead to reduced colonisation possibly due to the masking of the surface properties of the surface and reducing the potential for conditioning (Pringle & Fletcher, 1986). This would suggest that the conditioning film is defined by the properties of the surface. Therefore, assuming that the surface is relatively homogenous, it follows that the pellicle will be relatively similar with each renewed coating.

1.3.1.3. Nutrient availability.

Nutrient availability is a major factor of primary colonisation. Any environment with a significant limitation or over-abundance of one or more specific nutrients will set natural limitations to the niche and in so, the colonisation by certain species. For example, oral commensal *Streptococcus mutans*, widely considered the main protagonist in dental caries, metabolises sucrose into glucans and fructans which constitute the bulk of the exopolysaccharide (EPS) matrix (Kreth *et al*., 2008). However, in the absence of sucrose, Kreth *et al*. were able to show that *S. mutans* was out-competed by rival oral streptococci (*Streptococcus oralis, Streptococcus mitis, Streptococcus sobrinus, Streptococcus gordonii*),

and was unable to establish observable colonies. However, on the introduction of sucrose (at increasing concentrations 0.1% - 0.5%) *S. mutans* gained a clear advantage such that, after washing in PBS, competing microcolonies of streptococci were washed away leaving only *S. mutans* microcolonies attached to the substrate (Kreth *et al.*, 2008). In this case, the availability of sucrose provided *S. mutans* with a biochemical edge, improving its footing within the community while simultaneously destabilising its competitors.

Such interactions are not restricted to oral microbiota. In *E. coli,* it was found that flagella synthesis and the mediation of mechanisms essential for the export of EPS from the cell are linked to the availability of glucose in the environment. Bacterial 'base-pairing small-RNA' (sRNAs) are small (40 – 500 nt) molecules that regulate expression of target mRNAs and/or proteins by binding to specific sites, either pre or post-transcription, and blocking or increasing access to ribosomal binding sites (Cao *et al.*, 2010; Vogel & Wagner, 2007). In a 2012 study, Thomason *et al*. observed that an *E. coli* sRNA 'IS061/IsrA' was upregulated when glucose became limited. IS061/IsrA, which is upregulated during the stationary phase of the bacterial growth cycle, was also found to be linked to flagella synthesis which is known to be a major factor in biofilm formation for *E. coli* (Pratt & Kolter, 1998). In addition, IS061/IsrA was also found to regulate the synthesis of a porin which functions to export an adhesive polysaccharide, also associated with biofilm formation (Itoh *et al.*, 2005). Finally, Thomason and co-workers noted that down-regulation of IS061/IsrA resulted in poor biofilm formation whereas up-regulation improved biofilm stability indicating a direct association with nutrient-limited biofilm initiation (Thomason *et al.*, 2012).

In both examples, nutrient availability is indirectly linked to the overall architecture of the growing and maturing biofilm. There are three main structural models reported in the literature which describe generalisations regarding the 'wildtype' biofilm architecture. The 'penetrated water channel model' the 'mosaic model', and the 'dense confluent model' (Allan

et al., 2002; Evans, 2003; Keevil & Walker, 1992; Lawrence *et al.*, 1991; Nyvad & Fejerskov, 1989; Robinson *et al.*, 1984; Wimpenny & Colasanti, 1997).

The water channel model is perhaps the most widely accepted description and portrays the gradual succession of species attachment and with it, the expression of a shielding EPS which encapsulates the colonisers in mushroom-like towers. The continuous secretion of EPS leads to the development of stalks which gradually fuse, developing unoccupied channels which are thought to channel water, oxygen and nutrients into the structure while carrying away waste debris and metabolites (Lawrence *et al.*, 1991; Robinson *et al.*, 1984; Wimpenny & Colasanti, 1997).

The mosaic model, possibly the lesser known of the three was formulated by Keevil and Walker and describes stacks or towers of micro-colonies which are thought to interact with one-another but are metabolically separate from neighbouring stacks. At the base of the biofilm a thin confluent layer (approximately 5 µm thick) of cells coat the colonised surface. This basal film was observed to be grazed upon by protozoans which appeared to move between and around the stacks (Allan *et al.*, 2002; Evans, 2003; Keevil & Walker, 1992; Wimpenny & Colasanti, 1997) and may be a factor in their development. Finally, the 'dense', confluent conformation was described by Nyvad and Fejerskov as part of their observations of supra- and subgingival biofilm formation in the oral cavity (Nyvad & Fejerskov, 1989). In this study, transmission electron microscopy (TEM) was used to analyse biofilms from three different individuals and in each case the striking similarity was the absence of the water channels.

The link between these architectural observations, as proposed by Wimpenny and Colasant (1997) was the availability and replenishment of nutrients and subsequent production of EPS. Although over-simplified, it is not inaccurate to state that the rate and volume of EPS production is linked to the availability of the appropriate nutrient or nutrients (Chenier *et al.*,

2006; Costerton *et al.*, 1995; Sutherland, 2001). Wimpenny's review noted that highly channelled biofilms appear in nutrient-limited environments whereas the confluent dense structures are associated with much higher concentrations of nutrient replenishment. She describes the development of water channels as being the product of nutrient-associated limitations in EPS production. As microcolonies metabolise the available substrate they will continue to express the EPS envelope until the nutrient is exhausted. The channels which separate the microcolonies are therefore constructed by the incomplete connection of two adjacent EPS envelopes. When nutrients are not limited, such voids will be completely occupied thus preventing the development of such channels.

It worth noting that in Nyvad and Fejerskov's observations, specifically, the lack of channels in the dense biofilm model were debated as a possible by-product of dehydration steps associated with transmission electron microscopy (TEM) protocol which may have resulted in shrinkage and loss of observable channels (Cortizo & Lorenzo, 2007; Wood *et al.*, 2000). There have been numerous reviews of the suitability of different microscopy techniques published in the literature (Evans, 2003; Little *et al.*, 1991; Mukhopadhyay, 2003; Surman *et al.*, 1996; Sutton *et al.*, 1994) and while no one technique has been favoured over another, each method appears to lend some degree of suitability and restriction, depending upon the type of sample being studied (Mukhopadhyay, 2003).

Interestingly, Wimpenny & Colasanti (1997) cited three other works (Ganderton *et al.*, 1992; Stickler *et al.*, 1993a; Stickler *et al.*, 1993b) which described biofilms present on urinary catheters which were also observed as having no water channels. In each of these cases, biofilms were harvested from subdermal catheters associated with urinary infections, however, each of these findings were described after the use of SEM and TEM. Scanning electron microscopy (SEM), like TEM, requires a dehydration step prior to observation which may have resulted in the same fate as described by Nyvad (1989). However Sutton *et al*.

(1994) reviewed a number of microscopy techniques to assess their suitability for the viewing

biofilms. Here a 20 h Streptococcal biofilm was observed in a fully hydrated state, using

environmental scanning electron microscopy (ESEM), otherwise known as 'electroscan

imaging' and no pores or channels were observed. This ESEM technique allows the

observation of wet samples in their native state and without prior preparation (Donald, 2003;

Papthanasiou & Bardwell, 2001) which lends credit to the Nyvad (1989) observations. It must

be noted, however, that this is not a consistent finding as water channels have been observed

in oral biofilms previously (Wood *et al.*, 2000).

1.3.1.4. pH.

Differences in pH, as it has been previously described, can have a major effect on the

composition of the developing biofilm. The catabolism of sucrose to acid and subsequent

reduction in pH has a weakening effect on some acid-intolerant species. This leads to a shift

in the hierarchy in favour of more aciduric species (Bradshaw & Marsh, 1998), as

demonstrated by Kreth *et al.* (2008). Within the biofilm the production of weak acids such as

acetate, benefit the colony by buffering against extreme shifts in the environmental pH

(Margolis *et al.*, 1985; Vratsanos & Mandel, 1982; Walsh, 2006). However, on the

introduction of fermentable carbohydrates (specifically sucrose), the production of much

stronger acids, e.g. lactate, results in a rapid drop in pH (Walsh, 2006). Such conditions are

not habitable by many species which are subsequently lost, leaving only those which can

endure the acidic condition (Bradshaw *et al.*, 1989; Marsh, 1994; Svanberg, 1980; Walsh,

2006). However, as a species' niche is defined by processes of adaptation, the colonisation

process must also be affected by the acidity of the local environment. In fact, depending upon

the environmental conditions and the organism in question, changes in the local pH have a

marked effect on a number of physiological parameters including the internal pH, the pH

gradient (the difference between the external and internal pH), the membrane potential (the ion gradient across the cell membrane) also known as the proton motive force (Mara & Horan, 2003; Olson, 1993; Winn & Koneman, 2006).

In an investigation into the various factors affecting the irreversible attachment of *Pseudomonas aeruginosa*, Stanley (1983) demonstrated that, while flagellation was important for the colonisation of a surface, all samples, both motile and non-motile colonised best at conditions between pH 7 and 8. *Helicobacter pylori* is a highly persistent human commensal which colonise the gastric epithelia causing numerous health issues including gastric ulcers and potentially, gastric malignancies (Testerman *et al.*, 2001; Veerman *et al.*, 1997). During early colonisation, *H. pylori* maximises surface attachment at conditions as low as pH 2 (Nordman *et al.*, 1999) and it was therefore considered that environmental acidity could act as a signal for the up-regulation of adhesin expression (Testerman *et al.*, 2001). Interestingly studies noted that even oral *H. pylori* bound more effectively to surfaces at pH 5 – 6 (Veerman *et al.*, 1997).

Colonisation and survival signals are not restricted to *H. pylori*. *Streptococcus mutans* also appears to demonstrate signal–associated surface colonisation. In a study by Li *et al.* (2001) it was found that low pH survival mechanisms of *S. mutans* are mediated by a possible combination of colony density and cell to cell communication. High cell density throughout the exponential and stationary phases resulted in increased resistance to low pH compared to that which was observed in cells from low-density colonies. This was demonstrated when a *S. mutans* culture filtrate from acid tolerant, highly dense culture was neutralised and added to a *S. mutans* inocula at an early stage of a low pH (pH 3.5) killing assay. It was found that cultures treated with the filtrate were more resistant to low pH killing, suggesting that an extracellular component triggered an acid tolerance response. To further understand the implications of these extracellular components, Li treated similar cultures to increasingly

diluted, acid-tolerant filtrate and found that the benefits decreased in parallel with increased dilution. This suggested that the positive effects of the apparent 'signal molecule' was more effective in large quantities; further suggesting protection by high cell densities.

1.3.1.5. Temperature.

Temperature is also a major factor that defines an environmental niche and in such, will play a major role in biofilm development. However, the range of temperature tolerances is likely to differ both between species but also in the breadth of the ranges per species. This is because depending upon the species and the niche occupied, temperature fluctuations could vary dramatically.

An example of such a species is *Staphylococcus epidermidis*, a human skin coloniser, a strong biofilm former, a major perpetrator in nosocomial infections, particularly those associated with the use of sub-dermal catheter-associated infections (Dai *et al.*, 2012). In a 2005 study, Fitzpatrick *et al.* (2005) found that some isolates upregulated the production of intercellular polysaccharide adhesin (IPA) at 30°C whereas others upregulated at 42°C. This is interesting as human skin temperature is approximately 33°C and the author suggests that this may contribute to the persistence of *S. epidermidis*. However, the range of colonising temperatures observed in this study also hints towards the potential for adaptation to a number of anatomical sites, notably, external to subdermal, mucosal colonisation (Fey & Olson, 2010; Fitzpatrick *et al.*, 2005).

Similarly, *Enterobacter cloacae* are a commensal of the human gut and present in between 40 – 80% of the population. However, it has also been associated with the contamination of a number of food groups including cereal products, fresh and cooked market foods, 'ready-to-eat' foods and baby formula (Nyenje *et al.*, 2012; Nyenje *et al.*, 2013; Shaker *et al.*, 2007;

Tunung & Chai, 2008). Temperatures in the human gut are generally recorded at a steady 37°C although there is some small fluctuation during and after the consumption of water (Koziolek *et al.*, 2014; Merrell, 2013). However, with a range of potential niches outside of the gut you would expect a similar range of biofilm initiation temperatures. Nyenje *et al.* (2013) found that of 30 isolates, 100% formed biofilms after 48 h of incubation regardless of the temperature. After 24 h incubation however, the range of biofilms formed dropped to 60 – 90% when incubated at 25°C whereas, 93 – 100% of isolates formed biofilms after 24 h at 37°C. These results indicate that *E. cloacae* has a preference with regards to the temperature of their niche and biofilm formation is likely linked to both temperature and incubation time, though nutrient sources outside of the gut conditions could induce biofilm formation, possibly as a protective mechanism when conditions within the environment are sub-optimal.

The conditions described above represent a trend rather than the rule and serve to illustrate the possibility for a potentially limitless array of adaptations which define the bacterial niche. This means that there is little prospect of elucidating the single paradigm which encapsulates all developmental examples (Percival *et al.*, 2011; Pratt & Kolter, 1998). However Stoodley and colleagues (2002) constructed a five-stage model from the findings of numerous studies in order to define the developmental processes both single and mixed species biofilms.

As described, surface conditioning provides the substrate for cell attachment which is the first of five stages of bacterial colonisation. Pratt and Kolters (1998) described cell motility as a vital biofilm-initiation factor. Motility permits cellular migration to nutritionally suitable surfaces, the overcoming of surface-associated repulsive forces while also providing an anchor for surface attachment. In their studies, Pratt and Kolter observed that paralysed or 'surface attachment defective' (SAD, O'Toole & Kolter, 1998) strains of *E. coli* clustered into small, loosely adhered groups which did not appear to migrate into the surrounding abiotic regions. Alternatively non-chemotactic, motile species formed well distributed biofilms that

were indistinguishable from the wild type examples. This indicated that in *E. coli*, chemotaxis was not essential in the initiation of biofilm formation though motility in association with type I pili was critical. After attachment, nutrient availability promotes cell division and subsequently, Stage two; the production of extra cellular polysaccharides (EPS). Extra Cellular Polysaccharides form the bulk of the biofilm (Katsikogianni & Missirlis, 2004) and production occurs simultaneously with the aggregation of cells from the surrounding media which cluster together in micro-colonies, separated by channel-like structures. Continuous aggregation of cells and EPS production leads to the multi-layering and three-dimensional formation of the biofilm structure (Katsikogianni & Missirlis, 2004; Lappin-Scott & Costerton, 2003; Percival *et al.*, 2011). Stages three and four refer to the development and maturation of the biofilm architecture in which complex pores and water channels containing little or no cells are formed. The pores and channels within a biofilm form a kind of irrigation system and are constructed of more permeable matrix materials than the bulk of the EPS structure. Functionally, they are thought to permit the exchange of gas and nutrients to and from the external environment as well as acting as a drainage system for the removal of metabolic waste (Lappin-Scott & Costerton, 2003; Lawrence *et al.*, 1991; Percival *et al.*, 2011; Robinson *et al.*, 1984), as described above.

Finally, Stage five describes the dispersal or 'sloughing' of cells, either as individuals or in clusters, into the bulk phase liquid and subsequently, the surrounding environment (Trulear & Characklis, 1982). This mechanism has been linked to promoting genetic diversity via the release of viable cells capable of further colonisation in a more nutritionally favourable niche. It has also been suggested that this process may be linked to the reduction of colony numbers, perhaps due to the depletion of vital gasses/nutrients or waste-associated toxification of the biofilm; possibly indicating a survival mechanism (Percival *et al.*, 2011; Trulear & Characklis, 1982). It is noteworthy that this process may also be a product of mechanical shear due to fluid mechanics interacting with/against the denser biofilm structure.

The benefits of the biofilm existence, as opposed to planktonic suspension are highly dependent upon the local environment and species which make up the whole. However, studies have shown that micro-colonies encapsulated within the EPS matrix can be protected from a number of environmental threats including host immunity, phagocytic cells, grazing protists, a range of antibiotics, disinfectants, antiseptics and surfactants which are known to kill cells that are not protected by the slime matrix (Costerton *et al.*, 1987; Govan, 1975; Gristina *et al.*, 1989; Hahn M. W. *et al.*, 2004; Nickel *et al.*, 1985a; Nickel *et al.*, 1985b; Percival *et al.*, 2011; Schwarzmann & Boring, 1971).

1.3.2. The oral biofilm.

As described previously (section 1.3) a biofilm is a highly dynamic community of aggregated micro-colonies, irreversibly associated to a conditioned surface (Costerton *et al.*, 1995; Donlan, 2002; O'Toole *et al.*, 2000a; Palmer & White, 1997). Like the exogenous biofilm, the oral counterpart is a highly complex community of numerous bacterial species, enveloped and supported by an EPS matrix, co-aggregated into clusters and attached to the tooth surface. Tooth colonisation and subsequent biofilm development involves numerous reactions and interactions, both reversible and irreversible and some conserved while others are species-specific and all dependent upon the availability of nutrients, pH, temperature, surface conditions, bulk-phase liquid dynamics and the physical and metabolic composition of the surrounding colony and/or potential colonisers (Kostakioti *et al.*, 2013).

Biofilm accretion occurs at the enamel surface where bacteria bind to the proteinaceous pellicle. The two most commonly recognised plaque accumulations are known as subgingival and supragingival based their location on and around the tooth. Subgingival plaque accumulates below the gum line within the gingival pocket that houses the root and neck of the tooth and is not typically visible without a detailed examination. Supragingival plaque

refers to the biofilm which accumulates above the gum line, developing on the visible hard and soft surfaces including the teeth and gums (Reddy, 2008, Figure 2). The morphology and microbial composition of these two biofilms often differ quite extensively and like non-oral biofilms, dental plaque is not composed purely of bacteria but often include fungi, protozoa, mycoplasmas and viruses (Palmer & White, 1997; Reddy, 2008). In subgingival plaque the bacterial colonies are predominantly Gram negative, anaerobic, often motile with higher rates of protein metabolism whereas supragingival plaque is colonised by predominantly Gram positive, aerobic (depending upon the biofilm thickness) non-motile, carbohydrate-metabolising species (Reddy, 2008).

Primary colonisation of a tooth (or any substrate) is defined by the pioneers' capacity to survive within that particular niche (Rotimi & Duerden, 1981). The intimate positioning and subsequent interaction between heterogeneous species will modify the biochemical topography of the local environment (including pH, E_h (reduction potential), nutrient levels and the physical properties of the surface) and define the niche for subsequent colonisers (Diaz *et al.*, 2006; Li *et al.*, 2004; Marsh *et al.*, 2009; Sampaio-Maia & Monteiro-Silva, 2014).

Within the first few hours of life the mouth is colonised primarily by aerobes, facultative aerobes and aerotolerant species (Marsh *et al.*, 2009). As described earlier, this applies to the beginnings of all air-exposed biofilms. The capacity to tolerate an aerobic condition slowly gives way to colonisation by less aerotolerant species as the pioneers consume the available oxygen.

The oral cavity of a new born is colonised by significantly fewer species than that of an adult, a factor which may be associated with there being fewer types of surface to colonise, prior to the eruption of the deciduous teeth (Kononen, 2000). Until tooth eruption, the exposed surfaces of the oral cavity of the new born are shedding squamous epithelial and the pioneer

colonisers tend to be streptococci (specifically *S. oralis*, *S. mitis* and *S. salivarius*) and actinomyces (including *A. naeslundii*, Cortelli *et al.*, 2008; Gibbons & van Houte, 1971; Li *et al.*, 2004; Marsh *et al.*, 2009; Pearce *et al.*, 1995; Rotimi & Duerden, 1981; Smith *et al.*, 1993). These pioneers are typically associated with primary colonisation due to a specialised capacity for recognising pellicle-bound mucins, statherin, prolein rich proteins (PRP), alpha amylase, sialylated mucins and salivary agglutinins whereas the actinomyces recognise the PRP and statherin (Kolenbrander *et al.*, 2010; Palmer *et al.*, 2003).
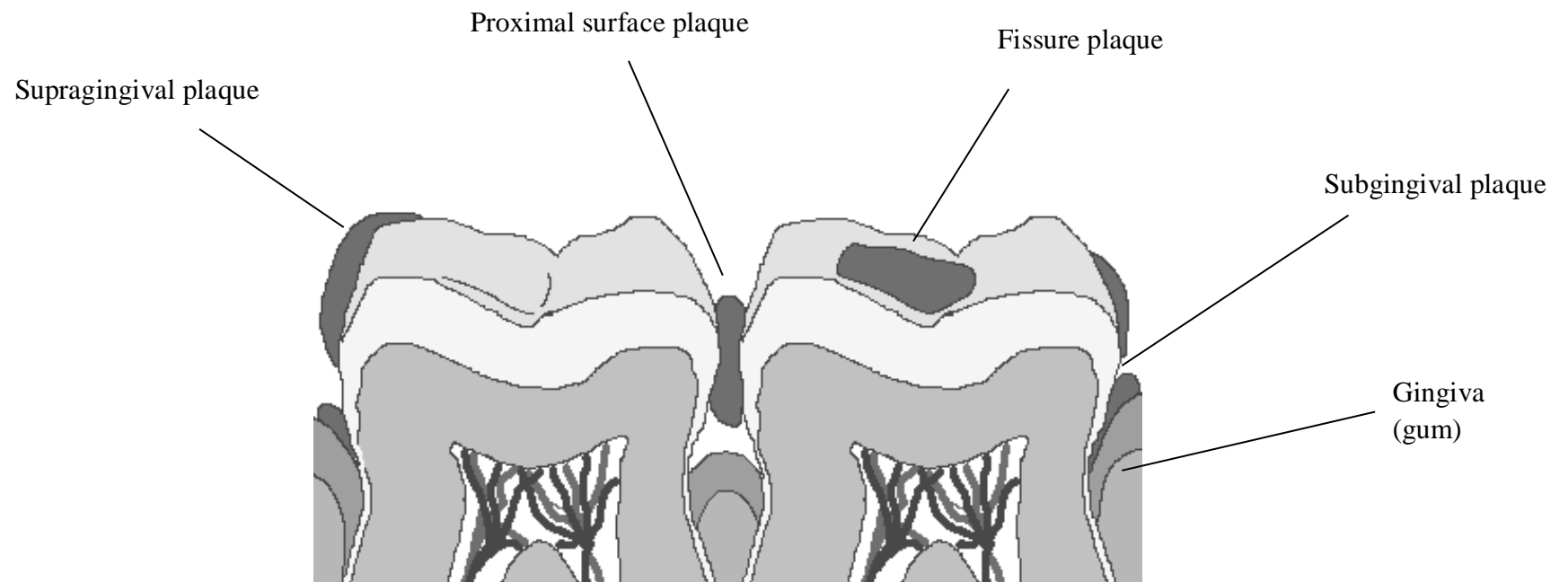
Figure 2 Tooth surface plaque distributions and associated nomenclature.

Image revised from Grassi and DeAngelo (2012) and Samaranayake, (2006)

Interestingly, one report found that approximately 70% of samples taken from neonates revealed no evidence of bacterial colonisation (10 min to 8 h after birth) which dropped dramatically to 0% between 24 h and 53 h after birth (Nelson-Filho *et al.*, 2013). Another found that staphylococci were the dominant coloniser of the oral cavity between two and six days of life (Rotimi & Duerden, 1981) which is supported by later reports (Nelson-Filho *et al.*, 2013). Variations are likely due to the route of contamination; neonates are likely to be contaminated not just by the local environment (hand to mouth transmission) but also due to person-to-person contact between family and medical attendees (Kononen, 2000; Makhoul *et al.*, 2002; Rotimi & Duerden, 1981), lending weight to the theory that the composition of the microbiome is unique to the host; at least at the species level (Bik *et al.*, 2010; Diaz *et al.*, 2006).

Within three months of life, species from the genera *Prevotella, Fusobacterium and Veillonella* are frequently isolated (Marsh *et al.*, 2009) however the eruption of deciduous teeth brings about a significant change in the topography providing a platform for significant and complex species diversification (Cortelli *et al.*, 2008; Crielaard *et al.*, 2011; Kononen, 2000). One study (Diaz *et al.*, 2006) found that retrievable enamel chips were 66 – 80% colonised by streptococci, the predominant species being *S. mitis* and *S. oralis*. Interestingly, they also noted that while bacterial profiles may have presented similarly at the phylum level, specific phylotypes were unique to the individual. Only 10 phylotypes were observed in all subjects, they were *Neisseria, Gemella* (x 2) and *Streptococcus* (x 7). This, it was thought, may be attributed to the fundamental beginnings of community development. The presence of aerobic *Neisseria* with aero-tolerant *Gemellae* and streptococci may define the foundation of a metabolically-beneficial partnership between multigeneric species, as described elsewhere (Cortelli *et al.*, 2008; Marsh *et al.*, 2009). Subsequently, members from the genera *Actinomyces, Capnocytophaga*, *Corynebacterium, Eikenella, Haemophilus, Lactobacillus, Neisseria, Prevotella, Propionibacterium, Rothia, Veillonella* and the *Fusobacterium* become

more abundant (Kolenbrander *et al.*, 2010; Marsh *et al.*, 2009; McCarthy *et al.*, 1965). Fusobacteria share interaction with many oral species and for this reason they are generally referred to as the 'bridge species' as they bridge the gap between early and late colonisation (Bradshaw *et al.*, 1998; Kolenbrander *et al.*, 2010). Late colonisers include examples from the genera *Prevotella*, *Aggregatibacter*, *Eubacterium*, *Treponema*, *Tannerella*, *Porphyromonas*, and *Selenomonas* (Al-Ahmad *et al.*, 2009; Kolenbrander & London, 1993; Kolenbrander *et al.*, 2002; Kolenbrander *et al.*, 2010; Nyvad & Kilian, 1987, Figure 3)
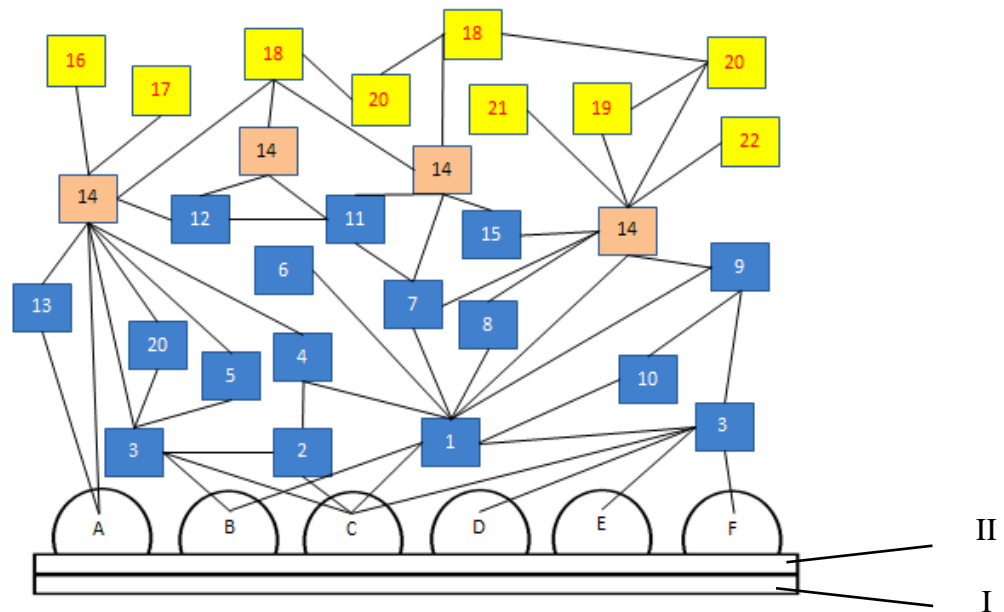
Figure 3. Bacterial colonisation of the dental surfaces.

The tooth surface (I) is coated with the salivary pellicle (II) which carries a unique composition of proteins and cellular debris which are rapidly adsorbed to the tooth. These include (A) Statherin, (B) Bacterial cell fragments, (C) Sialylated mucin, (D) Salivary agglutinin (E) α-Amylase, and (F) Proline-rich proteins. Early colonisers (▢), (1) *S. oralis* and *S sanguinis*, (2) *S. mitis*, (3) *S. gordonii*, (4) *Capnocytophaga ochracea*, (5) *Propionibacterium acnes*, (6) *Haemophilus parainfluenzae*, (7) *Prevotella loescheii*, (8) *Veillonella* spp., (9) *Actinomyces oris* and *A. naeslundii,* (10) *Eikenella corrodens*, (11) *Actinomyces israelii*, (12) *Capnocytophaga gingivalis*, (13*) C. sputigena*, (15) *Prevotella denticola*, and (20) *Porphyromonas gingivalis* are followed by the 'bridge' organisms (▢) (14) *Fusobacterium nucleatum* which link early colonisers to the later colonisers (▢) (16) *Aggregatibacter actinomycetemcomitans*, (17) *Eubacterium* spp., (18) *Treponema denticola*, (19) *Tannerella forsythia*, (20) *Porphyromonas gingivalis*, (21) *Prevotella intermedia*, (22) *Selenomonas flueggei*. The lines connecting the representative species to one-another and to the salivary pellicle represent receptor recognition and adhesion within the multi-species colony. Modified from Kolenbrander *et al*. (2010).

The bacterial layout presented in Kolenbrander's model may be somewhat misleading as the colonisation of enamel is not so uniform. A recent study (Crielaard *et al.*, 2011) compared the oral microbiota of children between the ages of 3 and 18 years old with a similar study of adults (Keijser *et al.*, 2008). Crielaard *et al.* (2011), as in some previous studies (Bik *et al.*, 2010; Diaz *et al.*, 2006) found that while specific species showed some variability, particularly when analysing the results sample by sample; the percentages of specific phyla remained stable between the ages of eruption and puberty and differed from adulthood, though whether the difference was significant was not reported (Figure 4). This, it was proposed, may be an indicator that the oral microbiome undergoes a process of maturation. As previously stated, the oral cavity as a habitat, changes throughout host maturation due to a combination of factors, not least the eruption of teeth and an evolving diet, but also as a result of puberty which is thought to enrich bacterial nutrient sources contributing to fluctuations in the oral microbial diversity (Nakagawa *et al.*, 1994; Sampaio-Maia & Monteiro-Silva, 2014).
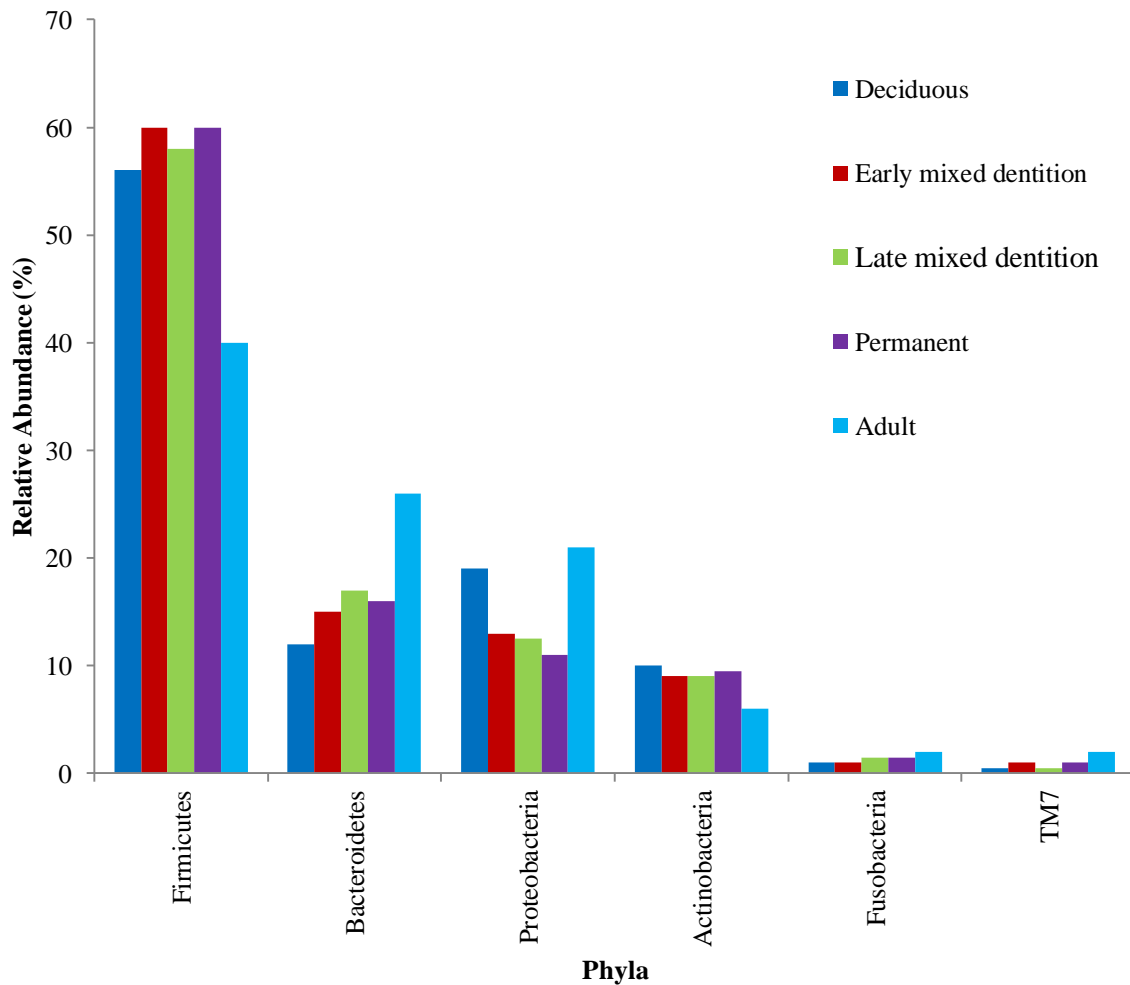
Figure 4. Relative abundances of the six most abundant phyla collected from children (aged 3 – 18, and adults).

The graph illustrates changes the phylogenetic profile from the childhood deciduous teeth, throughout early teens where mixed dentition is dominated by deciduous teeth in the early stages and by the permanent throughout the late stages, then finally, into the permanent dentition. Data was reproduced from Crielaard *et al*., (2011) compared to an adult profile from a similar study Keijser *et al*., (2008)

The liquid phase of the dental biofilm is made up of approximately 80% water, the remaining 20% (dry weight) is composed of 50% bacterial and salivary proteins, 25% carbohydrates (predominantly glucans, fructans and bacterially synthesised heterosaccharides), lipids, and 5-10% inorganic materials (mainly calcium, phosphates, magnesium and DNA, Flemming & Wingender, 2010; Rajendran *et al.*, 2009). The proportion of components which make up the EPS matrix is highly variable and is dictated by the resident microbes in relation to the host and their local environment (Avila *et al.*, 2009; Branda *et al.*, 2005); glucans and fructans however, are considered to be major virulence factors in oral biofilm development.

Glucans are polysaccharide chains which are synthesised by extracellular enzymes known as glucosyltransferases (GTF, Devulapalle & Mooser, 1998). These are thought to be constitutively expressed enzymes and although upregulated when fermentable carbohydrates are in excess, they are synthesised under all growth conditions and are present in whole saliva and the salivary pellicle (Hiremath, 2007; Lamont & Jenkinson, 2010). There are two types of glucan and a fructan polymer associated with biofilm accretion. Synthesised from sucrose (fructose-glucose disaccharide), the linear soluble glucan and fructan provides a fermentable energy source whereas the branched insoluble, sticky glucan is more complex, take longer to denature and are a major component in the accumulation and fortification of the plaque matrix.

Newbrun (1974) recorded that GTFs are produced by a number of species, typically associated with human dental plaque, which include *S. salivarius*, cariogenic *S. mutans*, *S. sobrinus*, *Actinomyces* spp. and *Lactobacillus* spp, and caries-linked *S. sanguinis* (Bowden and Koo, 2011). Once expressed, GTFs are readily adsorbed to the salivary pellicle, acting as a stable substrate for bacterial colonisation (Devulapalle & Mooser, 1998; Hojo *et al.*, 1976; Lamont & Jenkinson, 2010; Schilling & Bowen, 1988; van Houte, 1994). Similarly, extracellular fructosyltransferases (FTF) have been identified in both the saliva and

immobilised in the pellicle (Steinberg *et al.*, 2002) and lead to the production of 1-3 β-linked fructans which act as a nutrient reservoir for the surrounding colonies (Baird *et al.*, 1973; Jacques, 1993; Kopec *et al.*, 1997; Lamont & Jenkinson, 2010; Wenham *et al.*, 1979).

Interestingly, extracellular DNA (eDNA) is thought to play a vital role formation and integrity of biofilms on the oral cavity (Jakubovics & Grant Burgess, 2015). Concentrations of eDNA in the various mucosal linings (e.g. gastrointestinal and lung) are thought to reach into the hundreds of micrograms per millilitre (Finkel & Kolter, 2001). The sources of eDNA may include one or more or a number specific processes that include the passive lysis and death of a cell, possibly linked to a recent drop in pH, and subsequent release of DNA. Another source may include bacteriophages or recently lysed host cells (Jakubovics *et al.*, 2013; Jakubovics & Grant Burgess, 2015). Extracellular DNA provides a number of functions in the biofilm (Chiang *et al.*, 2013; Finkel & Kolter, 2001; Jakubovics & Grant Burgess, 2015); one study found that there was a significant increase in surface attachment of *S. mutans* cells in the presence of eDNA (Das *et al.*, 2010). Extracellular DNA is also thought to act as a nutrient source (Finkel & Kolter, 2001), while the horizontal transfer of genetic materials has been shown to propagate beneficial traits and develop entirely new genetic combinations (Madsen *et al.*, 2012; Tribble *et al.*, 2012). The possibility of eDNA inferring protective traits within the biofilm may be the first consideration however, Doroshenko *et al.*, (2014) reported that the affinity vancomycin has for DNA is one hundred times greater than of its intended cellular target meaning that eDNA may also provide a shielding mechanism against some antimicrobials.

Currently, there have been approximately 700 species associated with the oral cavity, spanning nine phyla*; Deferribacteres*, *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *OP11* and *TM7* (Aas *et al.*, 2005; Diaz *et al.*, 2006; Zijnge *et al.*, 2010). Most plaque forming bacteria are non-motile and are derived,

passively from saliva. This means that the larger accumulations of plaque tend to occur at sheltered or stagnant sites away from environmental shear or those sites which are regularly exposed to hydrodynamic, biochemical (saliva) or mechanical disruption, allowing for community establishment and growth (Marsh, 1994; Marsh, 2003).

As described earlier, indigenous species are equipped with cell surface adherence proteins (adhesins) which recognise molecular receptors such as proteins and glycoproteins in the acquired pellicle. This is of particular importance for non-motile species which utilise such substrates for co-aggregatory colonisation (Kolenbrander & London, 1993) and accumulation in dental plaque (Bradshaw *et al.*, 1998).

Bacterial coaggregation is defined as the adhesion of genetically distinct species, into a mutually beneficial colony. Similarly, co-adhesion is the mechanism of recognition and binding to microorganisms already adhered to a given substrate; in this case, enamel or gingival tissue (Bos *et al.*, 1995; Kolenbrander & Andersen, 1986; Kolenbrander, 1989; Kolenbrander & London, 1993). The process is energy independent and imitates a lectin-like (a proteins that binds to sugars/carbohydrates) interaction (Kolenbrander & London, 1993; Palmer *et al.*, 2003; Sharon & Lis, 2004).

Alternative mechanisms of attachment are thought to include ionic interactions when the negatively charged pellicle and bacterial cell membranes (possibly linked to the negative charges of lipopolysaccharides in Gram negative bacteria and the phosphates in teichoic acids of Gram positive bacteria) are bridged by positively charged ions in the saliva, such as calcium (Cisar *et al.*, 1979; Jin & Yip, 2002; Reddy, 2008; Scannapieco, 1994; van der Mei *et al.*, 2007).

Like most resident flora, dental biofilms typically exists in harmony with both the host and its immunity, providing competitive inhibition from invasion by exogenous pathogens (Avila *et al.*, 2009; Marsh, 2000). However, as with most ecosystems, the introduction of an

environmental bias (such as a specific type of nutrient) may bring about unbalance in the system. For example, dietary carbohydrates (such as sucrose) are utilised by some species of oral bacteria as a carbon source. Anaerobic metabolism of sucrose within the biofilm leads to the production of acids (principally, lactic acid) by cariogenic species, resulting in a localised drop in pH. Continued exposed to such sugars and subsequently low pH, destabilises the biofilm causing the proliferation in aciduric (acid tolerant) species and the demineralisation and potential loss of dental enamel (Marsh, 2009).

1.4. The Aetiology of Caries.

Caries (tooth decay) is possibly the most prevalent transmissible disease of both adults and children and is characterised by the gradual demineralisation and loss of tooth enamel though repeated and/or prolonged acidification of oral biofilms (Arora *et al.*, 2011; Bonifait & Grenier, 2010; Campbell & Farrell, 2011; Colby & Russell, 1997; Jeon *et al.*, 2011; Ruby *et al.*, 2010; Schlafer *et al.*, 2011; Torlakovic *et al.*, 2012). However, the complexity of the disease has been the focus of scientific investigations for years. Keyes and Jordan (1963) identified a correlation between the types of food consumed and the degree of enamel demineralisation. Their findings proposed that both the bacteria and their contribution to the habitat must be described collectively with the host's diet, general health, salivary composition and the structural condition of the tooth, if the disease was to be fully understood.

Today, caries represents a major world health issue. The US Surgeon General's report (Satcher, 2000) described that in the US, early childhood caries (ECC) was five times more common than asthma and seven times more common than hay fever. Additionally, the cosmetic nature of poor dental health impacts social development while the associated pain and discomfort has an impact on learning and general behaviour (Casamassimo *et al.*, 2009).

The ubiquitous nature of caries and poor oral health also has major economic implications. It is thought that, in industrialised counties between 5 – 10% of public spending is on oral health (Fejerskov & Kidd, 2008). Widström and Eaton (2004) recorded that in 2000, the total population of the European Union was approximately 456 million and the total cost of oral healthcare was approximately €54 billion. However, despite the prolific nature of caries in modern society, in most cases it is an avoidable disease. Aside from the metabolic activities of oral bacteria, contributing factors to the continuation of caries include the increase in dietary refined sugars and a poor knowledge of oral health. Therefore, any reduction in recent years is due to modern hygiene products, techniques and increased education (Campbell & Farrell, 2011).

There are a number of theories which, over the years, have formed our current understanding of cariogenic disease and its association with plaque biofilms. Loesche (1976), cited in Marsh (1994) and Rosier *et al*. (2014), described the 'specific plaque hypothesis' in which the onset of disease is linked to a small number of specific species (e.g. *S. mutans*). In 1986 the 'non-specific plaque hypothesis' suggested that the progression from a healthy status into symptomatic disease is linked to the increased accumulation of biofilms, possibly from supragingival accumulations, which 'down grow' into and colonise the gingival pocket interacting with the host, (Theilade, 1986) cited in Marsh (1994) and Rosier (2014). Combining these two ideas with observations made in the laboratory, the 'ecological plaque hypothesis' (Marsh, 1991) theorised that symptomatic cariogenesis was a product of the destabilisation of biofilm ecology. A downward shift in one or more of the primary environmental factors, for example, pH, will drive the biofilm into a state of destabilisation which favours species capable of surviving at acidic conditions (Marsh, 1991; Marsh, 1994; Marsh & Bradshaw, 1997; Paes Leme *et al.*, 2006).

In the majority of publications *S. mutans* and *S. sobrinus* are cited as being the major cause of caries (Aas *et al.*, 2008; Lemos *et al.*, 2005; Preza *et al.*, 2008) though they have also been isolated from enamel surfaces which are free of lesions (Marsh, 2006; Marsh, 2009; Milnes & Bowden, 1985) suggesting that they can also exist in biofilms without inducing cariogenic conditions, which is an important factor when addressing caries at an ecological level. Similarly, streptococci which may not be typically associated with caries, such as *S. orals*, *S. mitis* and *S. salivarius* can also metabolise sugar to acids resulting in a reduction in environmental pH and contributing to cariogenic conditioning (de Soet *et al.*, 2000; Drucker *et al.*, 1984). Other taxa that have been strongly associated with aciduricity and caries include predominantly *Lactobacillus* but also *Bifidobacterium* spp. *Atopobium*, *Actinomyces* spp. and *Veillonella* spp. which are considered to be a marker for caries as they produce weaker acids than required for enamel dissolution but utilise lactic acid as a carbon sources (Becker *et al.*, 2002; Brailsford *et al.*, 1999; Kianoush *et al.*, 2014; Nakajo *et al.*, 2010; Peterson *et al.*, 2013; Piwat *et al.*, 2010).

In a balanced, non-cariogenic biofilm some bacteria produce beneficial weak acids such as acetate which buffer the colony against extreme pH fluctuations (Margolis *et al.*, 1985; Vratsanos & Mandel, 1982; Walsh, 2006). However, sucrose is rapidly metabolised into stronger acids such as lactate resulting in a rapid decline in pH to values as low as 4, where it can remain for several hours depending upon dose and repeated exposure (Walsh, 2006). The prolonged catabolism of dietary sugars positively selects for aciduric species, such as the mutans streptococci (MS) group, which are capable of continued carbohydrate fermentation within an acidic environment. Sustained acidification of the biofilm results in the loss of species which are incapable of surviving at low pH, thus, reducing microbial diversity within the biofilm and with it the capacity for a rapid, more effective recovery (Bradshaw *et al.*, 1989; Crielaard *et al.*, 2011; Marsh, 1994; Svanberg, 1980; Walsh, 2006).

The MS group are a subdivision the viridans streptococci (VS) group and are characterised by their ability to ferment artificial sweeteners such as mannitol and sorbitol (Olmsted, 1953). Viridans streptococci are a heterogenetic group of haemolytic (and some non-haemolytic) commensals which derive their name from the greenish discolouration of blood agar, brought about by alpha-haemolysis of red blood cells (Coykendall, 1989; Sharma & Gupta, 2014). However, the production of acids via the catabolism of sweeteners is greatly reduced when compared to sucrose, glucose and/or fructose, as is the production of structural 'glucans' (Boonyanit *et al.*, 2011; Caufield *et al.*, 2000; Janda & Kuramitsu, 1978; Renschler, 2006). For greater detail on glucans, see section 1.2.2. 'The Oral Biofilm'.

The species' generally associated with the MS group are *S. mutans* and *S. sobrinus* (Lamont & Rosan, 1990; Law *et al.*, 2007; Okada *et al.*, 2010) and although *Staphylococcus cricetus*, *Staphylococcus rattus* and *Staphylococcus downei* are also included, they are rarely isolated from human samples (Colby & Russell, 1997). Furthermore, while cariogenic MS can maintain a metabolic turnover at conditions as low as pH 4, numerous VS species (including *Staphylococcus anginosus*, *S. sanguinis*, *S. oralis*, *S. gordonii*, *S. mitis* and *S. salivarius*) can be isolated from conditions as low as pH 5 (Harper & Loesche, 1984; Svensäter *et al.*, 2003; Walsh, 2006). This indicates that while, strictly speaking, VS are not cariogenic they are likely to contribute to the acidification of the biofilm during the early stages of caries development.

The oral environmental conditions, defined by changes in pH, temperature, protein and mineral composition, diet, age, hydration and health are in constant fluctuation which, in turn, influences the status of dental enamel that is in a constant state of cyclic demineralisation and remineralisation (Cole, 1993; Hegde & Moany, 2012; Rabinowitz *et al.*, 1996; Slade *et al.*, 1996; Surdacka *et al.*, 2007), See Figure 5.

Under physiological conditions and when in contact with water, small concentrations of dental enamel is dissolved, separated into its ionic components (specifically, calcium, inorganic phosphates and hydroxyls) and released into the surrounding saliva and fluid-phase biofilm. This will continue until the surrounding medium becomes saturated with the mineral components of the enamel. This is known as the 'critical demineralisation threshold' and is reported to range between approximately pH 5.5 to 5.7, although, depending upon the mineral component of the surrounding solution and that of the solid phase enamel, this figure may have a wider range (Dawes, 2003). At the critical pH, the rate of dissolution is equal to the rate of re-precipitation and the demineralisation and remineralisation process is said to be in a state of equilibrium (Cury & Tenuta, 2009; Dawes, 2003; Hegde & Moany, 2012; Shipman *et al.*, 2009; Stoker, 2008). However, the composition of hydroxyapatite also includes variable concentrations of additional impurities including carbonate, sodium, magnesium and fluoride. The concentrations of these impurities differ between individuals and therefore the susceptibility to dissolution is due, in part, to the measure of demineralised enamel in the surrounding fluid phases and the composition of the enamel, which differs from tooth to tooth and person to person (Amaechi & van Loveren, 2013; Dawes, 2003; Fejerskov & Kidd, 2008; Stoker, 2008).
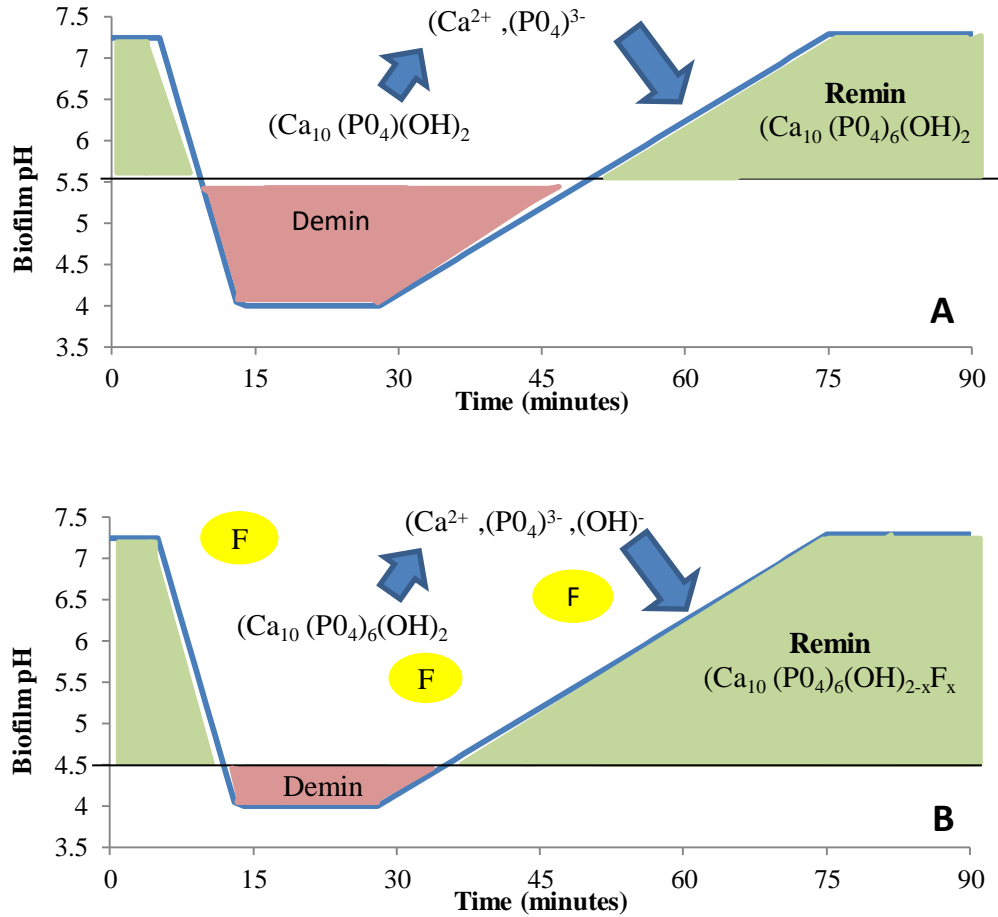
Figure 5. Schematic illustration of the demineralisation ('DEMIN') and remineralisation ('REMIN') of hydroxyapatite (HA), in the absence of fluoride (F).

A: During demineralisation, hydroxyapatite is broken into its ionic components which are released into the bulk phase saliva/plaque and later precipitate back into the enamel surface. B: In the presence of fluoride the remineralisation of OH is replaced by F where $0 < X < 2$, potentially reducing the demineralisation threshold to pH 4.5 and below. Images do not account for elemental loss due to hydrodynamic clearance. Figure reproduced from Amaechi and van Loveren (2013) and Shafiei et al. (2012).

Below the critical demineralisation threshold, calcium ions, phosphate and hydroxyl are released into the surrounding saliva. Hydroxyls and phosphate ions bind with the hydrogen ions to form water and hydrogen phosphate respectively, reducing ionic availability, the rate of precipitation and in turn, driving the continuation of enamel dissolution towards cavity formation (Arnold *et al.*, 2006b; Dawes, 2003; Fejerskov & Kidd, 2008).

1.4.1. Fluoride, water fluoridation and topical dentifrices.

There is now little doubt that the addition of topical fluoride to dental hygiene routines and potable water supplies positively affects the quality and stability of tooth enamel (Arnold *et al.*, 2006a; Arnold *et al.*, 2006b; Cury & Tenuta, 2009; Lagerweij & Ten Cate, 2002; Ten Cate, 1990).

Fluoride (F) is the reduced (ionic) form of fluorine and exists as a weak hydrofluoric acid in an aqueous milieu or dissociated F^- (Thibodeau *et al.*, 1985). In nature, F compounds represent approximately $0.06 – 0.1\%$ of the earth's crust and are therefore present at varying concentrations in most naturally occurring water bodies (Fawell, 2006; Jiménez-Farfán *et al.*, 2011) .

Research into water fluoridisation began between 1909 and 1915 in Colorado Springs (Colorado, USA) by two dental researchers, Frederick McKay and Vardiman Black. Their research revolved around an observation that individuals with brown mottling on the teeth, then known as Colorado Brown Stain, were highly resistant to tooth decay. In 1923 McKay noted the same type of brown staining in townsfolk of the adjacent state 'Idaho' where recent improvements to the delivery of fresh water had resulted in the gradual mottling of enamel (Anon, 2011). Acting on McKays advice, the local townsfolk abandoned the new water supply and within a few years the mottling of teeth began to fade.

A later collaboration with US Public Health representative, Grover Kempf and the Aluminum Company of America's chief chemist, 'Harry', that a link between high concentrations of fluoride in the drinking water and the mottling of teeth, now known as 'fluorosis', was drawn (Anon, 2011; Arnold *et al.*, 2006a).

In 1931 a head dental hygienist at the National Institute of Heath, 'Henry Trindley Dean', began studying the epidemiology of fluorosis (Anon, 2011). His results showed that artificial fluoridation of water supplies to levels as low as 1ppm caused little or no enamel discolouration in the population's majority. He was able to bridge the apparent safety of consuming small quantities of fluoride in water with McKay and Black's observations of the apparent reduction in tooth decay and sought out permission to test his theories on a larger scale. In 1944 the City Commission of Grand Rapids, Michigan USA, granted permission allowing Dean to test his theory and in 1945 they became the first city to use artificially fluoridated water supplies (Anon, 2011; Horowitz, 1989); when compared against a similar survey in the non-fluoridated ($\leq$ 0.2ppm, Arnold *et al.*, 2006a) city of Muskegon, Michigan. The fifteen year study surveyed the continued dental health of approximately 30,000 children and noted that the incidence of caries in children born before the water fluoridation had fallen by approximately 26% with a reduction of over 60% in those born after artificial fluoridation (Anon, 2011; Arnold, 1957). The results of the Grand Rapids investigations were so impressive that in 1951, the people Muskegon requested a release from its status as the control group and proceeded to fluoridate its water supplies (Arnold, 1957; Horowitz, 1989).

Similarly, in the UK a dentist by the name of Norman Ainsworth, who was aware of McKays work, compared water samples within districts of Essex, UK, against a study of over 4000 children. What he found reflected the Grand Rapids study, in that high concentrations of waterborne F (4.5 – 5.5ppm) resulted in enamel mottling and low incidences of caries, whereas low concentrations (0.5ppm) resulted in reduced staining and higher rates of caries

(Mullen, 2005). In the UK, specifically, England, F adjustments in drinking water are permitted by parliament and regulated by the 'Drinking Water Inspectorate'. A report published by Public Health England in 2014 noted that, while the maximum F concentration permitted is 1.5ppm, schemes in England typically aim for a standard 1ppm (Public Health England., 2014).

Today, there are numerous vehicles for the delivery of fluoride to the general public. Other than through potable water supplies which, when consumed result in a systemic F reservoir, there are also topical products which refer to those applied to the exposed surfaces of the enamel (toothpaste) and are not intended for ingestion (Naumova *et al.*, 2010). Modern dentifrices come in a range of different formulas, the most frequent being sodium fluoride, sodium monofluorophosphate and amine fluoride (Arnold *et al.*, 2006b). Many vehicles for topical fluoride result in up to 1000-fold increase in transient fluoride that may remain significantly higher than the baseline, for up to 60 min (Ekstrand, 1987; Zamataro *et al.*, 2008). Much of the fluoride is suspended in saliva and is cleared from the oral cavity by swallowing. That which remains localised may, potentially, re-precipitate on to the enamel surface or become concentrated within dental plaque as calcium fluoride, dissociating slowly and acting as a reservoir (Arnold *et al.*, 2006b; Ekstrand, 1987; Tenuta *et al.*, 2008; Tenuta & Cury, 2010).

Regardless of the mode of delivery, the benefits of F use in dental care are undebatable. Public Health England (2014) reported that children under 5 years old, living within fluoridated areas are 15% less likely to suffer from tooth decay than those from non-fluoridated areas. For children of around 12 years old that figure is approximately 11%. The most striking statistic, however, is that of children between 1 and 4 years old, there are approximately 45% fewer hospitalisations due to caries-associated symptoms (generally, in

requirement of surgery under general anaesthetic) in fluoridated areas, when compared to non-fluoridated areas.

Medicinal F is administered via a number of routes, principally, water treatments (approximately 1 ppm, Public Health England., 2014) and commercial and professional dentifrices, (toothpastes, mouthwashes and varnishes, ≤ 1.5 ppm, when sold as a cosmetic product, (Anon, 2008). However, F cannot enter the fluid phase of enamel or plaque directly, bioavailability (the rate and completeness to which a drug has been absorbed and, subsequently, becomes available at the intended site of action, Ekstrand, 1987; FDA, 2011) requires that it is must first be dissolved in saliva (Arnold *et al.*, 2006b; Ekstrand, 1987). Saliva and the fluid-phase of plaque are considered to be supersaturated with F at concentrations as low as 0.02ppm (1µM, Tenuta & Cury, 2010).

As described earlier, demineralisation occurs when the local pH falls below approximately 5.5. Ionic calcium, phosphates and hydroxyl precipitate into the surrounding saliva and are either lost via hydrodynamic clearance, due to the formation of water and hydrogen phosphates (Arnold *et al.*, 2006b; Dawes, 2003; Fejerskov & Kidd, 2008) or precipitate back onto the tooth surface during remineralisation. When made available i.e. via the use of topical dentifrices or systemically available due to the consumption of treated water, F is incorporated in the enamel during remineralisation (Figure 5), by replacing hydroxyl ions in hydroxyapatite and forming fluorohydroxyapatite (partial replacement of hydroxyls) and/or fluorapatite (complete replacement of hydroxyls). These compounds are less soluble than hydroxyapatite, having a dissolution threshold of approximately pH 5 - 4.5, between 0.5 and 1 pH unit below the average enamel dissolution threshold (Amaechi & van Loveren, 2013). In addition to this, ionic fluoride binds with calcium ions to form reservoirs of calcium fluoride in the fluid phases of saliva, the biofilm and at the enamel surface, breaking down slowly to release fluoride after topical application (Arnold *et al.*, 2006b; Darroudi *et al.*, 2010; Dowker

et al., 1999; Fejerskov & Kidd, 2008; Hiremath, 2007; Levine, 2011; Roberts & Wright, 2009; Wiegand *et al.*, 2005; Yip *et al.*, 2006). With continued exposure to F (particularly, in water supplies) concentrations can accumulate within the body's hard tissues, such as teeth and bones, in a process known as fluorosis (Jiménez-Farfán *et al.*, 2011).

Fluorosis is an irreversible, dose-dependent hyper-mineralisation of tooth enamel and bones and can, in very high doses, affect the development of pre-erupted teeth (Figure 6, Haufe & Tressaud, 2008; Hiremath, 2007). The severity of fluoride accumulation depends on the degree of exposure during enamel development, however, it may also be influenced by some genetic susceptibility as both human subjects and mice display variations in accumulation, even when subjected to the same concentrations of F (Brudevold *et al.*, 1978; de Almeida *et al.*, 2007; Everett *et al.*, 2002; Vieira *et al.*, 2004; Vieira *et al.*, 2005). The symptoms of fluorosis include white or brown flecks or striations across the tooth enamel which may merge, depending in the level of exposure and resulting in surface enamel loss and variable 'pitting', (Anon, 2011; Arnold *et al.*, 2006b; Haufe & Tressaud, 2008).

Figure 6. Dental Fluorosis.

Examples of mild (A top), moderate (B middle) and severe (C bottom) fluorosis. Images

taken from Centers for Disease Control and Prevention (CDC, 2012).

In spite of the cosmetic implications of fluoride accumulation during enamel development, community-wide protection can be no better demonstrated than in the fluoridation of water supplies in order to combat caries (Tenuta & Cury, 2010). Studies have shown that not only is the artificial fluoridation of water the simplest method for the delivery of community-wide protection but it is safe, inexpensive and, with lifelong consumption, provides lifelong benefits (Arnold, 1957; Arnold *et al.*, 2006a; Horowitz, 1989).

1.5. Antimicrobial effects of fluoride.

1.5.1. Fluoride bioavailability in saliva and plaque.

The addition of fluoride to the dental hygiene routine is positively correlated with reduced cariogenic demineralisation of dental enamel (Arnold *et al.*, 2006a; Arnold *et al.*, 2006b; Cury & Tenuta, 2009; Ingle *et al.*, 2014; Lagerweij & Ten Cate, 2002; Mohammed *et al.*, 2014; Ten Cate, 1990; Vogel, 2011). However, in spite of the numerous studies and reviews which have reported the antimicrobial effects of F on some metabolic and homeostatic processes in oral bacteria (Guha-Chowdhury *et al.*, 1997; Marquis *et al.*, 2003; Sutton *et al.*, 1987), the specific interactions between fluoride and oral biofilms *in vivo* remain unclear (Burne & Marquis, 2000; Buzalaf *et al.*, 2011).

Fluoride bioavailability in the oral cavity has been shown to depend on a number of factors, not least the delivery vehicle (i.e. toothpastes, rinses and drinking water), the volume and regularity of use, salivary flow rates and age (Ingle *et al.*, 2014; Naumova *et al.*, 2010; Naumova *et al.*, 2012a). One study of found that baseline concentrations of F in a healthy individual with regular access to fluoridated topical products (within a catchment of 0.2 ppm fluoridated water) was approximately 0.4 – 0.5ppm. After treatment with various fluoridated

dentifrices, salivary concentrations had returned to baseline within 2 h; similar results confirming this data were reported elsewhere (Ingle *et al.*, 2014; Naumova *et al.*, 2012a; Naumova *et al.*, 2012b; Phan *et al.*, 2001; Vale *et al.*, 2015).

As previously described the addition of fluoride to drinking water and topical dental products is positively associated with reduced incidence caries within a given community. In addition, however, there is mounting evidence indicating a negative correlation between concentrations of oral fluoride and the bacterial catabolism of dietary sugars (Barboza-Silva *et al.*, 2005; Barboza-Silva *et al.*, 2009; Hata *et al.*, 1990; Hüther *et al.*, 1990).

In vitro, the inhibitory effects of F appear to be due to a direct association, of either fluoride and/or hydrogen fluoride (HF), with a number of enzymes which are linked to bacterial homeostasis and include (but are not limited to) enolase, urease and the arginine and agmatine deiminase systems as well as ATPase and catalase (Barboza-Silva *et al.*, 2005; Bunick & Kashket, 1981; Hannuksela *et al.*, 1994; Marquis *et al.*, 2003; Thibodeau & Keefe, 1990) each of which will be described here.

1.5.2. Enolase

Glycolysis is the catabolism of glucose and other simple sugars into CO_2 and pyruvate and lactate under aerobic and anaerobic conditions respectively (Davidson & Sittman, 1999; Garrett & Grisham, 2010). Enolase (2-phospho-D-glycerate hydrolyase [E.C.4.2.1.11]) is a highly conserved glycolytic enzyme (Balzar Ekenbäck et al., 2001) which reacts with 2-phosphoglycerate to produce phosphoenolpyruvate in the ninth step of the glycolytic pathway (Brewer & Weber, 1968; Gropper *et al.*, 2009; Qin *et al.*, 2006).

The inhibition of enolase by fluoride results in the accumulation of substrate, specifically, 3-phosphoglycerate (3PG) and 2-phosphoglycerate (2PG) and a subsequent reduction in the

enzymatic by-product, phosphoenolpyruvate (PEP) availability. This leads to an arrest in further sugar-uptake (via the phosphoenolpyruvate-dependant transport system) resulting in a compounding inhibition of glycolysis (Bunick & Kashket, 1981; Guha-Chowdhury *et al.*, 1997; Levine, 2011; Marquis *et al.*, 2003). This was demonstrated in a study by Maehara *et al.*, (2005) on the synergistic effects of fluoride and xylitol on glycolysis in *S. mutans*. Similarly with xylitol, which inhibited sugar-uptake and resulted in the increase of most glycolytic intermediates, the inhibition by fluoride resulted in a build-up 3PG and 2PG, and a reduction in cellular concentrations of PEP, indicating enolase inhibition.

Synergistic inhibition is not restricted to xylitol. In a study by Guha-Chowdhury *et al.* (1997) enolase inhibition was achieved with F concentrations of between 16 and 54 µM when in the presence of 5 µM organic phosphate and 2 mM magnesium.

This was explained by Huther *et al.* (1990) who suggested that the inhibition of enolase by F was competitive whereas the addition of phosphate changed the inhibitory action to a non-competitive process. Competitive inhibition describes the inhibitor molecule competing with the substrate for the enzymes active site. Whereas in non-competitive inhibition the inhibitor binds to another site on the enzyme and disables further action, possibly due to a change in protein conformation (Hüther *et al.*, 1990; Roberts *et al.*, 2000). This means that while F alone will be an effective inhibitor, the catabolism of 2PG may continue, albeit at a reduced rate. The combination of F with phosphate, therefore, may be more effective as it would remove the enzyme substrate as a meaningful factor within the equation. Although the method of inhibition was not determined, the increased effectiveness of F when combined with phosphate has been observed by other authors (Kaufmann & Bartholmes, 1992). Furthermore, a study by Qin *et al.* (2006), using crystallography, noted that the enolase-fluoride-phosphate complex imitated the substrate-product (2-phosphglycerate – phosphoenolpyruvate) structure, suggesting a competitive inhibition.

The effects of F on enolase and the inhibition of glycolysis are thought to be gradual and pH-dependant. In a review of the literature, Marquis *et al.* (2003) reported that the inhibition of glycolysis by F at near-neutral pH required concentrations of around 10 mM whereas at pH 4, only micromolar concentrations of F were required. Interestingly, Bunick and Kashket (1981) suggested that internalised F concentrations are unlikely to differ, regardless of external pH conditions (specifically between 6-7), but because glycolysis was impaired by the reduction in cytoplasmic pH, inhibition of enolase by F was more likely to have an observable impact on the cellular viability because glycolytic turnover was reduced when compared to that at pH 7. This was confirmed by Belli *et al.* (1995) who noted that the inhibitory effects of F were more likely to be due to the cytoplasmic acidification by HF, as they noticed a sharp reduction in glycolytic flux as the pH was reduced from 6 to 5.

Fluoride enters the cell in association with HF on a pH gradient that has a detrimental effect on enzymatic processes which include glycolysis (Germaine & Tellefson, 1986; Hamilton, 1990). Under acidic conditions, the passive accumulation of HF and subsequent dissociation ($F^- + H^+$), acidifies the cytoplasm accounting for the gradual inhibition under pH-dependant conditions.

Thus, previous research indicates that, at least *in vitro*, the antimicrobial effects of fluoride on enolase are variable. Importantly, there are also variations in the sensitivity to F between species (e.g. *Actinomyces naeslundii*, *Lactobacillus rhamnosus*, *S. mutans*, *S. salivarius* and *S. sanguinis* (Guha-Chowdhury *et al.*, 1997)). This suggests that the effect, *in vivo* is likely due to the cumulative effect of a combination of factors rather than any one action, leading to short-term destabilisation in the biofilm community.

1.5.3. Urease

One of the primary factors which determine the pathogenic potential of a given species within the oral environment is pH and the ability to survive extreme pH fluctuations (Burne & Marquis, 2000; Curran *et al.*, 1995). The enamel demineralisation phase is followed by a period of alkalinisation, during which time demineralisation is reversed and the elemental components of hydroxyapatite precipitate out of suspension back onto the tooth surface.

The increase in pH has been attributed, to some degree, to the hydrolytic breakdown of urea and arginine to ammonia by some bacteria and this has positively associated with dental health (Burne & Marquis, 2000; Clancy *et al.*, 2000; Morou-Bermudez *et al.*, 2011b; Nascimento *et al.*, 2009; Van Wuyckhuyse *et al.*, 1995).

The urease system functions to protect cell viability by reducing cytoplasmic proton accumulation and subsequent acidification (Marquis *et al.*, 2003; Mobley *et al.*, 1995; Todd & Hausinger, 2000; Zotta *et al.*, 2008) and in some bacteria, urease expression is actually repressed at neutral pH (Liu *et al.*, 2012). Urea is actively transported in to the cytoplasm (though passive transport also occurs) and is broken down to produce NH_3. The NH_3 molecule is converted into NH_4^+ in a process that consumes environmental protons ($NH_3 \leftrightarrows NH_4^+$, Kidd, 2011; Sawyer, 2008). To understand the implications of ammonia production on cariogenesis, Clancy and Burne (1997) transformed urease-negative *S. mutans* with a plasmid containing the urease locus from *S. salivarius*. The experiment showed that, while urease (urea amidohydrolase, EC 3.5.1.5) activity did not affect the catabolism of sugars via glycolysis, it was effective in reducing the depth and duration of the glycolytic pH drop, indicating that urease was likely to be a relevant factor in the control of biofilm cariogenicity. These results were reflected in a study by Morou-Bermudez *et al*. (2011a) who further deduced that a negative correlation existed between urease activity and cariogenicity due in part to the increased amounts of sugar consumed by caries-active/risk individuals. This was

thought to produce an aciduric niche and an associated reduction in ureolytic, indigenous species (Morou-Bermudez *et al.*, 2011a)

There are a number of bacteria which are indigenous to the oral environment (e.g. *S. salivarius*, etc.) and utilise urease to hydrolyse urea to form ammonia and CO_2 (Burne & Marquis, 2000; Clancy *et al.*, 2000), as shown in the following equation (Vilaichone & Mahachai, 2013).

$$H_2O$$

$$CO(NH_2)_2 \longrightarrow CO_2$$

$$2NH_3$$
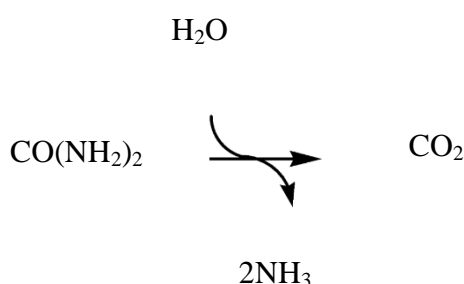
Burne and Marquis (2000) reported that urease is highly sensitive to fluoride, with 50% inhibition by concentrations as low as 0.3 mM. The inhibition of the urease enzyme by F is pH-independent however, like enolase inhibition the inhibition of urease by F *in vivo* appears to be gradual and pH-dependant. This suggests the necessity for transport and dissociation of F from HF (and subsequent cytoplasmic acidification), indicating that the inhibitor is the F anion rather than an association with HF (Barboza-Silva *et al.*, 2005; Marquis *et al.*, 2003; Todd & Hausinger, 2000).

Although urease activity is a protective mechanism at a bacterial level, increased activity at the host level, has been associated with cariogenic risk, particularly in the presence of carbohydrates (Chen *et al.*, 1998; Morou-Bermudez *et al.*, 2011b). This was thought to be due to a combination of both sucrose availability (and subsequent acid production) and urea-

limitations in the saliva. Cariogenesis results in cytoplasmic protonation which in turn increases urease activity. This utilises available urea, which then becomes limited, exposing the teeth to cariogenic demineralisation in the event of repeated sucrose exposure.

1.5.4. The arginine and agmatine deiminase systems.

1.5.4.1. The arginine deiminase system.

The highly conserved 'Arginine Deiminase system' (ADS) also increases cytoplasmic and extracellular pH via the accumulation of ammonia which is subsequently converted to ammonium, decreasing proton concentrations in the cytoplasm (Casiano-Colón & Marquis, 1988; Liu *et al.*, 2012; Marquis *et al.*, 2003). Collectively, the ADS utilise three main enzymes, arginine deiminase (EC 3.5.3.6), ornithine carbamoyltransferase (EC 2.1.3.3) and carbamate kinase (EC 2.7.2.2), to metabolise free arginine and saliva derived arginine-associated peptide 'sialin' to produce ammonia and ATP (Casiano-Colón & Marquis, 1988), as shown in Figure 7

Arginine

Arginine deiminase   NH$_3$

Citrulline

ornithine carbamoyltransferase

Ornithine + Carbamoyl

ADP

carbamate kinase

ATP

NH$_3$ + CO$_2$
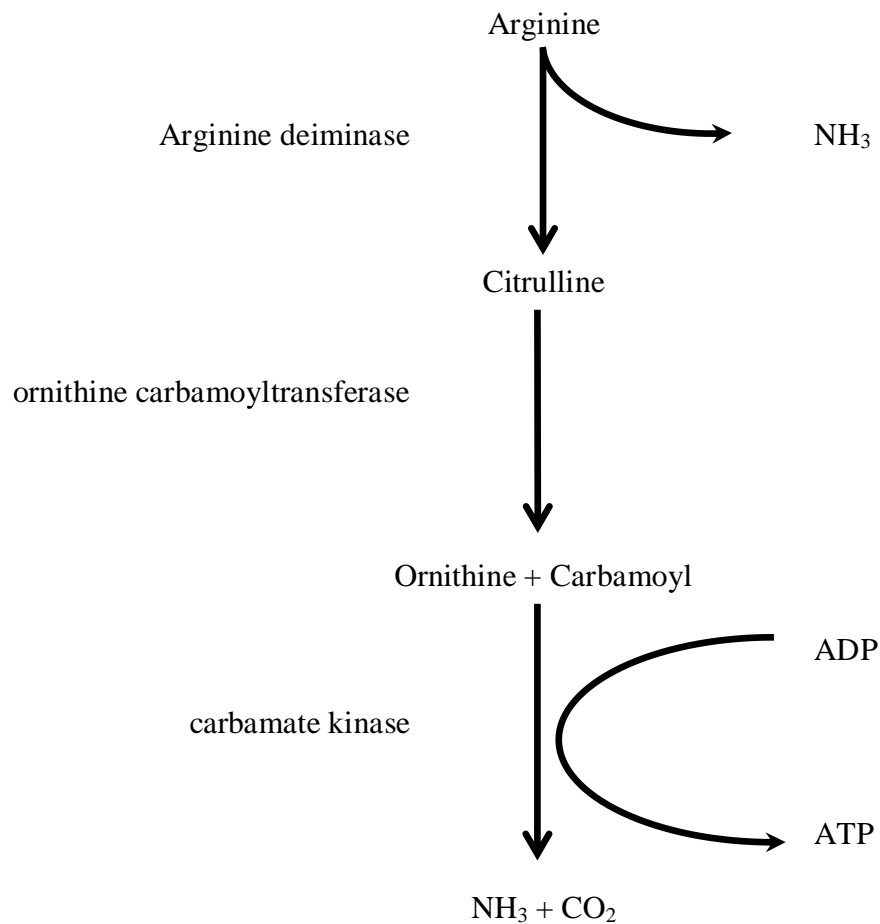
Figure 7. Arginine deiminase system.

Arginine is converted to citrulline and ammonia (NH$_3$) by arginine deiminase. Citrulline is then converted to ornithine and carbamoyl phosphate by ornithine carbamoyltransferase. The carbamoyl phosphate is converted to NH$_3$ and CO$_2$ with the phosphorylation of ADP to form ATP. Figure Modified from Ferro *et al*. (1983).

The ADS is a highly acid-tolerant process and is induced in the presence of arginine and low pH (Liu *et al.*, 2012). Arginolysis will continue at 10% output at pH values as low as 2.1 and in such, ADS-positive bacteria (e.g. S. *mutans*, *Lactobacillus casei*) have been shown to

survive for up to 6 h at pH 3.5 – 4, after treatment with as little as 2.9 mM arginine (Casiano-Colón & Marquis, 1988). These results were mirrored in other studies (Marquis *et al.*, 1987).

ADS activity in plaque samples was shown to be significantly greater in caries-free patients when compared to caries active. Furthermore, the use of arginine-positive topical dentifrices significantly increased ADS activity in caries-free subjects while causing a shift in the microbial communities of caries-active patients toward that similar to caries-free profile (Nascimento *et al.*, 2014). The presence of sodium fluoride was found to be largely ineffective against ADS although the inhibitory effect was greater on suspended cells than it was on intact biofilms (Barboza-Silva et al., 2009).

1.5.4.2. The agmatine deiminase system.

Similarly, the agmatine deiminase (EC 3.5.3.12) system (AgDS) in *S. mutans* is optimised at low pH and catalyses the conversion of agmatine to putrescine, yielding CO_2, ammonia and ATP, as illustrated in Figure 8.

It has been postulated that, as the AgDS does not produce extra-cellular concentrations of ammonia that would affect the environmental pH, it may not be an important factor within a biofilm community (Griswold *et al.*, 2004; Liu *et al.*, 2012). In fact, the AgDS was identified in numerous MS species, including *S. mutans* and *S. sobrinus* and *S. downei* and *S. rattus* and is considered to be one of the primary methods by which such acidogenic species control cytoplasmic acidity. This system, therefore, provides a significant physiological advantage to such species which occupy a potentially acidic niche, making it a major factor in AgDS-positive pathogenicity (Griswold *et al.*, 2004; Griswold *et al.*, 2006; Griswold *et al.*, 2009)

Arginine   Arginine
                  decarboxylase

Arginine deiminase
                                        CO_2

$\boxed{\text{Citrulline} + NH_3}$ Agmatine

        Agmatine
        deiminase
                                        NH_3

                                   Carbamoyl
                                   putrescine

Putrescine carbamoyltransferase   Putrescine

                                   Ornithine
                                   Carbamoyphosphate

                                                  ADP

Carbamate kinase

                                                  ATP

                              $NH_3 + CO_2$
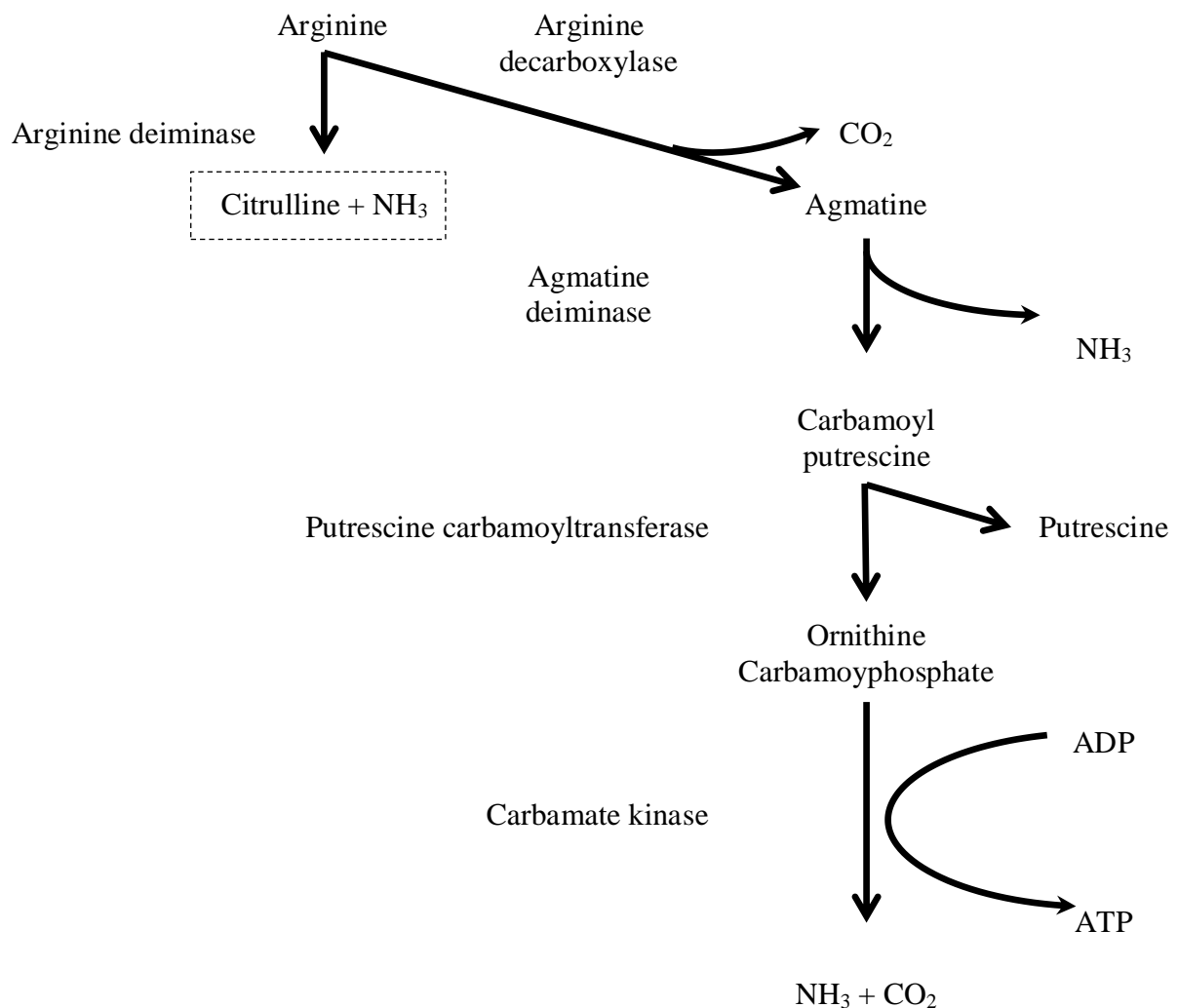
Figure 8. Agmatine deiminase system.

The Agmatine deiminase system is highly similar to the ADS. Arginine is converted to

agmatine and CO_2 by the arginine decarboxylase, which is subsequently converted to

carbamoyl putrescine and NH_3 by agmatine deiminase. In the next step putrescine carbamoyl

transferase converts carbamoyl putrescine to ornithine carbamoyphosphate which is converted

to $NH_3 + CO_2$ by carbamate kinase in a reaction accompanied by the phosphorylation of ADP

to form ATP. Modified from Griswold *et al*. (2006) and Liu and Burne (2009).

There are few references to the effects of fluoride on the AgDS, however, Jeon *et al.* (2009) looked at the effects of naturally occurring agents on the expression of genes associated with *S. mutans* pathogenicity. Of specific interest were their results pertaining to the inhibition of aguD with 250 ppm sodium fluoride. The operon 'aguBDAC' encodes for four genes (including *aguD*) which express for the AgDS (Griswold *et al.*, 2004; Liu *et al.*, 2012). Jeon *et al.* (2009) found that treatments with fluoride (250 ppm), twice a day, significantly reduced the expression of *aguD* in an *S. mutans* biofilm at 49 h (approximately pH 5.5 – 6) and 97 h (approximately pH 6) corresponding to early stage and late stage biofilm development respectively. The author provided no specific theory as to why or how this measure of inhibition took place other than as a result of rapid acidification of the cytoplasm. Agmatine deiminase activity has been shown to have an optimum activity at approximately pH 5. Studies (Griswold *et al.*, 2006) have shown a four-fold increase in activity-inhibition at pH values between 5.5 and 6 which could be linked to the AgDS running above sub-optimally and provide an answer for the results observed by Jeon *et al.* (2009)

1.5.5. F-ATPase.

The ability of cariogenic species (*S. mutans* and *S. sobrinus*) to maintain glycolytic turnover at low pH gives it a clear edge over health-associated species. Glycolysis has been shown to have an optimal cytoplasmic pH of 7 and an extracellular pH of 6. The aciduricity of these species is partly due to a proton-extruding mechanism called the '$F_O F_1$ – ATPase', also known as F-ATPase or ATP synthase (Dashper & Reynolds, 1992; Nascimento *et al.*, 2004). Investigations observing the cellular physiology of oral streptococci have shown that the cell membrane permeability, specifically the transport of protons to and from the cytoplasm, plays a major part in the homeostatic stabilisation (Bender *et al.*, 1986; Booth, 1985) of the cell.

F-ATPase is a ubiquitous, bi-directional transmembrane protein that utilises the proton motive force (ion gradient) to synthesise ATP from ADP and inorganic phosphate (Pi, Hogg, 2005; Oster & Wang, 1999). The multi-unit enzyme complex is composed of a transmembrane subsection, 'F_O' ('Factor Oligomycin', which functions as the proton translocator) and an 'internal' unit 'F_1' (Factor 1, the ATPase, Capaldi & Aggeler, 2002; Stock *et al.*, 2000, Figure 9) and can be found in the plasma membrane of bacteria, in the inner mitochondrial membrane of plants and animals and the chloroplast thylakoid membranes of plants (Capaldi & Aggeler, 2002; Oster & Wang, 1999).

The F-ATPase apparatus is fully operational at pH values of 5 and in some species can continue functioning at 40% that of the optimum at pH 4. The F-ATPase functions by channelling the passage of protons through the F_O, transmembrane subunit. This causes F_O to gradually rotate, generating a 'torque' (a rotational force) in a proteinaceous 'shaft' that connects F_O and F_1. This causes a conformational change in F_1 and the phosphorylation of ADP to ATP (Bender *et al.*, 1986; Capaldi & Aggeler, 2002; Nascimento *et al.*, 2004). As a regulatory mechanism, ATPase can also be reversed. ATP is hydrolysed in a reaction that reverses the rotation of the protein, pumping protons out of the cell and thus, acting to de-acidify the cytoplasm and creating a proton gradient (Das & Ljungdahl, 1997; Oster & Wang, 1999).
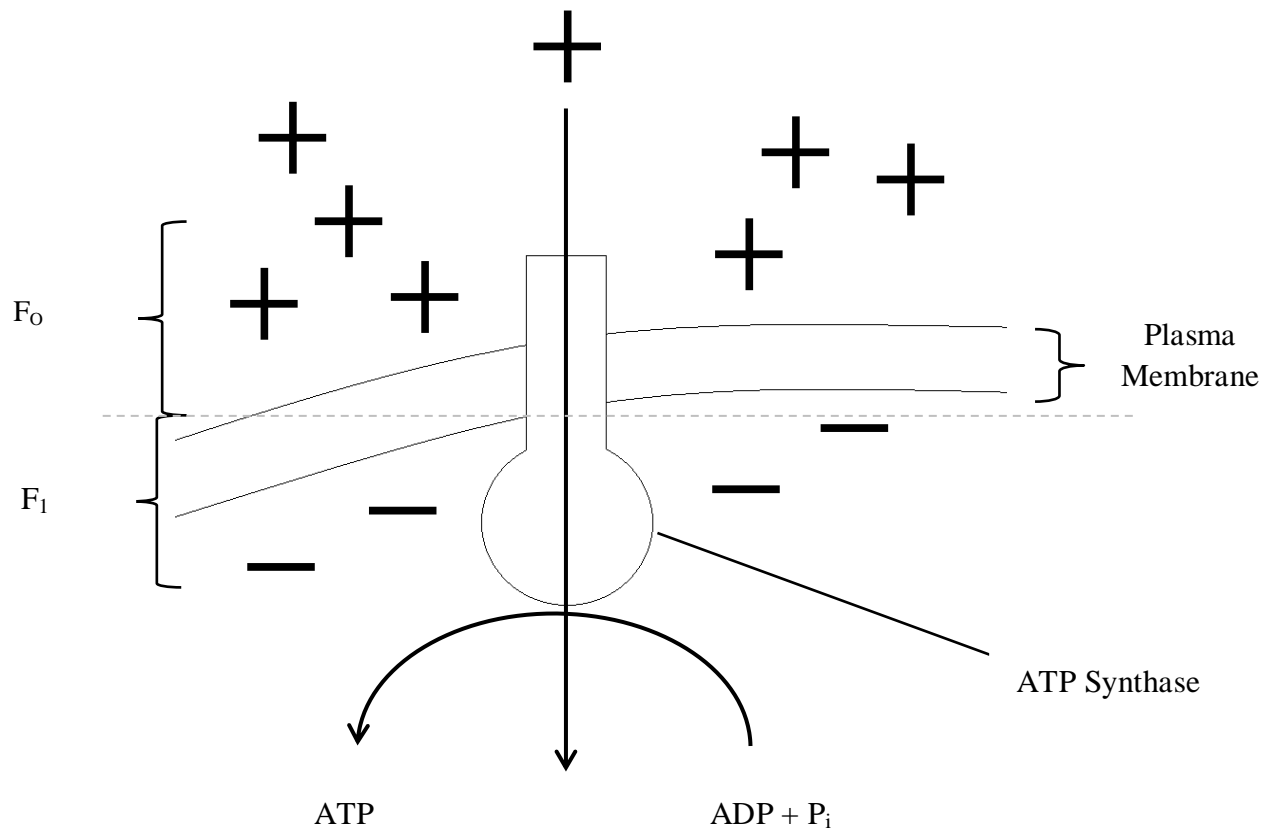
Figure 9: Schematic of the F-ATPase.

Protons move across the cell membrane, driven by a gradient, causing a partial rotation in the F_O portion, building a torque in the shaft connecting the F_O and F_1 portions. This induces a conformational change in the cytoplasmic F_1 portion resulting in the phosphorylation of ADP to produce ATP. Diagram reproduced from Hogg (2005), Oster and Wang (1999) and Oster (2000).

The inhibition of F-ATPase by fluoride is thought to be a result of the transition of HF over the cell membrane, resulting in the collapse of the proton gradient and complete metabolic breakdown (Ferro *et al.*, 1983). This was confirmed in a study (Sutton *et al.*, 1987) which found that the inhibition process was linked, not to the inhibition of ATP production but to the

inhibition of ATP-hydrolysis and subsequent proton-extrusion and may have been due to some interference with the proton pore on the cytoplasm-side of the protein. Sutton *et al.* (1987) found that while there was no direct link between pH and enzyme inhibition, the inhibition of ATP-hydrolysis of cariogenic species (e.g. *S. mutans* and *L. casei*) was found to optimise at pH 6. Because inhibition in associated the F_O portion of the enzyme, *in vivo* the cell must be in the process of concentrating HF in the cytoplasm. Therefore, F inhibition of ATPase is likely to occur at low pH. The concentration of F which inhibited *S. mutans* and *L. casei* F-ATPases by 50% was 3 mM and 25 mM respectively. Although *Lactobacillus* is known to have some resistance to fluoride (Hamilton *et al.*, 1985; Yoshihara *et al.*, 2001) the sensitivity of *S. mutans* proton extrusion may indicate that F is a significant factor in controlling species numbers with the biofilm.

1.5.6. Catalase.

Many microorganisms utilise catalases as a defence mechanism against hydrogen peroxide (H_2O_2), a by-product of host macrophages during phagocytosis, as well as aerobic metabolism in bacteria (Babior, 1984; Forman & Torres, 2001; Phan *et al.*, 2001; Taha *et al.*, 2012).

As a member of the ubiquitous 'haem peroxidase superfamily' (Peroxibase, 2012; Zamocky & Obinger, 2010) catalase provides a defensive mechanism against H_2O_2 by scavenging for oxygen and breaking down hydrogen peroxide to water and oxygen (Bol & Yasbin, 1990; Roos *et al.*, 1980), as shown in the following reaction (Alfonso-Prieto *et al.*, 2009).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$
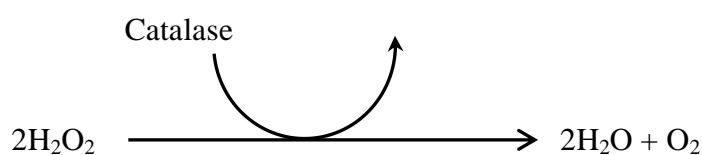
Within a mixed species biofilm, it is evident that catalase-positive species (e.g. *S. sanguis*, *S. sobrinus*, some *Actinomyces*, *Haemophilus* and *Naeslundii* spp.) can protect catalase-negative species, such as *S. mutans*, possibly by consuming, and in such, reducing the concentration of local H_2O_2 (Phan *et al.*, 2001). However, Marquis *et al.* (2003) reported that catalases are not typically transported out of the cell, meaning that H_2O_2 must first penetrate the cell membrane in order to induce catalase expression. This indicates that in order for F to be available for inhibition at meaningful concentrations, it must also cross the membrane and will likely do so in the form of HF and at low pH. Fluoride sensitivity has been shown to occur at millimolar concentrations (2.5 – 10 mM, Marquis *et al.*, 2003; Phan *et al.*, 2001; Thibodeau & Keefe, 1990) and the protection of neighbouring catalase-negative species compromised in the presence of fluoride (Phan *et al.*, 2001), meaning that catalase inhibition is very likely a factor in bacterial colonisation.

The potential benefits of F as an antimicrobial agent are as complex as they are varied. Those described above represent a small number of examples within a range of possibilities, and while the results of numerous reports of tests carried out *in vitro* appear to indicate a positive antimicrobial effect, the true impact of F on bacterial and biofilm homeostasis *in vivo* are still unclear. From the examples described above (as well as those reviewed elsewhere, Clinch, 2010; Liang *et al.*, 1995; Marquis *et al.*, 2003; Nouri & Titley, 2003; Rošin-Grget & Linčir,

2001; Yoshihara *et al.*, 2001) it is apparent that with regular access to and use of the many forms of fluoridated topical products, F can be maintained at cariostatic concentrations in the oral cavity but the clinical impact is diluted by a multitude of variables (including dosage, diet and the age and health of the host) such that the clinical implications still remain debatable.

1.6. Aims and Objectives.

The objectives of this study were to first, generate and optimise a method for the collection and analysis of oral bacterial samples from children (aged 3 and below, who were enrolled on an NHS-associated clinical study) to compare the potential benefits of improved education (behavioural) and chemical (fluoride) interventions over the standard NHS dental service. Second, data generated from the clinical study were to be compared to an *in vitro* study which analysed the bacterial distribution of biofilms cultured in the presence and absence of topical fluoride varnish. Collectively this combination of observations aimed to define the effects of improved behaviour and the use of fluoride varnish on the development and metabolic activity of oral biofilms.

2. Materials and methods.

2.1. Obtaining ethical clearance for the collection of dental plaque from children and adults.

2.1.1. Bright Smiles Study.

Bright Smiles, (A comparison of community based preventive services to improve child dental health. NIHR programme: PB-PG-0808-17228) was devised to analyse the potential benefits of novel preventative services on children in Salford and Greater Manchester. These preventative services included increasing parental understanding of caries and methods of improving dental hygiene. And secondly, the biannual use of fluoride varnish as a method in order to reduce the incidence of caries via a chemical intervention at the enamel surface.

Approximately 400 children were recruited and randomised into three test groups. Test group 1 (the Behavioural study) attempted to increase parents' knowledge of caries and oral hygiene over 10-12 annual sessions. Through informal classes, parents were taught tooth brushing techniques, about which products to use and methods of reducing sugar in the child's diet. For test group 2 (the Fluoride study), children aged between 12 and 30 months were treated with two applications of fluoride varnish per year and advised to follow a healthy diet and to brush twice a day with a fluoridated toothpaste. Finally, the control group (test group 3) would receive the standard NHS dental service but would able to access this service through at their Sure Start centre. Detailed information on the Bright Smiles study can be found in appendix VII.

The main objective of this study was to analyse the oral microbiology of a sample set of children registered onto the Bright Smiles clinical trial in order to identify trends that could

indicate the potential antimicrobial effects of fluoride varnish with particular reference to biofilm development and the reduction of cariogenic acids. Prior to the outset a number of defining considerations needed to be addressed.

2.1.1.1. Defining the primary method for plaque collection.

Firstly, due to financial restrictions there would be no funds available for the hiring of medically trained staff (dentists, dental nurses or clinicians) to aid in the collection of plaque samples from children. Although trained staff would often be present at sampling session, it was not considered possible to ensure attendance at all meetings and so the method would need to be fully accessible to the non-medically qualified user while remaining legally viable and safe.

Secondly, most samples were taken at the Sure Start centre of the volunteer's choice, and not necessarily at the centre to which they were registered. This also meant that should the volunteer require it, sampling could potentially be carried out at their home. This meant that any method used for the collection of plaque would need to be portable, contain no toxic materials, the necessity for additional auxiliary apparatus or require cold storage units such as liquid nitrogen or ice boxes. Sampling at the volunteer's home also meant that there would be instances when it would not be deemed appropriate for a male member of staff to attend alone, particularly without a female staff member in attendance. This was largely a security issue but also set in place to safeguard potential cultural or religious implications. In addition, the sampling methods would need to demonstrate flexibility in the protocol. Due to the nature of the trial, specifically the assessment of samples harvested from young children from different sites within Salford, it was paramount that once collected the sample could remain untreated and in transit for periods of up to a working day without risk of degradation and loss. This was vital in order to maintain the maximum cohort and prevent, where possible,

withdrawals or removals of registered volunteers. Furthermore, as the parent or guardian dictated the dates, times and locations of a sampling session it was assumed that sampling could be subject to a last-minute cancellation or alterations in times and/or locations. Such amendments would, it was supposed, result in one or more previously collected samples not being returned to the laboratory for a number of hours. Therefore, the method would need to be suitable for transporting sampled materials between multiple sessions throughout a day without risk of degradation or loss while being cost effective, for the same reasons. In addition to this, the potential for session cancellations was high (estimated by trial associates to be around 50%) which meant that the chosen reagents for sample collection and storage would need to be reusable and/or highly cost effective.

Thirdly, the chosen method would need to prepare the samples for use in subsequent analyses, e.g. PCR or microarray analyses. If live (plaque) samples were collected, the sampling method would need to provide a period of metabolic stasis between collection and storage, such that the bacterial components would not continue to grow/metabolise after collection, thus providing an inaccurate reading. However, should the method be one that lysed all samples immediately providing static view of the bacterial composition at the time it was taken then any subsequent analyses would be restricted to one or more molecular methodologies. This being the case, the DNA in the lysed sample would also need to remain stable without the need for immediate refrigeration or freezing after collection, a major factor when dealing with genomic materials. Given the flexibility required for sampling, as described in parameter above, the chosen method would likely be one where the plaque was lysed and DNA stabilised.

Fourthly, it was considered that the extraction of a plaque sample from a non-cooperative child would require a pain-free and rapid methodology which not only permit the entire

sample to be taken but would also not discourage repeat sampling over the entire study period.

To summarise, prior to the recruitment of volunteers from the existing Bright Smiles cohort, a sampling method would need to be devised and optimised. This method would need to be portable, and fully functional in the absence of additional processing materials and apparatus, e.g. DNA purification kits, cold storage units, centrifuges, water baths etc. It would need to be rapid, safe, ethically viable, scientifically reproducible and cost effective while being operable by untrained individuals.

2.1.2. Contacting the participant and collection of informed consent.

A parallel consideration was that, only once the method had been fully optimised could it be presented 'viable participants' (defined as, recruited onto the Bright Smiles trial but not yet exposed to clinical interventions) already registered on to the Bright Smiles cohort.

Contacting potential participants required complete methodological transparency such that individuals of all racial, cultural and educational backgrounds were able to comprehend the information and make the same informed decision. This meant that language would need to be clear and exclude unnecessary scientific terminology. This also meant that any diagrams would be informative and exclude lengthy descriptive text. Finally, each of these targets were to be achieved within the minimum possible word count. The National Research Ethics Services (NRES) guide to the preparation of information leaflets and gaining informed consent (NRES, 2011) advises the use of 'non-technical language suitable for general understanding, specifically no more incomprehensible than a non-prescribed medical insert or publically available tabloid.

2.2. The collection of human plaque and saliva for *in vitro* testing.

In order to accurately replicate the oral environment for clinical comparisons with laboratory investigations it was necessary to stockpile raw materials, specifically mixed human dental plaque and saliva, for the production of oral biofilms *in vitro*. As described, saliva is a fundamental precursor in the adherence of biofilms to the tooth surface and to ensure scientific consistency it was important that all *in vitro* testing was carried out using the same pooled raw materials. Furthermore, it was important that these raw samples were pooled from a number (> 2) of volunteers, including a range (≥ 2) of ethnic backgrounds and genders. This was done so to ensure that the specific phenotypic landscape of any one individual sample was diluted out, thus creating a more 'average' condition (Kendziorski *et al.,* 2005; Zhang and Gant 2005).

2.3. Specific requirements for the microbiological study, as described.

Further obligations including those described above, were required prior to the recruitment of volunteers and the progression of clinical and *in vitro* experimentation and are summarised as follows.

2.3.1. The completion of an enhanced disclosure (criminal records bureau).

2.3.2. The completion of a research passport (NRES).

2.3.3. The completion of a one day course covering good clinical practice (GCP, NRES).

2.3.4. To complete the optimisation of a bacterial DNA isolation protocol for the collection of dental plaque from children (1 – 3 years).

2.3.5. To produce patient information leaflet (PIL) which would lay out the specific of the microbiological study.

2.3.6. To apply for sub-amendment of the microbiological arm of the study (clinical and in vitro) to the previously approved BS trial. (Research, Innovation and Academic Engagement Ethical approval panel (University of Salford).

2.3.7. To apply for ethical approval of the microbiological arm of the study (clinical and in vitro) by the School of Environmental Life Sciences (University of Salford).

2.3.8. To confirm insurance cover for the study described.

2.4. Method discovery and optimisation.

A number of methods for the analysis of human oral bacterial samples were considered but using the Isohelix genomic DNA (gDNA) swab system (Cell Projects, Kent. UK) fulfilled many of the required criteria. Numerous sources (Quevedo *et al.*, 2011; Schaechter, 2004; Tannock, 1995) have reported that there are between 10^{10} and 10^{11} bacterial cells per gram of human oral plaque, therefore the first priority was to ascertain whether the Isohelix system was capable of sampling and stabilising potentially large quantities of genomic material with

particular reference to the morphological differences between Gram positive and Gram negative species, considering the relative abundances associated with supragingival plaque, without becoming biochemically saturated. Therefore the first round of experiments would determine whether the Isohelix kit was suitable to collect and stabilise increasing volumes of Gram positive genomic DNA, *E. coli* (NCIMB 8879) rDNA was amplified for use as a PCR positive control.

2.4.1. Isohelix kit: suitability for the collection of increasing loads of Gram positive cells.

2.4.2. *E. coli* culture

The culture of *E. coli* (NCIMB 8879) was carried out as follows. For the preparation of nutrient agar (Oxoid CM0003) 28g of nutrient agar powder was mixed per 1 litre of distilled water (as described in the user manual), autoclave-sterilised (121°C, 15 psi, 15 min), plated onto disposables petri dishes [Fisher Scientific PDS-140-045V] and cooled. Maximum Recovery Diluent (MRD, Adams & Moss, 2008) was prepared (0.05 g peptone; 0.425 g NaCl; 50 ml Distilled Water) and autoclaved under conditions described above. Ten millilitres of MRD was inoculated with a single loop of *E. coli* each, briefly vortexed (30 sec) and incubated (37°C) in an orbital shaker (G24 orbital shaker/incubator, New Brunswick Scientific) at 150rpm for 16-24 h. Suspensions were removed from incubation, briefly vortexed and measured for optical density (CamSpec 330M spectrophotometer) at 600nm, to achieve an OD of approximately 0.4. The overnight culture was then serially diluted in MRD at 10-fold increments until 10^{-7} (approximately 10^2 CFU/ml) was reached. From each dilution 100 μl aliquots was spread onto nutrient agar and incubated at 37°C for 24 h, after which all plates were counted and CFU/ml determined.

2.4.3. *Staphylococcus aureus* culture

Gram positive *Staphylococcus aureus* (8325.4) was used as the test organism for Isohelix optimisation. For culturing *S. aureus*, Nutrient agar and MRD solution were prepared and cultures were incubated and enumerated as described above for *E. coli*.

2.4.4. Polymerase chain reaction.

2.4.4.1. Primers.

Two sets of broad range primers (see Table 1), suitable for the amplification of 16S rDNA were used; set one: Forward primer 8FPL (Tm 53.7), and reverse primer, 806R (Tm 55.3), were compared for match suitability with the rRNA gene using the NCBI basic local alignment search tool, BLASTN, (Altschul *et al.*, 1997). Forward primer, 8FPL matched 16/18 (89%) base pairs between 4038760 to 4038777 with a mismatch at nucleotides nine and ten. The reverse primer 806R matched 20/20 (100%) base pairs between 4039556 to 4039537 in NCBI *E. coli* complete genome entry NC_010473.1 (Durfee *et al.*, 2008). These sequences correspond to conserved regional bases 10 – 27 and highly conserved regional base pairs, 878 – 806 respectively within the rRNA gene map (Case *et al.*, 2007).

Set two: Forward 515LP (Tm 60.5) and reverse 13B (Tm 61.4) primers promote the amplification of an 875bp molecule and were selected based on similar melting temperatures. A BLASTN (Altschul *et al.*, 1997) comparison of 515LP and 13B against NCBI *E. coli* complete genome entry NC_010473.1 (Durfee *et al.*, 2008) and returned a 100% matches between bases 4039266 and 4039283 and 4040140 and 4040121 respectively. Similarly with 806R and 8FPL, 515LP and 13B ranged between 18 and 20 nucleotides in length, had acceptable numbers of guanine and/or cytosine within the GC clamp however, the GC% was a

little higher than the previously described primers, which promoted a higher melting temperature, 60.5 and 61.4 respectively.

Name	Sequence (5'→ 3')	Reference
8FPL	AGTTTGATGCTGGCTCAG	(Eden *et al.*, 1991; Persing, 1993; Weisburg *et al.*, 1991)
806R	GGACTACCAGGGTATCTAAT	(Persing, 1993; Relman *et al.*, 1992)
515LP	TGCCAGCAGCCGCGGTAA	(Persing, 1993; Relman *et al.*, 1990; 1992)
13B	AGGCCCGGGAACGTATTCAC	(Persing, 1993; Relman *et al.*, 1990; 1992)

Table 1. Primers and sequences used for the amplification of NCBI *E. coli* complete genome entry NC_010473.1 (Durfee *et al.*, 2008)

2.4.4.2. DNA purification and amplification of *E. coli* for a PCR positive control.

An overnight culture of *E. coli* was prepared as described above. After the optical density was confirmed the remaining 9 ml of overnight culture was centrifuged (7830 rpm) for 10 min, the supernatant discarded and the pellet re-suspended in 1ml MRD. The suspension was then transferred to a 1.5 ml reaction tube and isolated using Gram negative conditions, as directed in the GeneJet (ThermoFisher K0721) user manual. Purified samples were labelled 'EgDNA1' (*E. coli* genomic DNA-1) and stored at -20°C.

Seven polymerase chain reactions were carried out with decreasing quantities of template EgDNA1 which was diluted to 20:1, 40:1, 60:1 and 80:1 and the PCR was prepared thus: 2 x MyTaq red master mix (Bioline, Bio-25041); 20 μM forward primer 8FPL (eurofins); 20 μM reverse primer 806R (eurofins); 1μl template (either 20:1, 40:1, 60:1 or 80:1); 3 x negative controls were included where the template was substituted with sterile water (to 50μl) and

cycled (Robocycler gradient 96 – 'Vicky') under the following conditions 1 x 95℃ 4 min; 30

x [95℃ 1 min, 55℃ 1 min, 72℃ 1 min]; 1 x 72℃ 5min. Upon completion, all amplified

materials were transferred to an adjacent molecular laboratory for gel electrophoresis

quantification and amplicon analysis (see section 2.4.4.3).

2.4.4.3. Non-denaturing gel electrophoresis.

A 30 ml non-denaturing gel was prepared; briefly, 0.45g agarose (Bioline BIO-41026), 3 ml,

10x TBE (Tris Borate EDTA solution, Severn Biotech Ltd - 9932), and 27 ml water were

mixed in a conical flask and boiled until all components had fully dissolved and then cooled

on an orbital shaker. To this, 3 µl of 'Gel Red' (x10, 000, Biotium 41003) nucleic acid gel

stain was thoroughly mixed in and the gel was poured into electrophoretic tank cast (Horizon

58 - Life Technologies) with the well-comb secured into place. Once set, 10x TBE running

buffer was poured into the bath (so that the surface of the gel was submerged by

approximately 1mm), the casting comb was removed and the system was left for 30 min to

equilibrate. Samples were run at 70V (~10V/cm) for approximately 1 h. DNA was quantified

in base pairs (BP) using the HyperLadder I (Bioline Bio-33053).

2.5. Gram positive gDNA isolation using Isohelix swabs.

An overnight culture of *S. aureus* 8325-4 was prepared as described previously. Overnight

suspensions were briefly vortexed, measured for optical density and serially diluted (10-fold

increments, in triplicate) in sterile MRD until 10^{-7} was reached. From each dilution 100 µl

aliquots were spread onto nutrient agar (prepared as described) and incubated at 37℃ for 24

h, after which all plates were counted and CFU/ml calculated.

The remaining *S. aureus* dilutions were briefly vortexed and allowed to settle. A single sterile swab (CellProjects - Isohelix DDK50/SK-2 kit) was placed into the suspension and gently swirled for one minute (Maximum sampling time as directed in the user manual, Cooper & Hole, 2011) and then transferred to the lysis and stabilisation solution, sealed and mixed by inversion. All samples were stored upright at room temperature out of direct sunlight for approximately 72 h. Isolation and purification procedures were carried out as directed in the user manual (Cooper & Hole, 2012b).

2.5.1. Gram positive DNA isolation analysis via PCR.

Once optimised, twelve PCRs were carried out in order to amplify genomic materials isolated from *S. aureus* dilutions (see section 2.5). This included the stock concentration plus 10^{-1} – 10^{-7} dilutions, 1 x positive control ('EgDNAH', see section 3.2.1) and 3 x template-free negative controls. The PCR was prepared thus; 2x MyTaq Red master mix (Bioline, Bio-25041); 20 μM forward primer 8FPL (eurofins); 20 μM reverse primer 806R (eurofins); 1 μl template (negative control templates were substituted with water); water (to 50 μl) and cycled (Robocycler gradient 96 – 'Vicky') under the following conditions 1 x 95°C 4 min; 30 x [95°C 1 min, 55°C 1 min, 72°C 1 min]; 1 x 72°C 5 min. Upon completion all amplified materials were transferred to a separate lab for gel electrophoresis and run under conditions described earlier.

2.6. Human oral microbe identification microarray (HOMIM).

HOMIM was a high-throughput identification array which used 16S rRNA probes to simultaneously identify the most predominant 300+ bacterial species associated with the oral cavity (Paster & Dewhirst, 2009). Briefly, species-specific 16S-targetting, reverse capture

hybridisation probes were printed onto a glass slide (Fouad, 2009; Preza *et al.*, 2009) and using broad-range primers, 16S rRNA genes within the unknown clinical sample were amplified and labelled with a cyanine (blue-green) dye molecule (Paster & Dewhirst, 2009; Preza *et al.*, 2009). The labelled amplicons were hybridised overnight to the slide-printed probes and then scanned (532nm) to detect Cy3-amplicon hybridised probes. Signals were then translated into a 'barcode' format where intensities could be read within a range of 0 (species unidentified), 1+, 2+, 3+, 4+ and 5+, the lower limit (1+) corresponding to approximately 10^4 cells (Colombo *et al.*, 2009).

Depending upon the line of enquiry, HOMIM raw data was sub-categorised into phylum genera or phylotype and compared at inter- and intra-group levels (i.e. Control, Behavioural and Fluoride) using Microsoft Excel. The relative abundance (%) was calculated by dividing the individual hybridisation intensity (HI) by the total (phyla, genera etc.) and then multiplied by 100. Results were then compared to similar studies in the literature (Crielaard *et al.*, 2011) for confirmation of category distribution. Statistical analyses were carried out as described in section 2.8.

2.6.1. Proposed Isohelix protocol and Isohelix-HOMIM trial.

The final stage of the optimisation process was to test human oral samples collected using the Isohelix system for species-specific bacterial load using HOMIM analysis. This required an alteration to the final step of the Isohelix gDNA purification protocol, specifically the resuspension of genomic materials in TE buffer (in readiness for downstream reactions) with the freezing and lyophilisation of sample materials for overseas transport. This step was complementary to the HOMIM protocol as resuspension of clinical samples prior to hybridisation was done so in TE solution. Therefore, the developed Isohelix protocol was carried out as follows.

One swab was taken from four persons (not associated with the Bright Smiles study) aged approximately 13 y, 8 y, 4 y and 2 y and assigned a unique serial numbers 2000-13.6-13, 2000-8.9-13, 2005-4.10-13, and 2005-2.5-13 respectively; the serial numbers were made up of three number sets indicating firstly the time of sample harvest (in 24 h), age of the volunteer (years and months) and then the time (hours and minutes) since previous oral clean. Samples were harvested from the posterior incisor and lateral incisor tooth surface, targeting supragingival plaque over a period of no less than 30 s. Samples were collected from the incisors and lateral incisors only, this was stipulated early in the study as these were the only erupted teeth available for sampling at Baseline but expanding the sampling sites based on newly available teeth may introduce numerous variables, reducing comparability between sample groups, in subsequent years.

The swab head was then submerged in the Isohelix lysis and stabilisation solution (Cooper & Hole, 2012a), mixed via inversion and stored at room temperature (21°C) for transportation to the lab. Upon arrival (approximately 72 h later), the swab head was removed from each sample, which were then mixed thoroughly and split in two; one half was archived at -20°C and the remainder of each sample was processed under manufacturers guidelines, stored at -80°C (72 h), freeze-dried (5 d) and despatched to the Forsyth Institute (Cambridge, MA. USA) for HOMIM analysis.

The process of splitting the samples involved one additional step after gDNA isolation. Genomic DNA was isolated as described by the manufacturer (Cooper & Hole, 2012a; Cooper & Hole, 2012b) however, the after the elution step each sample was vortexed briefly and half was transferred to a sterile tube and stored at -20°C. Sample splitting was carried out to ensure against lost or damaged samples or the need to re-assess. If samples posted to the USA were lost in transit there would be second half archived for back-up or repeat analyses. However, this posed the question as to whether sample splitting would results in the loss of

some vital data due to biases caused by handling. In order to identify any potential issues, internal controls were added to the Bright Smiles samples and analysed via HOMIM; this included both halves of one sample (see section 5.2), donated by the author which would indicate any biases in bacterial distribution.

2.6.2. The collection of oral bacteria for HOMIM analysis using the Isohelix DNA isolation system.

The finalised method for the collection of oral bacteria from the Bright Smiles cohort and subsequent analyses are as described in sections 2.1.1, 2.1.1.1, 2.1.2, 2.3 (2.3.4-5), 2.6, 2.6.1-2 and 2.8, however, briefly, Isohelix kits (DDK-50 DNA stabilisation and extraction kit; SK-3 individually wrapped swabs and sterile 2 ml screw cap tubes) were implemented as described by the manufacturer (Cooper & Hole, 2012a; Cooper & Hole, 2012b). Lysis solution was prepared, no more than eight hours prior to the sampling appointment. For each sample, 20 µl PK was mixed with 500 µl LS in a sterile 2 ml crew cap, briefly vortexed and stored at room temperature. Based on the success of the pilot study, described in sections 3.9., 3.12., 3.13., and 3.14, the sampling process was modified to include both the buccal and palatal surfaces of the upper incisors.

Immediately before sampling, the swab was removed from its packaging, taking care not to touch the swab head with hands, clothing or other equipment. Holding the swab by the handle and using firm circular motion, the swab head was rubbed over the inner and outer surfaces of the incisors and lateral incisors, (covering tooth surfaces and gingival margins) for no less than 30 seconds. The swab was immediately placed into the lysis solution. Care was taken not to contaminate the sample head, which was severed from the handle at the 'break point' allowing the tube cap to be screwed shut. The tube was briefly shaken to mix and then stored at room temperature. Finally, prior to HOMIM analysis all samples harvested from the

Bright Smiles cohort were split into two (section 2.6.1), one half was sent to HOMIM for composition analysis and the other was archived at -20°C. Species identification and distribution was carried out by HOMIM as described in section 2.6.

Hybridisation data was converted into three taxonomic levels, the phylum, genus and phylotype and mean profiles were analysed and compared for trends in biofilm development that could be linked to the implementation of described interventions. Specific analyses were carried out on the relative abundances of phyla, genera and phylotype at both inter- and intra-study categories which included intervention groups (e.g. Behavioural vs Fluoride) and sampling periods (e.g. Baseline vs. Round 1).

2.7. The plaque glycolysis regrowth methods (PGRM).

The plaque glycolysis regrowth method (PGRM) is a standardised protocol (FDA, 2003) devised to observe the anti-glycolytic effects of a given compound, with special emphasis on cariogenic biofilms. The protocol, as described in numerous works (Kasturi *et al.*, 1995; Lemos *et al.*, 2010; Liang *et al.*, 1995; White *et al.*, 1994; 1995) first requires the maturation of a test culture, be it mono- or metagenomic in nature, and in a sessile conditions or planktonic suspension although oral biofilms were the target of its conception. Samples were treated with the test compound prior to or during a sugar challenge in order to observe the differences in acid production. This study would analyse the potential benefits of FP varnish on the production of acids from oral biofilms *in vitro*.

The FP instruction manual required that teeth were professionally cleaned, rinsed and air dried. The varnish is then applied to all enamel surfaces and flossed in to include proximal surfaces and then permitted at least 60 sec to dry. Patients are then required to refrain from eating and drinking for at least 45 min after application. However early observations of FP

applications under trial conditions revealed that, because the children being treated were generally uncooperative, perhaps due to the taste of FP or treatment by an unknown adult, the manufacturer's protocol was rarely possible to complete with the accuracy described in the manual. It was therefore considered prudent to investigate whether FP had any anti-glycolytic effect under a range of partial and complete treatments.

The following protocols were used, firstly to optimise the PGRM and then re-adapted to include varying treatments of FP to assess the potential effects on biofilm development. The 0% FP control treatment was compared with a 100% coverage (expected to provide a result consistent with the manufacturers design), a 50% treatment (to provide evidence of fluoride-based inhibition after a good but partial application) and finally a 100% treatment in 'excess saliva' (to simulate a result consistent with an expected field treatment where applications are made directly over uncleaned, undried teeth).

2.7.1. Adsorption buffer (AB).

AB solution (Lemos *et al.*, 2010) was prepared as follows (molar values indicate final concentration); 50 mM KCl (Sigma-Aldrich 60137-1L), 0.35 mM K_2HPO_4 (Sigma-Aldrich P8584-1L), 0.65 mM KH_2PO_4 (Sigma-Aldrich P8709-1L), 200 µl 1 mM $CaCl_2$ (Sigma-Aldrich 21114-1L), 0.1 mM $MgCl_2$ (Sigma-Aldrich 63020-1L). The buffer was adjusted to pH 6.5 and topped up to the required volume and stored at room temperature out of direct sunlight.

2.7.2. Low molecular weight medium (LMW).

LMW medium with 1% sucrose and 1% glucose and sugar-free were prepared as directed by (Lemos *et al.*, 2010); molar values indicate final concentration. Twenty-five grams per litre

tryptone [Sigma-Aldrich T7293-250G] and 15 g/litre yeast extract [Sigma-Aldrich Y1625-250G] were dissolved into distilled water (ensuring that volumes were adjusted to account for buffer and sugar solutions, added later) which was then filtered through the Pellicon XL ultracell [Millipore UK Ltd PXC0 10C 50] until approximately half the desired permeate was collected. The filtered solution was then buffered with 25 mM KH_2PO_4 and 4 mM $MgSO_4$ and filtration was continued until the desired volume was reached. The permeate was divided into three equal volumes, mixed thoroughly and autoclaved at (121°C, 15 psi, 15 min). Once sterilised, each aliquot was mixed thoroughly and to the first, sucrose [Sigma-Aldrich S7903-250G] solution was added to a final concentration of 1% (LMWs). To the second, glucose [Sigma-Aldrich 49163-100ML] was added to a final concentration of 1% (LMWg) and to the third, sterile distilled water was added to equilibrate its volume and dilution with that of LMWg and LMWs (LMW_0). Each of the three media were sub-divided into 50 ml aliquots and stored at 4°C until required.

2.7.3. The collection of human whole saliva.

Adult volunteers, sourced at the University of Salford, were asked to avoid alcohol for 24 h prior to sample collection, not to brush their teeth or treat mouth with antibacterial products after 10pm the previous night and before lab presentation, and not to eat within 60 min prior to sample collection (Salimetrics, 2009). Volunteers that had completed a course of antibiotic treatment within 12 weeks prior to sampling were automatically excluded. Prior to sampling, volunteers were asked to rinse their mouth with bottled water in order to remove any food debris, swallowing the water to increase hydration. They then took a 10 min break after rinsing and before saliva collection to avoid sample dilution. Volunteers were then asked to chew on 5 cm^2 piece of parafilm [VWR 291-1214] to stimulate saliva flow, expectorating as necessary into a sterile 50 ml [VWR 525-0156] tube. The collection process started at

approximately 11.00 am so that saliva stimulation was associated with the chewing of paraffin and not due to the recent consumption of food (breakfast) or as a result of hunger for the next meal. Sampling was carried out for 45 min (or until 25 ml was collected) at which point a 30 min break with hydration was taken then sampling was repeated until a further 25 ml was collected. All raw saliva samples were stored, untreated at -20℃ until required.

2.7.4. Whole saliva clarification.

Saliva clarification and sterilisation was carried out as described by (Lemos *et al.*, 2010). All raw untreated saliva was pooled and mixed thoroughly with AB solution at a ratio of 1:1. Next, 500 µl/litre 0.1M pefabloc, (Roche Diagnostics Ltd #11429868001) protease inhibitor was added and mixed in thoroughly with a magnetic stirrer. The saliva was then divided into 50 ml aliquots and then centrifuged at 5,500 x g for 10 min until clarified, the supernatant was then aseptically removed and filtered through a low protein binding 0.22 µm filter [VWR 514-1117P] at approximately 4℃ and filtrates were immediately frozen and stored at -20℃ until required.

2.7.5. The collection of human whole dental bacteria.

Adult volunteers, sourced at the University of Salford, were asked to avoid alcohol for 24 h prior to sample collection, not to brush teeth or treat mouth with an antibacterial products after 10 pm the previous night and before lab presentation and not to eat within 60 min of sample collection. In the interests of reducing genotypic and phenotypic anomalies associated with using a single plaques sample, bacterial flora intended for culture was pooled from two or more individuals, as previously described (Lemos *et al.*, 2010; White *et al.*, 1995).

Volunteers were asked to roll a sterile cotton swab [Fisher Scientific DIS-290-019] over the entire dentition for 60 sec, the swab head was aseptically cut off and placed into a 50 ml tube containing 5 ml, LMWg pre-warmed to 37°C and vortexed for 20 sec to re-suspend all plaques. The swab head was then firmly rolled around the inside of the tube to squeeze out any liquid and discarded. Re-suspended plaques were subjected to a 60 s round of sonication (100W, Sonicor sonic bath SC-120TH) in order to break down the ESP matrix then briefly vortexed. One millilitre was taken from each sample, pooled and diluted 20:1 in sterile LMWg and cultured overnight at 37°C, 5% CO_2. Overnight cultures were briefly vortexed, then diluted in fresh sterile 37°C LMWg with 10% glycerol [Sigma-Aldrich G901-2] to achieve and OD of 0.5, as described previously (Söderling *et al.*, 2011) and split into 1 ml aliquots and stored at -80°C

2.7.6. Salivary pellicle formation (sHA).

Hydroxyapatite (HA) discs (Clarkson Chromatography, South Williamsport, PA. USA) were treated with clarified human saliva in order to produce an artificial salivary pellicle, as described elsewhere (Lemos *et al.*, 2010). Discs were aseptically placed into custom-prepared holding brackets, prepared from orthodontic wire [DB orthodontics, DB01-116R], as described by Lemos *et al*. (2010) and transferred to the pre-warmed culture dish [Fisher Scientific TKT-190-010Y). Each disc was submerged in approximately 2.8 ml (or until fully submerged), clarified human saliva and incubated at 37°C for 1 h in an orbital shaker. The HA disc was then removed from incubation, shaken to remove excess saliva (sHA) and dipped once in sterile AB solution.

2.7.7. The inoculation of sHA discs with human mixed oral bacteria.

PGRM tests were set up as directed by Lemos *et al*. (2010). A frozen, over-night, mixed oral bacterial culture (OD 0.5, as described above) was thawed on ice then diluted to 1:250 in fresh 37°C LMWs, permitting 2.8 ml/disc, approximately 5×10^6 CFU/ml. To each disc, 2.8 ml of bacterial-inoculated LMWs was added and the culture was incubated at 37°C, 5% CO_2 for 24 h. All discs were aseptically transferred to fresh LMWs every 24 h for 4 d, at which point the biofilm was considered to be mature.

On day five prior to PGRM testing, and taking care not to damage the matured biofilm, discs were removed from the LMWs and gently submerged in fresh 37°C LMW_0 and incubated for 30 min to allow the biofilm equilibration to fresh media. A micro pH probe [InLab Micro pH probe 51343160] was set in place, ensuring that the reference junction faced in towards the centre of the disc and was clamp-secured to prevent movement and thus minimising shear damage to the culture. The biofilm was then treated with 200 mM sucrose (final concentration) and the pH measured every 30 sec for the initial 5 min and every 5 min for the remaining 85 min. In each case, PGRM cultures were run in duplicate in order to provide untested, undamaged biofilms HOMIM analysed.

2.7.8. PGRM: The antimicrobial effects of Fluor Protector (FP) Fluoride varnish testing.

2.7.8.1. Complete (100%) FP coating.

Prior to pellicle processing (as described above) discs selected for 100% varnish testing were held fast using ethanol-flame sterilised forceps and using the manufacturers application brush, treated with FP on all exposed surfaces. Discs were air-dried for 60 s, as described by the

manufacturer (Anon, N.D.), secured in a sterile orthodontic bracket and placed into the culture well and treated with saliva, as described in section 2.7.6.

2.7.8.2. Partial (50%) FP coating.

Prior to pellicle processing, discs selected for 50% varnish treatment were held fast using ethanol-flame sterilised forceps and using the manufacturers application brush, treated with FP on 50% of each face, across the diameter of the disc. Discs were air-dried for 60 s, secured in an orthodontic bracket and placed into a culture well ensuring that the varnish coating pointed towards the bottom on the well. This ensured, firstly that the varnish coating could be located throughout incubations and so that pH testing at day 5 could be taken from the untreated section of the disc face.

2.7.8.3. Control (0%) FP coating.

For the 0% FP control, no FP was added to the disc. Salivary pellicle processing was carried out as was the inoculation and incubation of oral biofilms as described (sections 2.7.6. and 2.7.7. respectively)

2.7.8.4. Complete coating (100%) in excess saliva.

For varnish in excess saliva, discs were treated with a salivary pellicle as described in section 2.7.6. However in this case, after 30 min incubation, discs were removed from the saliva and dried using a cotton bud, in an attempt to simulate the drying of the tooth surface, a necessity in the application of FP in the field. Fluor Protector varnish was then applied, covering 100% of the discs surface, as described previously. The disc was then immediately returned to fresh

saliva without the drying period and incubated for a further 30 min, with shaking to complete the pellicle production step. Once completed, the discs were shaken to remove excess saliva, dipped once in AB solution and inoculated with diluted overnight bacteria as previously described (section 2.7.7.).

2.7.8.5. Biofilm dry weight measurements.

Prior to biofilm PGRM testing, 1.5 ml reaction tubes [Greiner Bio-One 616 201] were sterilised by autoclave (121°C, 15psi, 15 min), their lids were fastened shut and the lid-hinge cut to allow complete removal during the lyophilisation process. With the lids in place, tubes were labelled and weighed to the nearest microgram.

Biofilm-treated sHA discs were carefully removed from the culture dish and gently dipped into 0.89% NaCl solution three times (to remove non-adhered cells) and dropped into a sterile 50 ml reaction tube, containing 1 ml of sterile 0.89% NaCl and taking care not to collide with the tube sides. A further 0.5 ml 0.89% NaCl was added and the tube (totalling 1.5 ml) which was placed in a sonic bath for 15 min. Post-sonication, discs were removed using a heat-sterilised (cooled) wire hook, checking thoroughly to ensure that all biomass was removed from disc face, which were then discarded.

The homogenate was then mixed via gentle pumping action and transferred to the corresponding (labelled) and pre-weighed 1.5 ml micro-centrifuge tube. Homogenates were centrifuged (13K rpm) for 5 min after which the supernatant was removed and the pellet was stored at -80°C for at least 24 h in preparation for the lyophilisation. After ≥ 24 h at -80°C, pellets were lyophilised for ≥ 2 d or until no moisture remained in the tube; tubes plus lid and pellet were then re-weighed. The original tube weight was subtracted from the resulting tube

plus pellet weight to ascertain the approximate biofilm dry weight. Pellets were then processed using the GeneJet gDNA purification kit, and dispatched for HOMIM analysis.

2.8. Statistical Analyses.

HOMIM data, specifically the HI, were converted to relative abundances by phyla, genera and species/phylotypes and were found to have a non-normal distribution using normality tests ($P < 0.01$) and were subsequently analysed using non-parametric testing. The relative abundances (%) of target groups were analysed using the Kruskal-Wallis test. This is a non-parametric test which permits two or more samples to be compared for rank distribution similarities. This was used for testing for significant differences between large groups of data such as the distribution of genera within a treatment group. The Mann-Whitney U test is a two sample rank test and a non-parametric, alternative to the student T-test. The test requires that the data was independent and continuous and, like the Kruskal-Wallis, provided a confidence of ranked medians. This was used to compare individual samples (e.g. bacterial profiles by phyla, genera or species) when larger group analyses provided P values close to the alpha value of 0.05 (Camelo-Castillo *et al.*, 2015; Zhang *et al.*, 2015).

The Analysis Of Variance (ANOVA), general Linear Model (GLM) permits the univariate (both descriptive and inferential) analysis of factors and covariates within a combined model. In this case the GLM was used to compare pH profiles of PGRM experiments which were of equal size and unbalanced and combined the variable changes in pH (the covariate) due to a sugar challenge and after the fluoride treatments (the factor) over time (Anon, 2014).

The two-sample T-test was used to compare the population means for normally distributed experimental triplicates of dry weight analyses of biofilms cultured for PGRM and HOMIM testing. The Shannon-Wiener indices are a measure of the diversity within a sample

community and are calculated using the following equation, as described previously, where pi in the proportion of individuals found in the sample (Gafan *et al.*, 2005; Jost, 2006).

$$H' = - p \sum i \ln pi$$

This was used to quantify the distribution of sample species in order to observe potential differences between sample sets which were divided into hours between brushing and sample collection. Where there were two or more samples occupying the time slot (between brushing and sampling) within the Shannon-Wiener indices per study group, a Mann-Whitney U test compared sample sets for significance between diversity distributions. All the analyses were carried out using Minitab 16 or using the Microsoft Excel spread sheet facility.

3. Results.

3.1. Preparation for the study.

Prior to the outset of this study there were a number of targets which needed to be met. These included the discovery and optimisation of a method suitable for the collection of dental biofilms from young children by non-medically trained staff, the acquisition of ethical approval and the subsequent recruitment of participants.

Primary contact with the potential volunteer was made with the Patient Information Leaflet (PIL, appendix II). As all potential participants were already registered on to the Bright Smiles study, recruitment was carried out by Bright Smiles staff via post and face to face, during the trial meetings.

Before any samples could be collected, volunteers needed to be fully informed on the project targets and consent to trial registration. Therefore, prior to their initial study-specific intervention, participants were sent the P.I.L. by post to ensure that they had sufficient time to read and understand the information provided. Volunteers were then approached during and prior to treatment sessions and asked if they were interested in participating in the microbiological study and, if they agreed, they were asked to sign an informed consent.

3.2. Obtaining ethical clearance.

The initial studies focussed on the optimisation of plaque collection methods in line with legal and ethical obligations which included the completion of background checks (Criminal Records Bureau; CRB), the attendance at courses which outline the legal and ethical requirements associated with a clinical trial (Introduction to Good Clinical Practice; GCP,

attended 07/12/2011, Appendix V) and the submission of documents of proof which indicated suitability for the role as a researcher (Research Passport). It also required an ethically appropriate method of communicating the chosen methods to the public (P.I.L.). All ethical requirements were met between May 2011 and February 2012. The following section highlights the details of each obligation as well as the dates of which each target was achieved with references to documentation in the appendix. Some of the documents were excluded from this thesis because they contained personal information about the author which was deemed inappropriate for publication; specific details to follow.

3.3. Obtaining a background check - Disclosure and Barring Service (DBS).

The Disclosure and Barring Service (DBS), previously known as the Criminal Records Bureau check (CRB) is a government run background check developed to safeguard recipients employers and employees associated with the delivery of public health or primary care (Anon, 2015). This is a background check which investigates any previous convictions and draws a link to the position or role the applicant is seeking to attain to assign the individuals ethical and legal suitability. This may apply to the child adoption process, individuals seeking employment in the care of vulnerable individuals or in this case, students taking part in a clinical trial.

Here an advanced disclosure was submitted which included all of the standard checks, specifically details regarding unspent convictions, cautions or reprimands and includes any information held by local police forces.

The enhanced disclosure was passed on May 14th, 2011. This document was not included in the publication of this thesis as it contains personal information about the author and employees of the University of Salford who assisted in the completion of the application.

3.4. Method development and the production of a Patient Information Leaflet (P.I.L).

The methods by which the potential trial volunteer was contacted required complete transparency. It was vital that the reader, being from any educational, cultural or racial background could comprehend the information and was able to make an informed decision before consenting to participate. This meant that all language used in the text excluded unnecessary scientific terminology while illustrations were comprehensible without specialist knowledge. The primary contact also included an informed consent form which the volunteer would sign, acknowledging that they fully understood the information and consented to be registered onto the trial.

The P.I.L. was the medium by which the potential participant would be introduced to the microbiological arm of the trial and needed to document a fully optimised and ethically approved methodology. Therefore the methods by which plaque would be collected and analysed needed to be fully identified and optimised before they could be presented to the Bright Smiles cohort. Therefore in order to comply with trial and ethical regulations pertaining to the collection of oral samples from children by untrained personnel, it was decided that the parent or guardian responsible for the child would be taught to collect the sample under the guidance and supervision of a fully trained trial-associated member of staff. This would not only ensure compliance with the law but would likely result in a better quality sample as the child would likely be less distressed during sample collection. In order to achieve maximum interest in the project and with the assistance form the Bright Smiles team the PIL was laid out under a set of sub-headings to include short paragraphs explaining why the individual had been invited, what their responsibilities would be should they accept the invitation, sampling methodology and what we (the research team) hoped to achieve. Each section was clear and concise so as to inform the individual without overwhelming them in

complexity. The PIL also included a set of contact details, encouraging their potential participant to make contact to discuss any parts of the project that they were unsure about.

The mixed species bacterial DNA isolation protocol (Appendix I 'Micro Protocol'), participant information leaflet (Appendix II 'PIL') and informed consent form (Appendix III 'IC form') were submitted simultaneously for ethical assessment by the National Research Ethics Service ('NRES', Northwest – Greater Manchester East) committee. Approval was granted and the details were sub-amended to the Bright Smiles approved study via the Integrated Research Application System (IRAS) November 1st, 2011 (Appendix IV 'sub-amendment Form 6, 24th Oct 2011').

3.5. Introduction to good clinical practice (GCP).

Good clinical practice training is a key requirement for any individual wishing to be involved in clinical research (NIHR, 2015a). The day session, prepared by the National Institute for Health Research and Clinical Research Network (NIHR CRN), included the following modules.

1. The value of clinical research and the role of the NIHR CRN.

2. GCP: The standards and why we have them.

3. Study set-up: Responsibilities, approvals and essential documents.

4. The process of informed consent.

5. Case report form, source data and data entry completion

6. Safety reporting in clinical trials.

Registration and completion of the Introduction to GCP was carried out on December 7th, 2011 (Appendix V - GCP Certificate)

3.6. Amendment to the Bright Smiles trial.

To gain access to the BS trial and its cohorts an additional application was made to the Research, Innovation and Academic Engagement, Ethical Approval Panel (College of Health & Social Care, University of Salford) in order to sub-amend the microbiological arm of the study to the previously approved School application; confirmation of approval was received December 19th, 2011 (Appendix VI 'amendment to REP10/32 - Approval').

3.7. Research passport.

The research passport system (NIHR, 2015b) provides a method for higher education research departments to share the experiences and qualifications held by their research students or staff with the relevant departments within the NHS, in which the research is to be carried out (NIHR, 2010). The document outlines the educational backgrounds which are relevant to the research programme at hand and requires the submission of an updated curriculum vitae and a medical questionnaire which is signed by the applicant's general practitioner.

Here the medical questionnaire was submitted with a research passport application and curriculum vitae to the NHS SalfoR+D for approval; confirmation was received January 12th, 2012. These three documents were excluded from this publication as they contained personal details, including those pertaining to the health status of the author.

3.8. Ethical clearance, University of Salford.

In order to gain full approval from the University of Salford, College of Science and Technology Ethics Panel (CST) it was important to outline the project along with full risk assessment as described previously.

In addition to the methodology for the collection of dental plaque for trial analysis, a second application was made to the school ethical committee in order to amend new methods for the collection of and use of human plaque and saliva for *in vitro* investigations to the previous application. For the development of human biofilms *in vitro*, it was necessary to stockpile and pool human plaque and saliva samples to ensure that all subsequent experiments carried out were done so using aliquots of the same stock materials. A specific example here would include the PGRM which first required that hydroxyapatite disc substrates to be treated with saliva in order to develop 'salivary pellicle', (a proteinaceous coating which is necessary for oral bacteria to adsorb to the enamel surface) which was then inoculated with oral bacteria under conditions which promote biofilm formation. The collection of oral bacteria and saliva from a number of volunteers was paramount as pooled sources comprised of samples from multiple donors would be more representative of an 'average' in that it will dilute any phenotypic anomalies associated with the individual which could alter the developmental processes away from the wild type. These consent forms were excluded as they contained personal information of colleagues and staff members from the University of Salford.

The application to the School of Environment and Life Sciences (Appendix VII 'ELS School Ethics Application') was submitted November 23th, 2011 with additional amendments were submitted January 17th, 2012 (Appendix VIII 'Risk assessments – amendments') with ethical approval being granted on February 7th, 2012 (Appendix IX 'School confirmation letter). In addition, it was vital to ensure that there was sufficient insurance cover for a Salford University-associated clinical trial, with particular reference to young children. An application

to U.M Associated Ltd (http://www.umal.co.uk/) for details regarding School research insurance cover was made on January 11th 2012 (Appendix X 'Clinical Trials Form CT09-10') and full confirmation including details of indemnity period was received January 17th, 2012 (Appendix XI 'Salford CT Q449 – The Antibacterial Effects of Fluoride').

3.9. The development of a method suitable for the collection of dental biofilms from young children.

The initial set of investigations was devised to ensure that the Isohelix system was suitable for the long term collection and subsequent analyses of dental biofilms from children and had to satisfy a number of primary requirements. Firstly, it has been shown that supragingival plaque is comprised mainly of Gram positive bacteria, predominantly streptococci and so it was essential that the kit was tested for the capacity to lyse and stabilise increasing loads of Gram positive cells. Secondly, once the above criteria had been satisfied the method and the process of analysis had to be optimised. It had been decided that the HOMIM facility would likely present the most efficient way of analysing large numbers of samples over a three year period. The final stage of the extraction and purification of gDNA from target cells using the Isohelix system was the resuspension of purified materials in TE buffer in readiness for downstream reactions. In the case of this study, it was intended that this step was removed and the DNA sample would be freeze-dried in preparation for delivery to the Forsyth Institute', as their initial resuspension step utilises TE buffer. This second investigation would also determine whether individuals who were unfamiliar with the Isohelix swabbing kit could be easily trained to collect a sample under the supervision of a qualified trial associate, and whether the kit could collect dental biofilm samples which were consistent in bacterial composition with the literature.

The Isohelix system was designed and developed for the collection of DNA samples for tests which include paternity investigations, forensic investigations and even veterinarian studies (Cooper & Hole, 2011) but to the authors knowledge and to that of the manufacturer, the system had never been used for drug trials and the effects on oral and dental bacteria. Therefore, a trial study was carried out using dental samples collected from children of a Salford University-associated parent who had volunteered to assist with the study optimisation. Samples were collected over 30 sec from the upper incisors and lateral incisors only, as Bright Smiles trial volunteers would probably only have these teeth at Baseline and to expand the range of sampling upon the eruption of new teeth may skew results in subsequent years. The parent was taught to take the samples which would then undergo DNA extraction, purification and lyophilisation and be delivered to HOMIM for analysis. The results of the HOMIM process were returned as hybridisation intensities for every phylotype identified per sample submitted which was then converted into relative abundances at different taxonomic levels, (including species, genera and phyla). It was these data that were used to confirm method suitability against sources in the literature.

Therefore, the first set of experiments would include a PCR study into the collection of Gram positive cells and required the optimisation of positive and negative internal controls for a PCR reaction. This would include the design and production of PCR primers which target 16S rDNA for gene amplification using PCR and amplicon quantification using gel electrophoresis.

3.10. The development of a positive internal control for a preliminary PCR study in the optimisation of the Isohelix system.

In order to optimise the PCR it is prudent to first secure the internal positive and negative controls. Four isolates of *E. coli* 16S rDNA template were amplified in decreasing dilutions

(stock concentration at 20:1; 40:1; 60:1 and 80:1) and run through a 1.5% agarose gel with the aim of optimising a standardised positive control for future PCRs. The genomic DNA from an overnight culture of *E. coli* was purified using the GeneJET genomic purification kit (Fermentas K0721) under Gram negative conditions, and the rDNA was amplified as described previously. Different dilutions used in the preparation of template DNA showed no quantifiable difference in amplicon size (> 100 ng) and all bands ran at molecular weights of approximately 800bp (Figure 10), consistent with the amplicon expected using primers 8FPL and 806R.
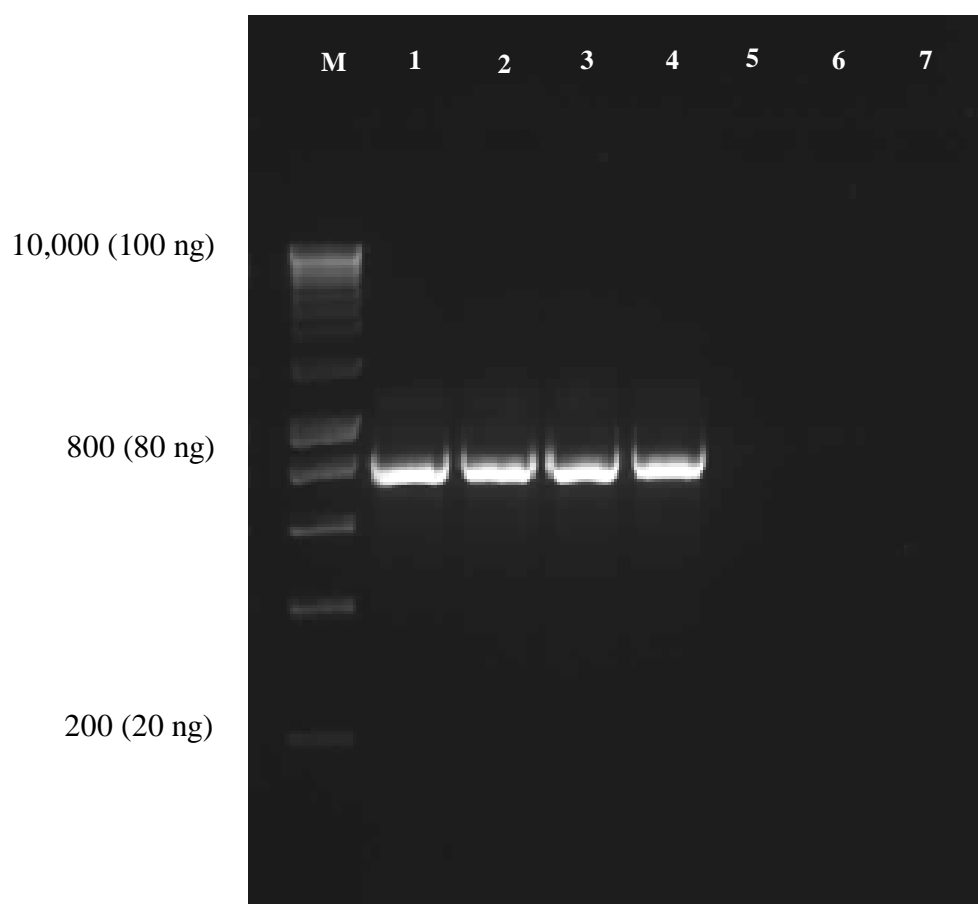
Figure 10. Agarose gel electrophoresis of PCR products for *Escherichia coli* 16S rDNA at different dilutions.

Numbering across the top of the figure indicate lane numbers and molecular weight in base pairs and band mass (ng) for mass determination (in brackets) are presented down the left side. Bands from left to right: M - Hyperladder I; 1 - EgDNA1(20:1); 2 - EgDNA1 (40:1); 3 - EgDNA1 (60:1); 4- EgDNA1(80:1); 5 - Template negative; 6 - Template negative; 7 - Template negative.

3.11. Gram positive gDNA isolation using Isohelix swabs.

In order to test the capability of the Isohelix system to deal with increasing dilutions of Gram positive organisms, overnight suspensions of *S. aureus* were diluted from stock concentration (approximately 10^9 CFU/ml) and then in ten-fold dilutions from approximately 10^8 to 10^2 CFU/ml. Suspensions were mixed immediately before sampling to ensure thorough suspension and sampled with a stirring motion over 60 sec, using the Isohelix kit swab. Genomic DNA was isolated using the kit's purification system and used as a template for the amplification of 16S rDNA by PCR (Figure 11). Clear bands decreased in mass, ranging from > 100 ng and provided a visual confirmation of the serially diluted template. Faint bands visible in template-negative lanes 11 and 12 may indicate low concentrations of bacterial contamination.
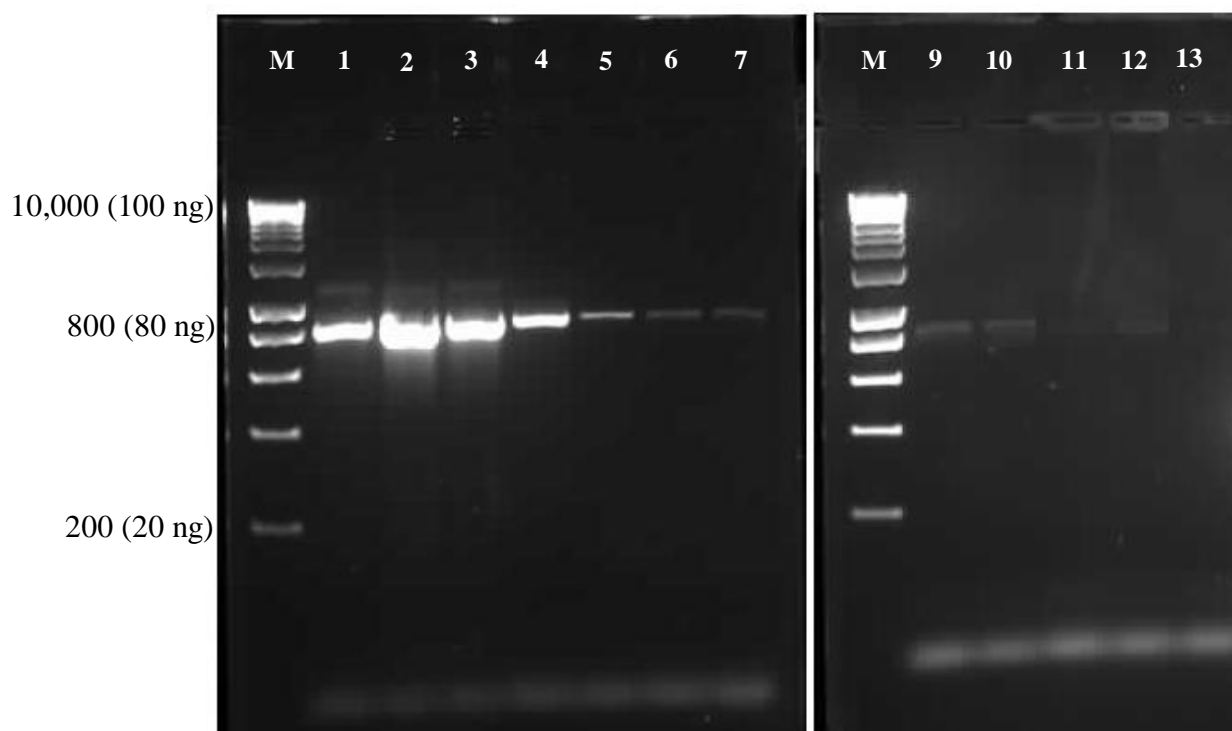
Figure 11. Agarose gel electrophoresis of PCR products for *Staphylococcus aureus* 16S rDNA at different dilutions.

Lane numbers are displayed across the top of the figure and molecular weight in base pairs and band mass for mass determination (in brackets) are presented down the left side. From left to right (dilution factor and [mean CFU/ml of suspension sampled]): M - Hyperladder I; 1 - EgDNAH; 2 – SA - Stock [$\approx 3.6 \times 10^9$]; 3 – SA - 10^1 [$\approx 2.9 \times 10^8$]; 4 – SA - 10^2 [$\approx 2.9 \times 10^7$]; 5 – SA - 10^3 [$\approx 3.5 \times 10^6$]; 6 – SA - 10^4, [$\approx 3.2 \times 10^5$]; 7 SA - 10^5 [$\approx 1.7 \times 10^4$] ; M - Hyperladder I; 9 SA - 10^6 [$\approx 3.6 \times 10^3$]; 10 SA - 10^7 [$\approx 2 \times 10^2$]; 11 negative control 1; 12 Negative control 2; 13 Negative control 3.

A separate investigation into the positive amplification of template-free, negative internal controls (Figure 11, lanes 11, 12 and possibly 13) was carried out over a number of weeks. Negative internal controls use sterilised DNA-free water as the target template so that any cross-contamination of DNA during the preparation process can be identified. Like the use of a positive control indicates that the PCR was successful, the use of water instead of a DNA template indicates that there is no DNA amplification as a result of poor sample handling or the presence of residual DNA in the water or amplification kit. In this case >20 ng bands are visible in lanes 11, 12 and possibly 13 which are consistent with the 800 bp primed target and the positive amplifications between lanes 2 - 10 with 16S target.

3.12. HOMIM analysis of initial plaque collection test from human subjects.

Bacterial gDNA was sampled and isolated using the Isohelix system and analysed via HOMIM (Figure 12). The youngest of the four volunteers was over two years and would most likely had all of his primary teeth whereas the two eldest of the volunteers were over 8 and over 13 years and may have had mixed dentition, between primary and permanent teeth with some gaps, though this could not be confirmed at the time. Hybridisation data was converted into relative abundances (Section 2.6). In total, seven of the ten phyla were identified and were distributed similarly throughout the data set; data labels and standard error bars were added to mean values for ease of interpretation. A preliminary Kruskal Wallis test (KWT) was carried out on the distribution of phyla throughout the whole test group and no significant difference between samples was observed (P = 0.95). A subsequent Mann-Whitney U test also found individual samples to be similar (P > 0.05) indicating that the distribution of phyla was equal throughout the test series and closely resembled the Crielaard (2011) data presented in Figure 4.

The abundace (%) of Phyla identified in samples collected from four children during the Isohelix-HOMIM pilot study
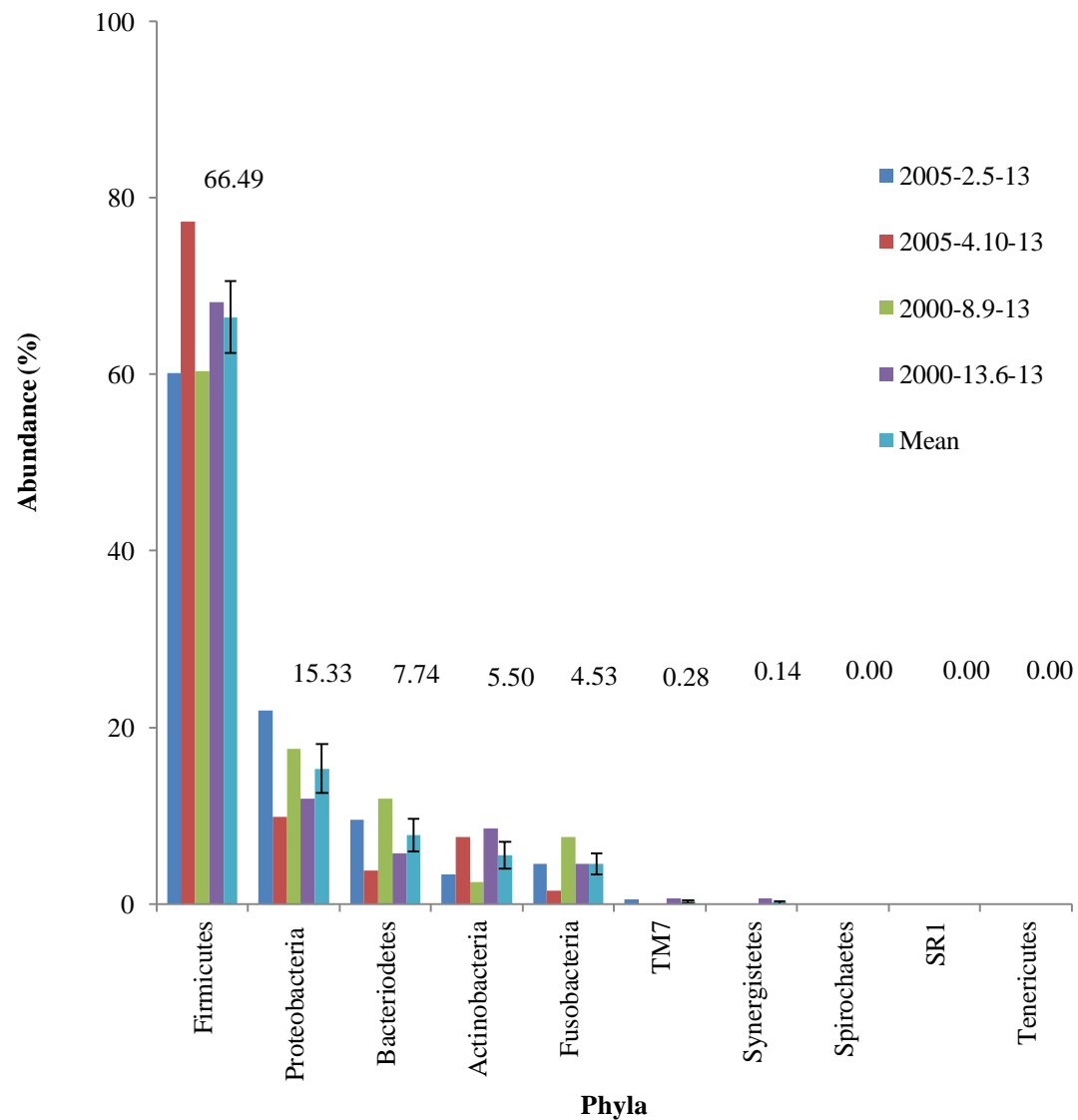
Figure 12. Mean abundance of 10 phyla most predominantly identified from the oral cavity, isolated from 4 children as part of a pilot study into the suitability of Isohelix swabs for sampling dental bacteria from children.

3.13. Genera identified in initial plaque collection test from human subjects using Isohelix.

Pilot data provided by HOMIM was clustered into genera and presented as percentage abundances (Figure 13). The number of genera identified within each sample was 2005-2.5-13, 23; 2005-4.10-13, 14; 2000-8.9-13, 20 and 2000-13.6-13, 28. Genera present in all four samples (alphabetised by phyla) were *Slackia* (*Actinobacteria*), *Capnocytophaga* (Bacteriodetes), *Prevotella* (*Bacteriodetes*), *Gemella*, *Granulicatella*, *Streptococcus* and *Veillonella* (*Firmicutes*), *Leptotrichia* (*Fusobacteria*), *Campylobacter*, *Haemophilus*, *Kingella* and *Neisseria* (*Proteobacteria*). A Kruskal Wallis test compared all four samples as a group and found that there was no significant differences between profiles (P = 0.35). Subsequent Mann-Whitney analyses compared all individual profiles and confirmed this finding (P > 0.12).

The abundance (%) of genera identified in samples collected from four children during the Isohelix-HOMIM pilot study
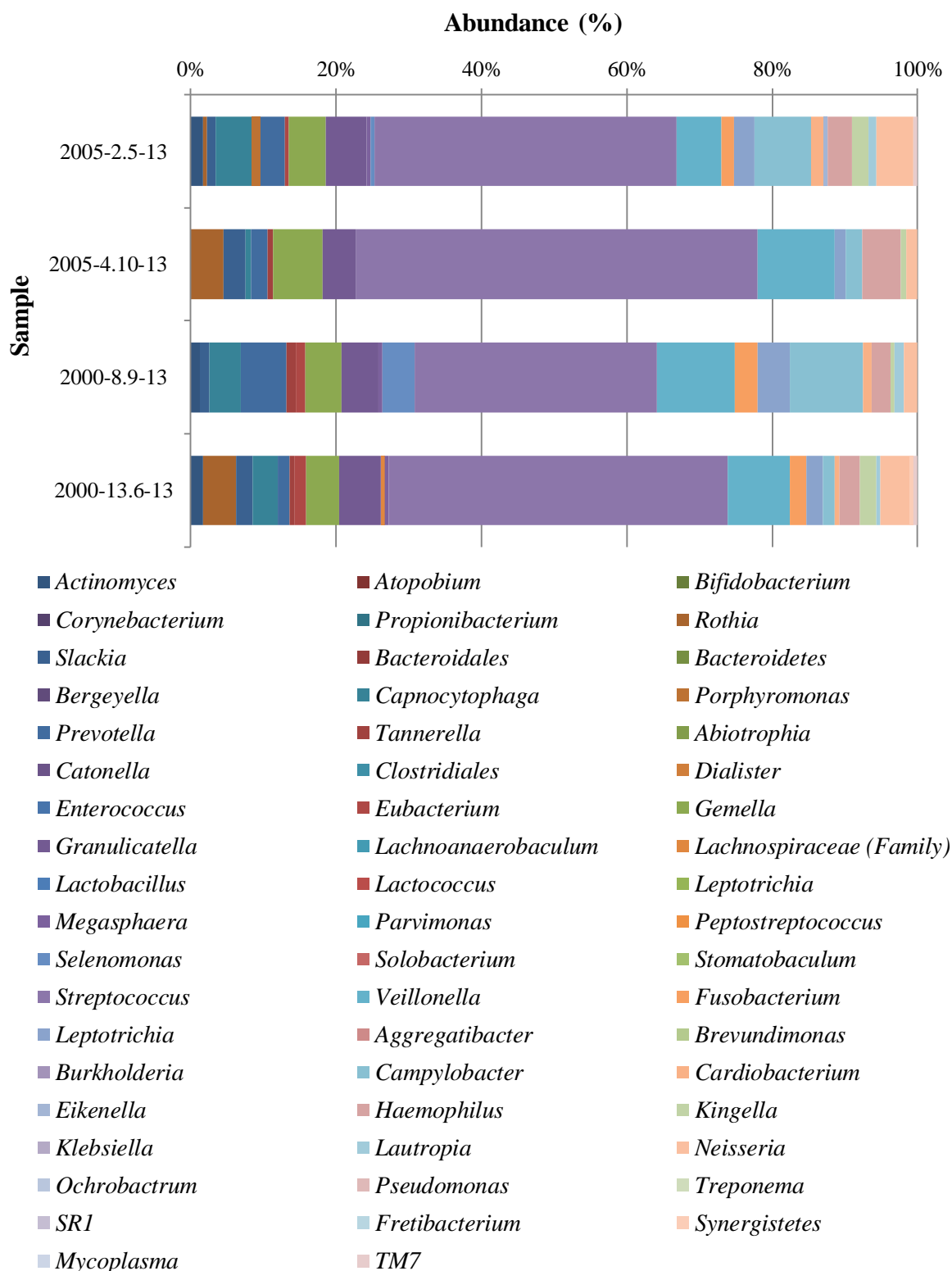
Abundance (%)

Figure 13. Data from HOMIM analysis of plaque samples from the pilot study clustered into genera.

Horizontal scale indicates relative abundance (%).

3.14 Discussion.

3.15. Obtaining ethical clearance.

In order to adhere to the law which governs clinical experimentation it was vital that all obligations were met with clarity and transparency. In this case the aim was to collect and analyse bacterial samples from the tooth surface and survey them for potential changes linked to the use of novel caries intervention services.

Submitting an application for advanced disclosure took approximately four weeks to complete as it required a background check for the previous five years of residency. However, successful completion (and clearance) of this obligation provides the holder with legal permission work with both vulnerable children and adults.

The timely discovery and optimisation of methods suitable for the collection and analysis of dental plaque from children was vital for project success. The reason for this was that a fully optimised method formed the core of the P.I.L. and would be the 'point of introduction' for the potential volunteer and would also have to be passed through ethical clearance before access could be granted for the recruitment of participants. In keeping with the total disclosure of all aims and methodology, it was vital that the volunteer was not in any way deceived or misled into agreeing to practices or treatments that were made unclear by the use of technical terminology.

The requirements of the chosen method were that it was suitable for the repeated collection of oral biofilms, that it was scientifically consistent, that it was lightweight and functioned without sample-loss due to the need for storage or DNA processing equipment or reagents while still in the field; and finally, that it was ethically suitable. In order to circumnavigate ethical restrictions which would have prevented the type of sampling required for the present

study, it was decided that the parent would be taught to collect the sample. This process was investigated during a pilot study (from section 3.12.) which combined the swabbing system with the HOMIM service, in order to evaluate the performance of the method.

3.16. The development of a method suitable for the collection of dental biofilms from young children.

This preliminary study was devised to investigate the suitability of the Isohelix system as a method for the collection of dental biofilm samples from children, as well as its compatibility with HOMIM as a method of sample analysis. This study began with a set of experiments which aimed to test the capabilities of the Isohelix swabbing system to collect and isolate increasing concentrations of gDNA from Gram positive organisms, which was quantified by PCR and the electrophoresis of amplified 16S rDNA.

In the first instance PCR primers, identified from Persing (1993), were obtained commercially (Eurofins. Ebersberg, Germany) for a set of ranging experiments designed to optimise a positive control for a PCR study. Here, an isolate of *E. coli* 16S rDNA template was diluted into ten-fold increments and amplified over 30 cycles. Amplified template produced clear bands at of >100 ng and approximately 800bp which was consistent with the literature (Persing, 1993). Based on the success of this initial study, which included band-free negative controls, future PCR internal control template were run at a dilution factor 1:80 and labelled 'EgDNAH'.

The isohelix DNA purification kit was originally designed to harvest human DNA and, as far as the manufacturer was aware the kit had never been tested on dental plaque (Cooper, 2011). For this reason, kit-capabilities with the processing of mixed bacterial samples were unknown and so the next set of experiments aimed to show the potential upper and lower limits of the

swabbing kit when sampling from a set of serially diluted Gram positive *S. aureus* suspensions.

HOMIM results, specifically Figures 11, 12 and 13 showed that the Isohelix system was capable of detecting Gram-positive as well as Gram-negative species at distributions representative of that presented in the literature (Figure 4). However, the presence of bands in the negative control (Figure 11) suggested a possible contaminant. Stringent asepsis during preparation reduced, though did not rule out the probability of a handling issue being the source of contamination, suggesting the possibility that it was an artefact arising from the contamination of one or more of the commercial reagents. DNA can enter the polymerase reaction at almost any step and as a result of potentially limitless handling errors. Ribosomal DNA genes are highly conserved and can be found at between 1 and 15 copies per bacterial genome and as a result, the contamination of polymerases from bacterial sources is inevitable and has been identified as an issue associated within molecular analyses of bacterial investigations previously (Corless *et al.*, 2000; Hughes *et al.*, 1994; Klappenbach *et al.*, 2001; Maiwald *et al.*, 1994; Mohammadi *et al.*, 2003; Rand & Houck, 1990). Further to this, it is possible that the source of the contaminant could have been the molecular grade water (e.g. Sigma W4502) which was found to have a bacterial contaminant specification of up to 10 CFU/ml (Sigma-Alderich, 2010).

The amplification of templates sourced from cell numbers ranging from 10^9 to 10^2 CFU/ml was considered a good indication that the isohelix DNA purification system was both robust and sensitive enough to process samples of predominantly Gram positive species at ranges consistent with cell counts expected from dental samples.

The final study in this group of experiments was devised to pilot the combination of methods which comprise the final protocol for the collection of dental biofilm samples within a clinical setting; specifically, the use of the Isohelix swab-optimised DNA isolation kit for bacterial

sampling, and the human oral microbe identification microarray as the system of sample analysis. Therefore, this investigation required the completion of three specific observations. Firstly, to determine whether the Isohelix system was simple enough to be taught to an untrained parent, and second, whether the kit could sample dental biofilms at bacterial distributions consistent with the literature at the phylum, genus and species level. And finally, whether HOMIM was a suitable method for the analysis of dental biofilms acquired using the sampling methods described above. In this case swabs were taken by the children's parent or guardian (who, in this case was employed as a nurse within the NHS but unfamiliar with the methods used) from palatal face of the upper incisors and lateral incisors of 4 children aged 3 to 13 years and the gDNA was isolated using the Isohelix purification kit as described previously. The data provided by HOMIM was returned as hybridisation intensities (numbered from 0-5) for each of up to 300 phylotypes associated with both oral health and disease and, once converted to phylogenetic abundances (Figure 12), indicated the presence of 7 phyla at percentage distributions which were consistent with the literature (Xu *et al.*, 2014).

The number of genera and phylotypes identified for each sample included all 8 of the most predominant genera identified in the literature (Peterson *et al.*, 2014). A Mann-Whitney U test was used to compare the four pilot samples and found three significant differences at the species level (data not shown as it included 233 phylotypes and was too large to convert into a graph format, see appendix XIII), specifically 2005-2.5-13 vs. 2005-4.10-13 (P < 0.01), 2005-4.10-13 vs. 2000-8.9-13 (P < 0.05) and 2000-8.9-13 vs. 2000-13.6-13 (P < 0.05).

Pairwise, all samples were found to be statistically similar at the species level with the exception of the profile produced from the 4 year old (2005-4.10-13) which may be as a result of differences in the diet, phenotypic variations attributed to genotype and age and combinations of the presence of primary, permanent or gaps, pre-eruption or a variation of some or all of these factors. Each of these elements are known to affect the developmental

processes of the oral microbiome but it is just as likely that of each of the four volunteers, the 4 year old showed more resistance to sampling and as a result rendered a smaller or inaccurate sample. However, differences in the bacterial profile between unrelated individuals has been observed previously (Bik *et al.*, 2010; Crielaard *et al.*, 2011) and therefore samples between different individuals are highly likely to differ, a factor which may have been the case here. However, a person to person comparison at the phylum or genus levels after tooth eruption and before physiological maturity is less likely to differ significantly. In this case, when the data were converted to relative abundances, calculations indicated that 7 phyla were isolated including the five most predominantly associated with the oral cavity. Although some variations between observations were expected a KWT, carried out to compare the median values between the distribution of phyla in each sample found no significant difference ($P = 0.95$) between profiles. In addition, a sample-to-sample comparison was carried out using the Mann-Whitney U test in order to elucidate any discrete deviations from the median also found no statistical differences between phylogenetic profiles ($P > 0.05$). Further deconstruction and analysis of the data at the genus level also found no significant difference between profiles (KWT, $P = 0.35$, MWU, $P > 0.05$).

This preliminary study was devised in order to provide evidence that the Isohelix gDNA isolation and purification kit was suitable for the collection of oral plaque samples from children under clinical conditions. The protocol for this kit was described previously, however, briefly, the sample lysis and stabilisation reagent is a combination of two commercially produced solutions, referred to as PK and LS solutions (specific ingredients are unavailable in order to protect commercial and intellectual property), which are combined no more than 8 hours prior to sample collection. The sample is then harvested from the upper incisors and lateral incisors only and placed directly into the lysis solution. The swab head

was broken away from the arm and the sample mixed by inversion. At this stage the sample was considered stable and could be stored for up to three years at room temperature.

The reagent preparation was a two-step procedure and sample tubes could be easily transported and used and there was no requirement for cooling or processing materials or apparatus. Assuming that all sampling was carried out during a single day the only drawback to the method was the necessity to mix sampling reagents under aseptic conditions within eight hours of the sampling session. Furthermore, the results described here indicated that this protocol was comprehensible enough to be taught to non-trained individuals and that a 30 sec sample produces mixed bacterial samples of a quality representative of those published in the literature.

HOMIM analysis identified mean values of 10 phyla, 56 genera and 233 species, across four 30 sec samples and as a result, the simplicity of this process provided greater scope for more thorough sampling in future studies and the protocol was modified to include the sampling of both buccal and palatal surfaces of the upper incisors and lateral incisors, to improve sample recovery.

Based on the simplicity of this protocol and the data provided it was concluded that, for the purposes of limited field sampling, Isohelix combined with the HOMIM facility was the best option available for the collection and analysis of large numbers samples.

4. *In vitro* studies

4.1. The Plaque Glycolysis Regrowth Method (PGRM): pH Profiles of 5 day old PGRM-processed oral biofilms

The Plaque Glycolysis Regrowth Method (PGRM) is a standardised protocol (FDA, 2003) which is used to test the cariogenicity of biofilms *in vitro*. In the following experiments PGRM was used to determine whether FP varnish treatments at variable stages of completion could alter the developmental processes of dental biofilms over 5 days. The aim of this study was to determine whether the FP varnish plays a role in reducing the production of acids in oral biofilms *in vitro*.

According to the FP instruction manual (Anon, N.D.), prior to application teeth should be professionally cleaned, rinsed and air dried. The varnish is then applied to all enamel surfaces and flossed in to the proximal surfaces and then permitted at least 60 sec to dry; patients are then required to refrain from eating and drinking for at least 45 min after application. However the reality of the application process for the young children was quite different. Personal observation showed that the patient was rarely cooperative and as such, the cleaning and drying of the teeth prior to application was difficult if not impossible. In addition, the application of varnish, which occurred only once every six months, was rarely applied correctly and often the enamel surface received little more than a single sweep of varnish across the buccal and/or labial surfaces.

This leads to questions as to what benefit could be expected from the varnish at a microbiological level, year on year. Certainly it was considered more likely that the patient would become more receptive to the treatment as the trial progressed and the child grew but

this gradual acceptance of treatment, and the subsequent increase in varnish coverage would provide inconsistency in the results.

Experimental design for the PGRM was to determine four specific outcomes which were designed to mimic *in vivo* application conditions. First, to observe the effects of FP on acid production and biofilm development when applied under manufacturer's directions; second, to observe the same effects after a partial application over a clean dried surface, as directed by the manufacturer. Third, to observe the effects of treatment when was applied over the top of salivary pellicle, as was likely the case in the field. And finally, to determine and compare with the outcome of a standard PGRM control in the absence of FP varnish.

As it was not possible to measure pH without disrupting the biofilm, once the PGRM had been optimised each pH profile was derived from the mean values of six replicates of each condition (0%, 50%, 100% and 100% (ES) FP). In a parallel study and run under identical conditions, biofilms from each replicate were removed from the disc surface and processed for dry weight calculations and HOMIM analysis. This was done specifically because it was not possible to use PGRM, pH-processed biofilms for dry weight determination as contact with the pH probe often removed visible biomass from the disc prohibiting use in further tests.

The six rounds from two experimental periods (May and August) were selected as they were the only two complete triplicate experimental sets; all other attempts were interrupted by mechanical faults (incubators), failed biofilm incubations or evidence of contamination within test samples. For the dry weight experiments and HOMIM analyses, of the 24 biofilms processed, three from each treatment group (i.e. 0%, 50%, 100% and 100% ES, totalling 12) were selected for analysis. The biofilms were selected from the highest pH ('Hi', the most basic), the mid values ('Med', the approximate mean) and from the low pH values ('Lo', most

acidic) in order to correlate the size and bacterial diversity of each treatment culture with a pH response.

Additional analyses compared the pH and the dry weight profiles as well as the distributions of phyla and genera from spring (May) and summer (August).

Once analysis had been completed it became apparent that there were variations in some of the results which were date-dependent, suggesting the impact of an uncontrolled factor not planned for in the methods; in most cases it was likely due to a loss in temperature control during incubation which sometimes led to fluctuations in CO_2.

4.2 Results

4.2.1 Effects of FP varnish on pH profiles of 5 day old mixed species biofilms, grown on hydroxyapatite discs and subjected to a sugar challenge.

Saliva coated hydroxyapatite discs were incubated with pooled human oral bacteria and incubated for 4 d in varying concentrations of FP, with media replenished every 24 h. On day 5 biofilms were placed in sugar free media and challenged with 200 mM sucrose and the pH was measured over 90 min. Mean values were compiled from 6 runs per treatment (May 7th-9th and Aug 6th-8th, Figure 14). The results indicated that the pH of mixed biofilms grown in 0% (control), 50%, 100% and 100% ES FP varnish dropped below 5.5 in approximately 10 min and decreased to below pH 4.75 within 75 min, regardless of treatment.

The mean pH profiles of 5 day old biofilms grown on sHA discs in the presence of Fluor Protector fluoride varnish (%)
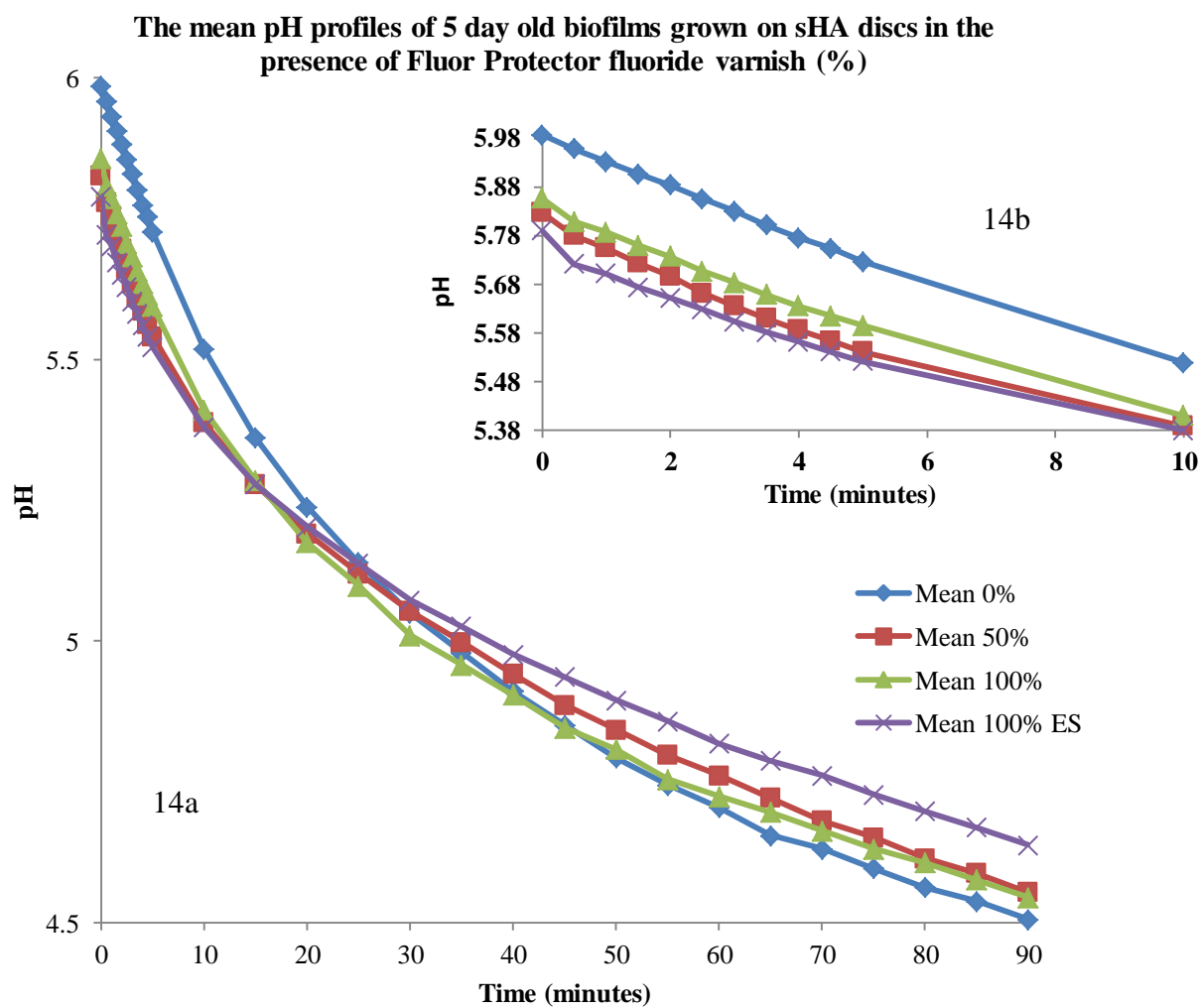
Figure 14a. The Mean pH profiles of 5 d old biofilms grown on hydroxyapatite discs in the presence of Fluor Protector varnish and 14b (inset) showing the initial 10 minutes of the reaction, amplified for clarity.

Graph shows mean pH profiles for sugar challenges carried out on 5 day old biofilms after 0% (control), 50% and 100% coatings with FP and 100% ES (100%) coating with FP varnish in excess saliva. The graph (14b) inset shows the same pH profile for the initial 10 min. Profiles are produced from six replicates of each condition and an ANOVA General Linear Modal (GLM) found that there was no significant difference between mean pH profiles (P = 0.96). The same statistical test was carried out on the initial 10 min of the mean pH demonstrating what appeared to be a marked difference between treatments. In the first 10 min of the reaction, The ANOVA GLM found a significant difference between 0% and 100% and 0% and 100% ES profiles only (P < 0.02).

4.2.2. PGRM – Individual pH profiles and related analyses.

Saliva coated hydroxyapatite discs were incubated with pooled human oral bacteria in the absence (0%) of FP with replenished media every 24 h. On day 5, biofilms were placed in sugar free media and challenged with 200 mM sucrose and the pH was measured over 90 min. Two triplicate experiments were run in May (7th-9th) and August (6th-8th). The pH profiles show some variability (Figure 15). Samples run on May 7th, and May 9th to Aug 8th fell below the demineralisation threshold (approximately pH 5.5) within 25 min of adding a sugar source, whereas the May 8th sample took approximately 45 min to reach pH 5.5. An ANOVA General Linear Model found no significant differences in the variations between May vs. May and Aug vs. Aug comparisons (P = 0.08, 0.96 respectively). However, May and August readings sit above and below the mean value (respectively) meaning that May profiles were less acidic at 0% FP varnish. Individual GLM comparisons found significant differences between May 7th and 8th and then May 7th - 9th and all of Augusts profiles (P < 0.02). Statistical summary of the spring-summer differences can be viewed in Table 2. From this set of profiles, samples were selected for dry weight and HOMIM analyses from May 7th, May 8th and Aug 7th.
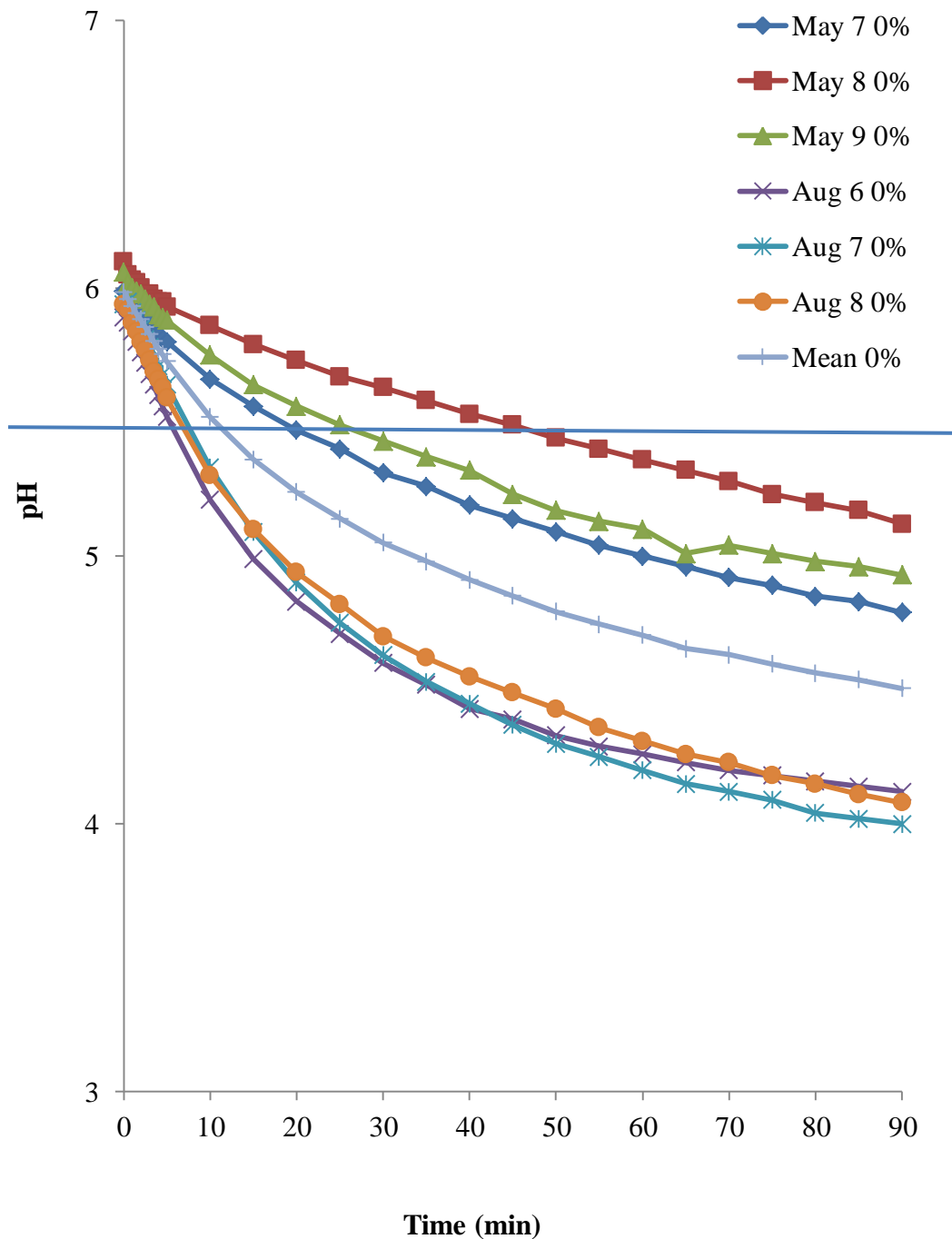
Figure 15 Individual pH profiles of 5 d old biofilms on hydroxyapatite discs with 0% Fluor-protector varnish (control), challenged with sucrose.

The blue line intersects the graph at approximately pH 5.5, the average enamel demineralisation threshold.

Individual profiles for the samples at 50% FP varnish coverage also show variability (Figure 16). May 7th and Aug 6th – 8th profiles fell below the pH 5.5 within 10 min whereas May 8th and 9th took approximately 60 sec to reach the same point. An ANOVA GLM found a significant difference between May comparisons (P < 0.01) whereas no difference was observed in the collective August profiles (P = 0.09). The same test was run against individual mean profiles and indicated a significant difference between each of the May profiles (P < 0.01) with the exception of May 7th vs May 8th (P = 0.87) and all May profiles differed significantly from each of the August profiles (P < 0.05). Within the summer profiles, Aug 6th differed from Aug 8th (P < 0.05) but there was no difference calculated between Aug 6th and 7th (P = 0.31) and 7th and 8th (P = 0.31). Statistical summary of the differences between samples can be viewed in Table 2. From these profiles, samples used for HOMIM analysis were May 7th, May 8th and Aug 6th.
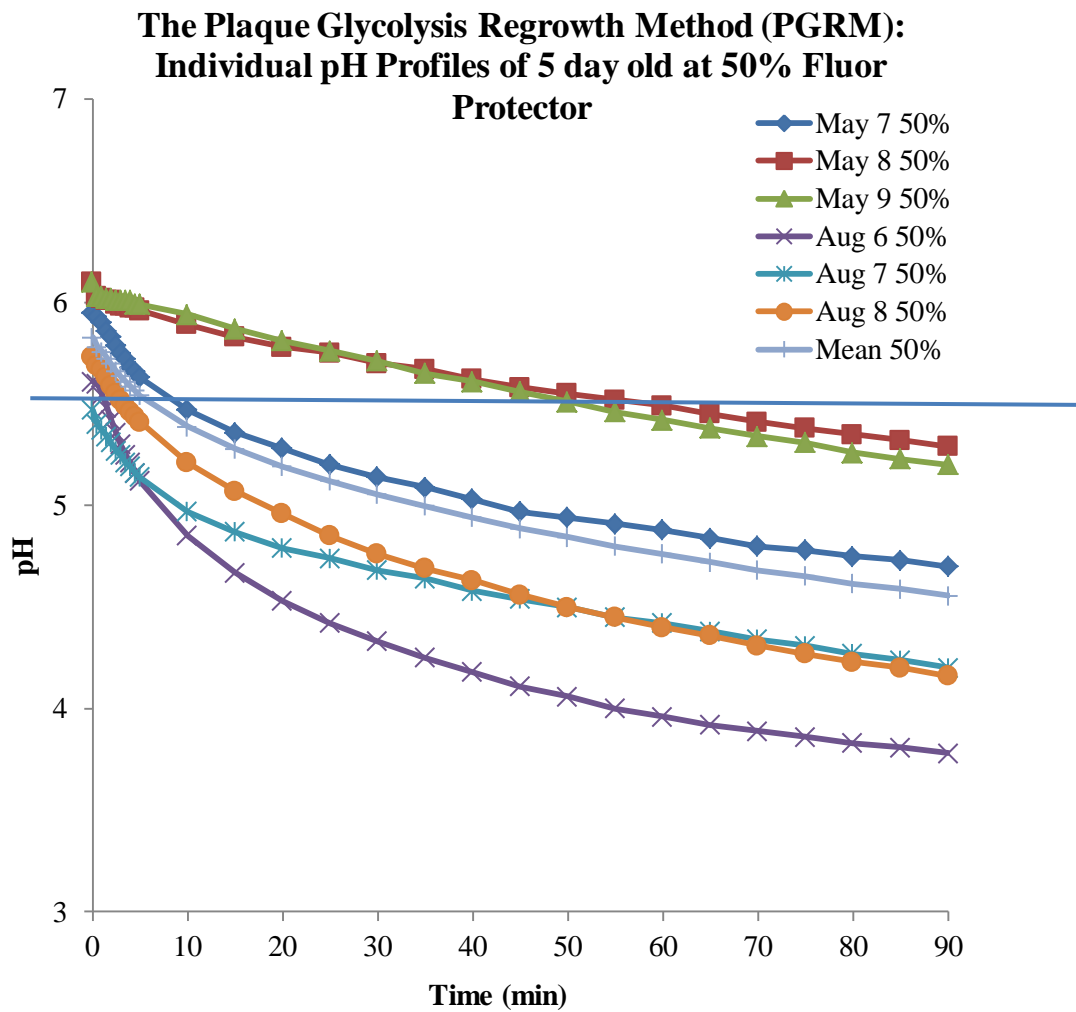
Figure 16   Individual pH profiles of 5 d old biofilms on hydroxyapatite discs with 50% Fluor-protector varnish challenged with sucrose.

The blue line intersects the graph at approximately pH 5.5, the average enamel demineralisation threshold.

The individual results for the samples coated with 100% of FP varnish are shown in Figure 17. On day 5 biofilms were placed in sugar free media and challenged with 200 mM sucrose and the pH was measured over 90 minutes. Two triplicate experiments were run in May (7th-9th) and August (6th-8th) and the pH profiles show some variability. An ANOVA GLM found no significance difference between combined May samples (P = 0.96) whereas August samples presented some difference in the combined analysis (P < 0.01). Samples run on May 7th – 8th fell below the pH threshold (approximately 5.5) within 15 min. Samples run on Aug 6th – 7th took 1.5 min and 0 min (respectively) and May 9th and Aug 8th took 30 and 50 min respectively. A GLM found a significant differences between all samples (P < 0.03) other than May 7th and May 8th (P = 0.41), May 7th and May 9th (P = 1.00) and Aug 6th and Aug 7th (P = 0.2). Statistical summary of the differences between samples can be viewed in Table 2. From this profile, samples used for HOMIM analysis were May 8th, Aug 7th and Aug 8th.

The Plaque Glycolysis Regrowth Method (PGRM): Individual pH Profiles of 5 day old at 100% Fluor Protector
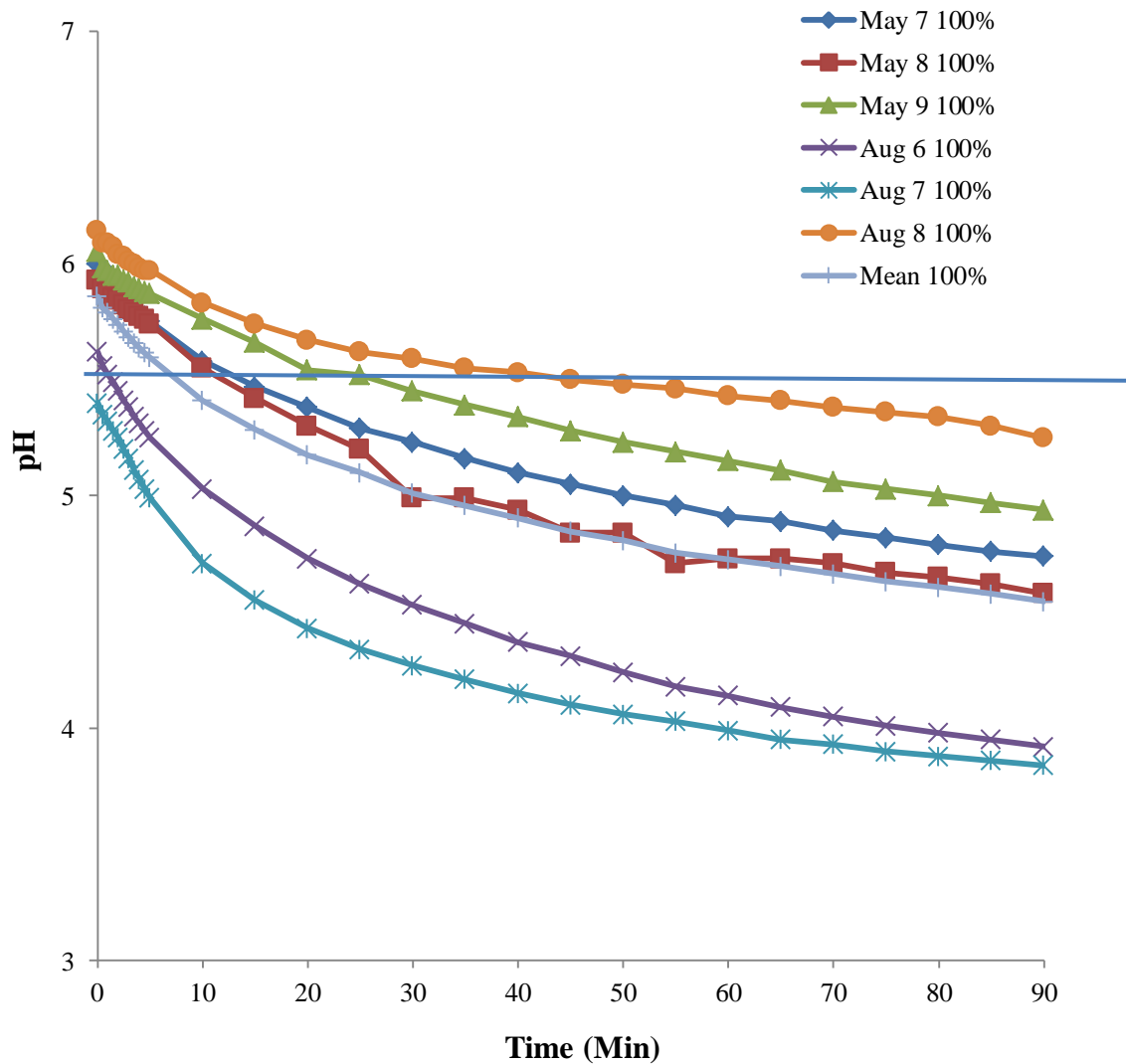
Figure 17   Individual pH profiles of 5 d old biofilms on hydroxyapatite discs with 100% Fluor Protector varnish challenged with sucrose.

The blue line intersects the graph at approximately pH 5.5, the average enamel demineralisation threshold.

The results of individual pH profiles of hydroxyapatite discs coated with 100% FP in excess saliva with 5 d old biofilms challenged with sucrose are shown in Figure 18. Two triplicate experiments were run in May (7th-9th) and August (6th-8th) and the pH profiles show some variability between profiles. Samples run on May 7th – 8th and Aug 6th fell below the pH threshold (approximately 5.5) within 25, 10 and 10 min respectively. The Aug 7th took 1 min whereas May 9th sample did not fall between pH 5.5 within the test period. An ANOVA GLM found no significant differences between May and August comparisons (P = 0.08, 0.96 respectively) although significant differences were observed between all individual samples (P < 0.01) with the exception of May 7th and May 8th (P = 0.07), May 7th and May 9th (P = 0.15) May 8th and Aug 6th (P = 0.45) and Aug 7th and Aug 8th. Statistical summary of the differences between samples can be viewed in Table 2. From this profile, samples used for HOMIM analysis were May 8th, May 9th and Aug 7th.

The Plaque Glycolysis Regrowth Method (PGRM): Individual pH Profiles of 5 day old at 100% ES Fluor Protector, in excess saliva
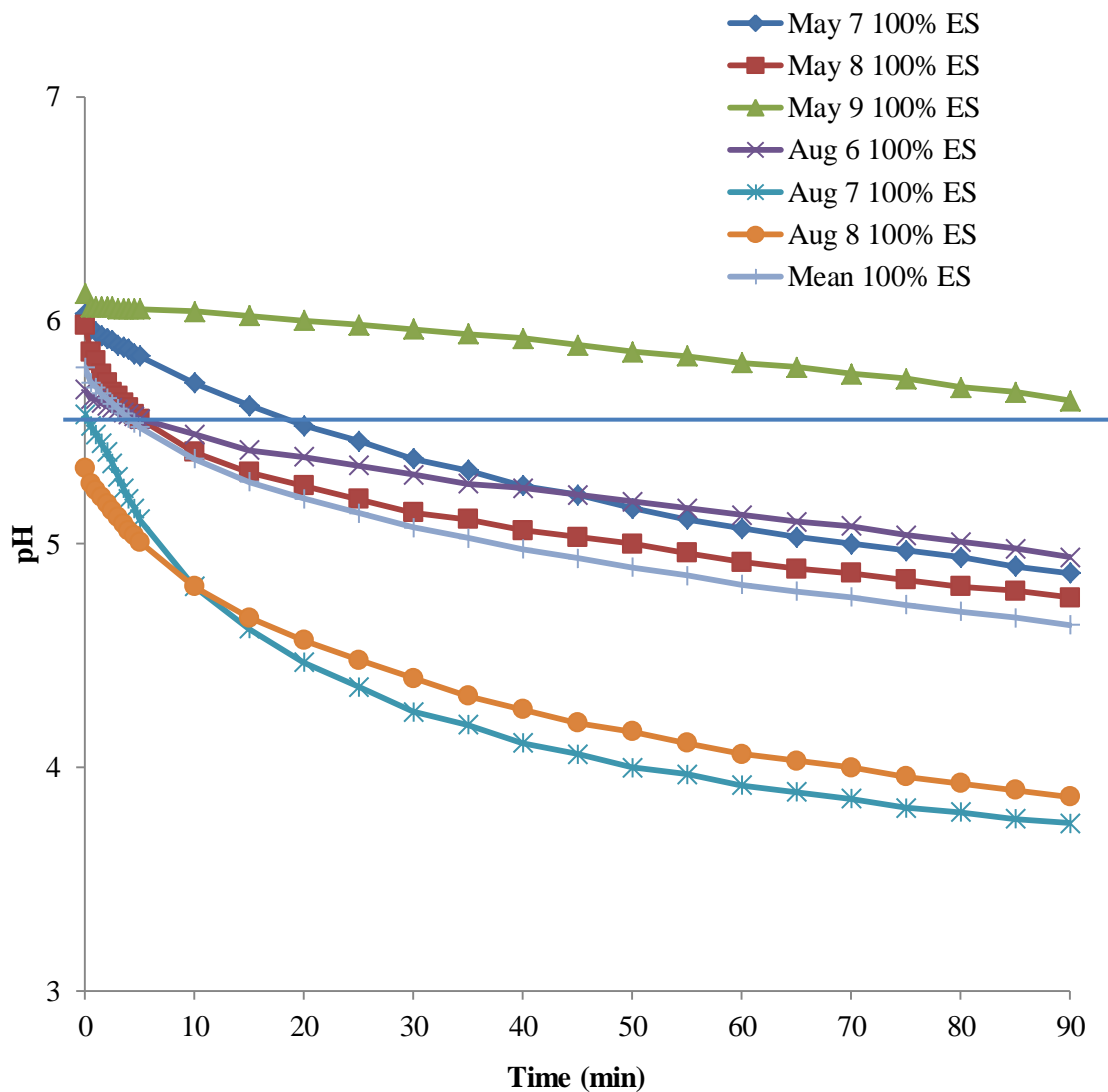
Figure 18 Individual pH profiles of 5 d old biofilms on hydroxyapatite discs with 100% Fluor Protector varnish in excess saliva challenged with sucrose.

The blue line intersects the graph at approximately pH 5.5, the average enamel demineralisation threshold.

		Kruskal Wallis test in May and August		ANOVA General Linear Model					
				May			**Aug**		
		May	Aug	7th	8th	9th	6th	7th	8th
0 % FP May	7th				*	0.4	*	**	**
	8th	0.08				0.14	**	**	**
	9th						**	**	**
Aug	6th							0.91	0.8
	7th		0.96						0.9
	8th								
50% FP May	7th				**	**	**	**	*
	8th	**				0.9	**	**	**
	9th						**	**	**
Aug	6th							0.19	*
	7th		0.09						0.31
	8th								
10% FP May	7th				0.41	1.00	**	**	**
	8th	0.96				*	**	**	**
	9th						**	**	0.06
Aug	6th							0.2	*
	7th		**						**
	8th								
100% ES FP May	7th				0.07	**	0.16	**	**
	8th	**				**	0.45	**	**
	9th						**	**	**
Aug	6th							**	**
	7th		**						-0.9
	8th								

Table 2. Summary of statistical analyses carried out for the PGRM pH profiles analysis.

Biofilms selected for HOMIM analysis are colour coded as Hi (GREEN), Med (BLUE) and Lo (RED). Unless stated, P values are indicated as < 0.05 (*) and < 0.01 (**).

HOMIM hybridisation data was converted to phylogenetic profiles as relative abundances (%) and compared for differences. the results are shown in Figure 19. The data shows the mean relative abundance of phylogenetic groups of biofilms grown in the presence and absence of FP varnish with standard error bars. A Kruskal-Wallis Test (KWT) carried out across the complete set of profiles found no significant differences between distributions (P = 0.98). A second KWT was run to test for differences within each FP treatment category and the same conclusion was drawn (P > 0.05) indicating that there were no significant differences between phylogenetic distributions regardless of FP treatment.

Mean Abundances (%) of Phyla identified within 5 day biofilms, grown on sHA discs in the presence of FP (%)
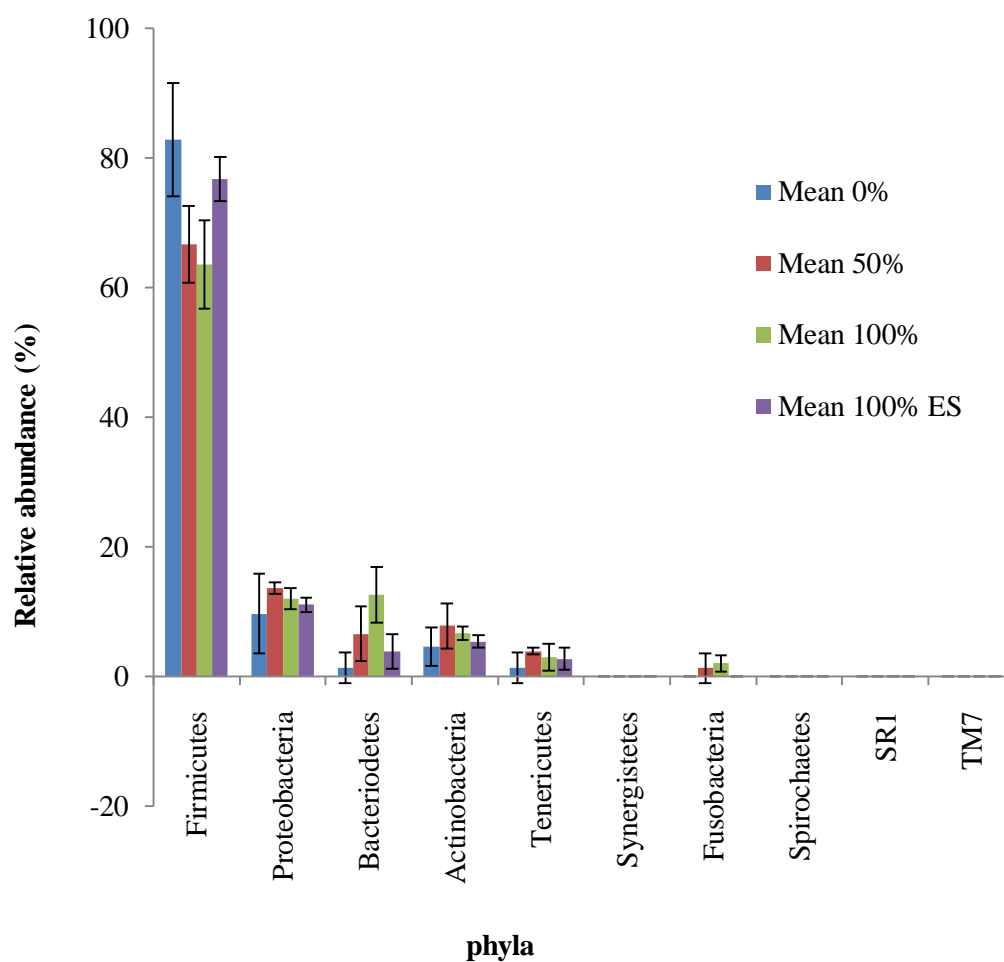
Figure 19. Abundance (%) of Phyla identified in five day old PGRM-processed biofilms as determined by HOMIM analysis.

The mean percentage abundance of genera identified in the four fluoride conditions as described by HOMIM is shown in Figure 20. The number of genera identified for each sample was mean 0%, 9; mean 50%, 15; mean 100%, 17; mean 100% ES, 10. A KWT showed that there was no significant difference between profiles (P = 0.051). Since the P-value fell just outside the alpha level (0.05), subsequent Mann-Whitney analyses were carried out and showed that there were a number of significant differences between inter and intragroup individual samples (alpha levels at 0.05 and 0.01), however, no significance differences were found between mean values (P=0.79), confirming the KWT.
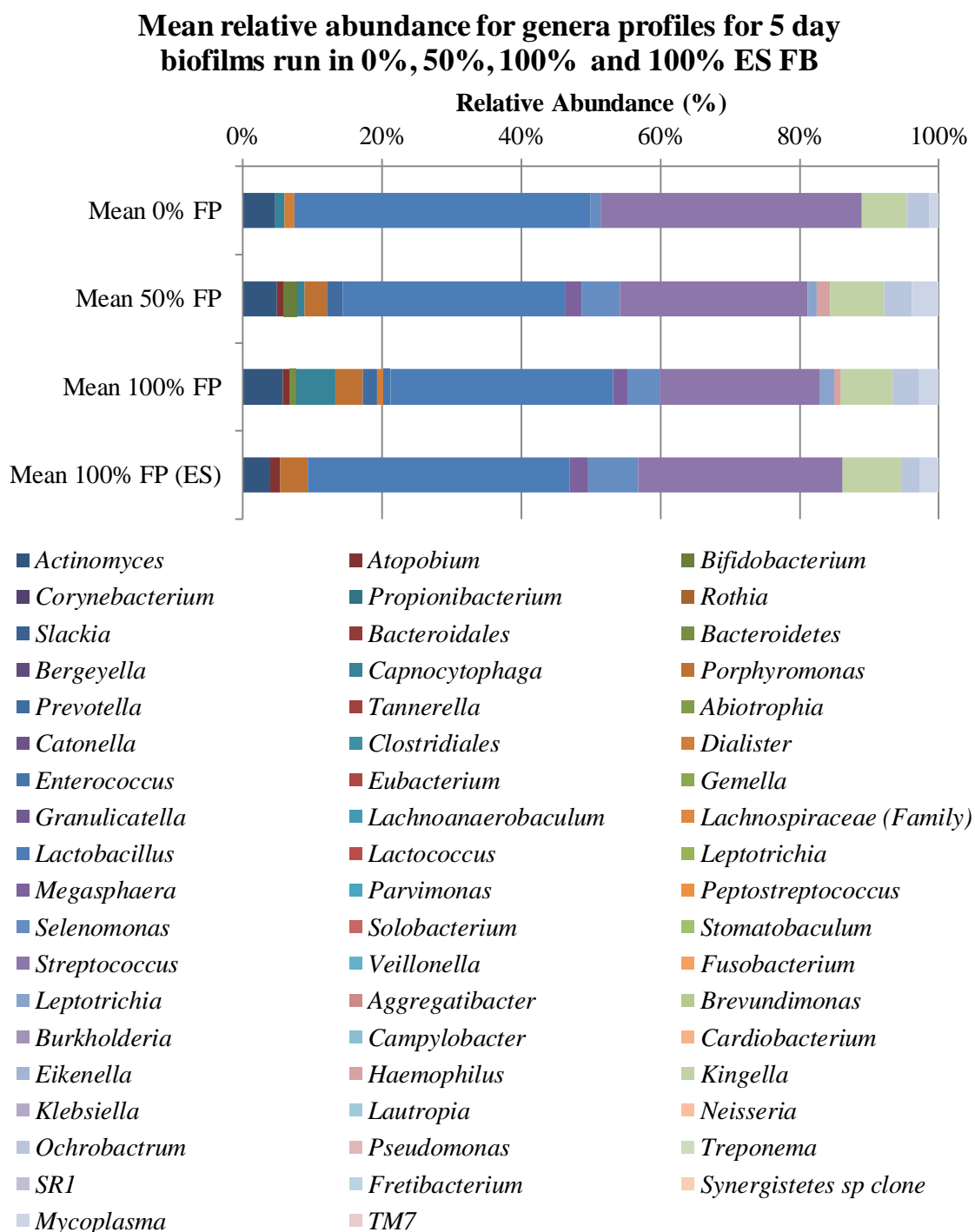
Figure 20. Mean percentage abundance of genera identified in the four fluoride conditions as described by HOMIM analysis.

Figure 21 shows the percentage abundance of genera identified in the individual 0% FP treated biofilms selected for HOMIM analysis. Sample labels indicate the relative pH profile based on high (Hi) median (Med) and Low (Low) pH range and each sample consisted of 2, 5 and 9 genera respectively. A KWT compared the group median and showed no significant differences within the group (P = 0.12). A Mann-Whitney analysis was used to compare individual samples and found that there was no difference between May 8th and May 7th or May 7th and Aug 7th profiles (P = 0.28 and 0.32 respectively) although the May 7th and Aug 7th comparison indicated a significant difference (P = 0.04)

Relative abundance for genera profiles for 5 day biofilms run in 0% FP
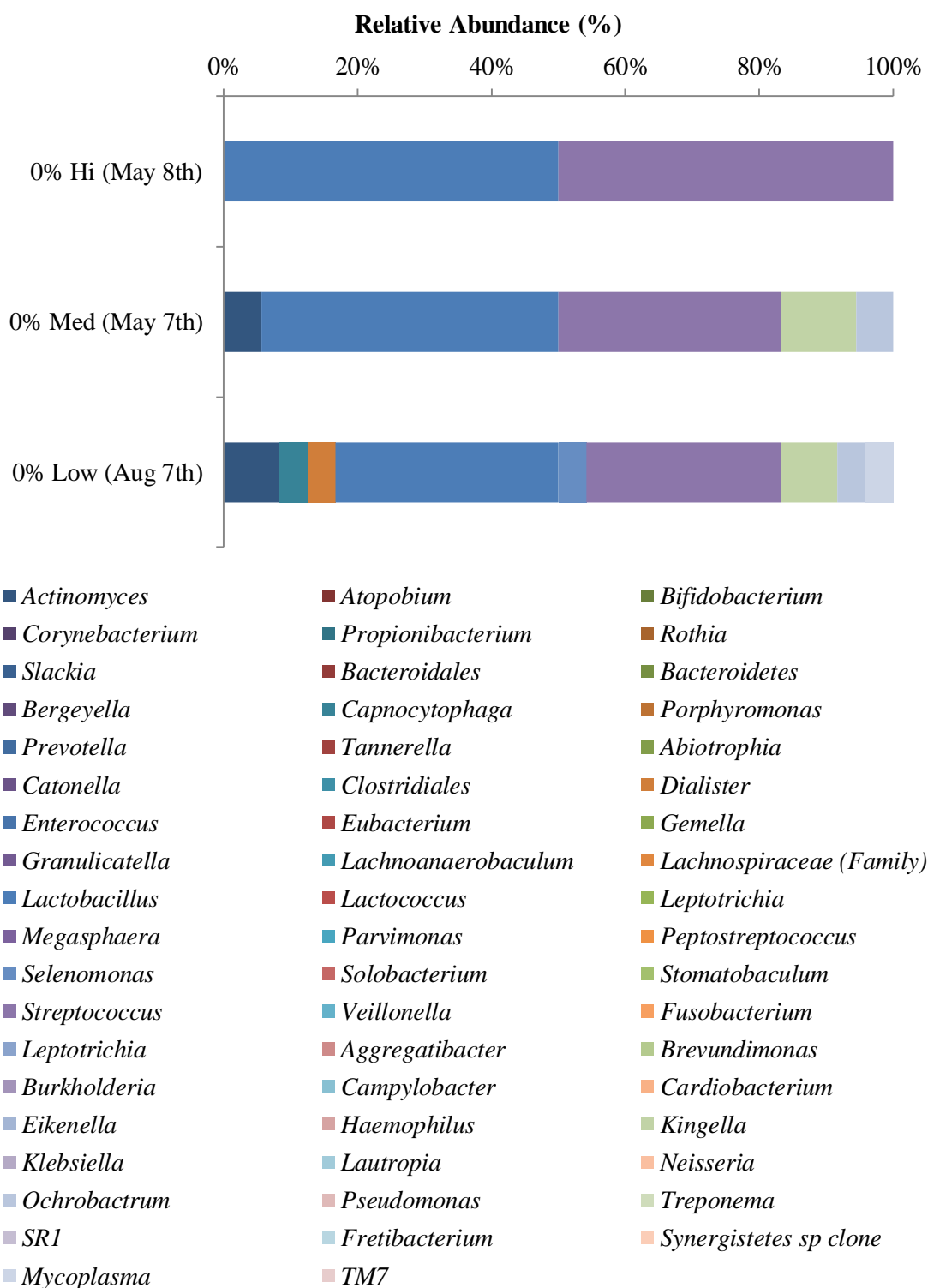
Figure 21. The abundance (%) of genera identified from 5-day old PGRM-processed biofilms run in 0% FP varnish (controls).

The results of HOMIM analysis of relative abundance (%) of genera in the 50% FP varnish biofilms are shown in Figure 22. Sample labels indicate biofilms selection based on high (Hi) median (Med) and Low (Low) pH range and each sample comprised 14, 11 and 7 genera respectively. A KWT compared the group median and showed no significant differences within the group (P = 0.37). A Mann-Whitney analysis was used to compare individual samples and also found no statistical difference between profiles (P > 0.05).

Relative abundance for genera profiles for 5 day biofilms run in 50% FP

Relative Abundance (%)
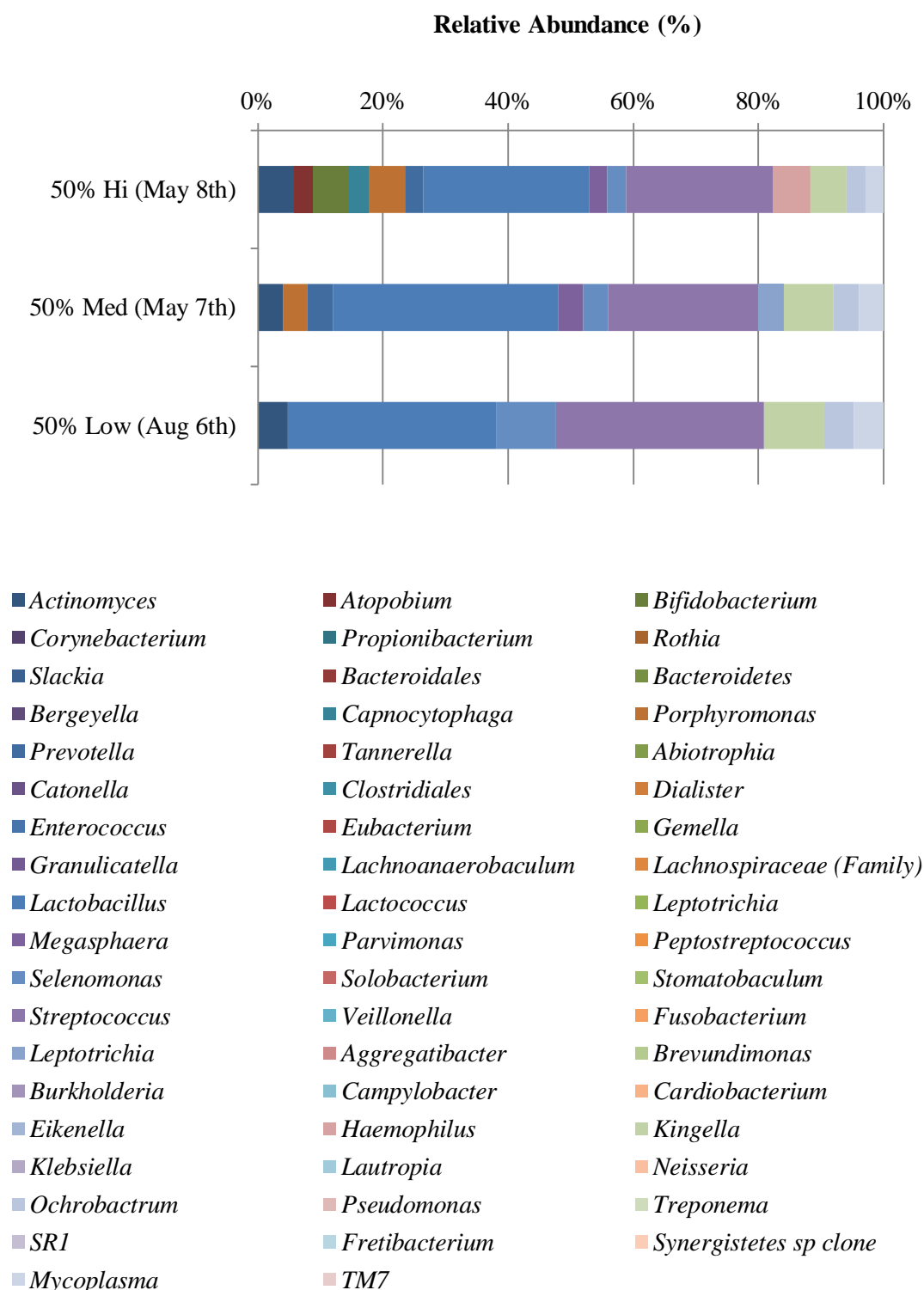
Figure 22. Relative abundance (%) of genera identified within individual 5 day old biofilms cultured after 50% FP treatment.

The relative abundances of genera detected by HOMIM analysis of individual 100% FP

varnish coated discs is shown in Figure 23. Sample labels indicate the selection category

based on high (Hi) median (Med) and Low (Low) pH range and each sample comprised 7, 12

and 17 genera respectively. A KWT compared the group median and found no significant

differences within the group (P = 0.15). A Mann-Whitney analyses was used to compare

individual samples and found there was a significant difference between Aug 8th and Aug 7th

(P = 0.04) but no other differences were observed (P > 0.05).

Relative abundance for genera profiles for 5 day biofilms run in 100% FP

Relative Abundance (%)
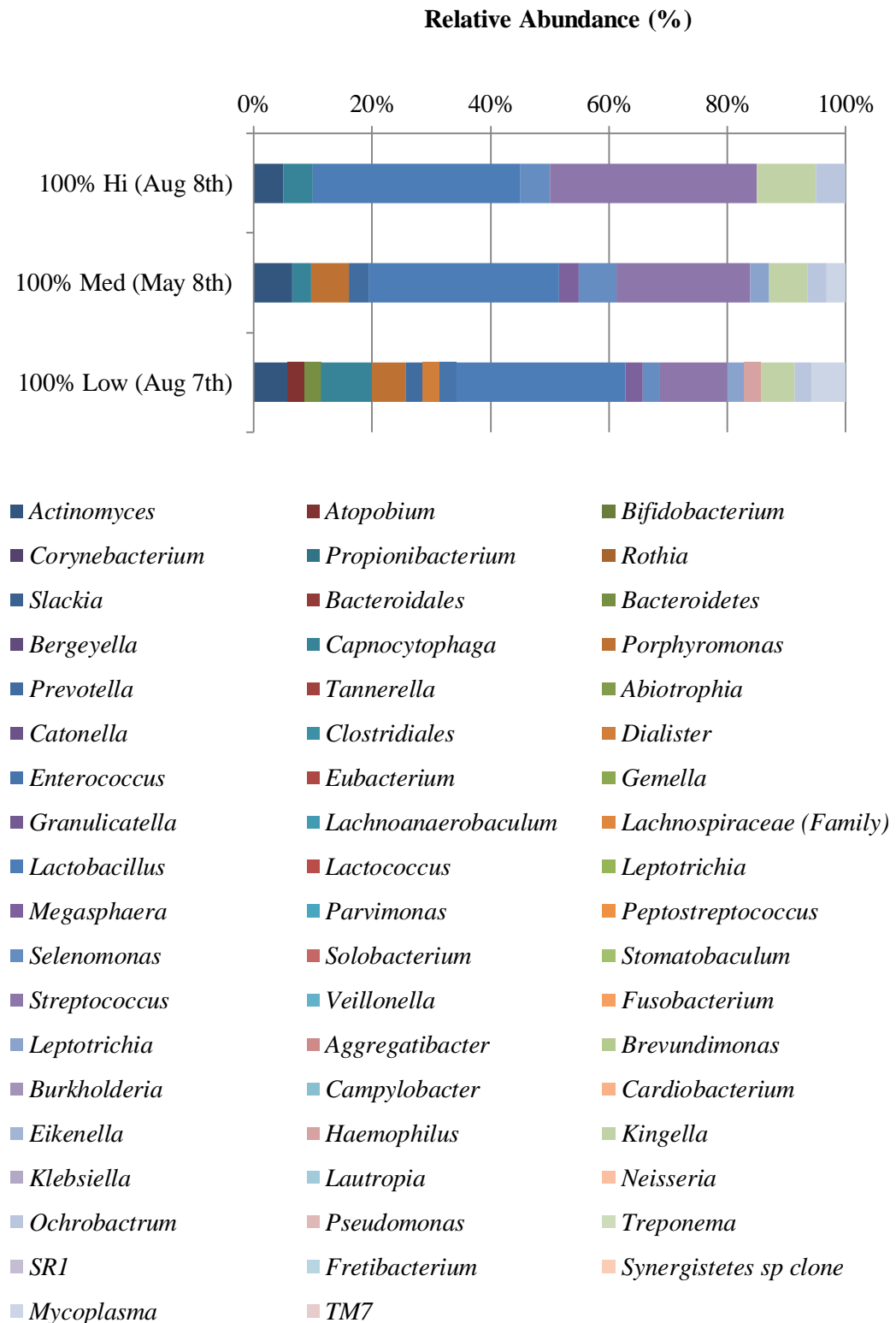
Figure 23. Relative abundance (%) of genera identified within individual 5 day old biofilms cultured after 100% FP treatment.

The relative abundance of genera detected by HOMIM analysis of 100% FP varnish treated

biofilms in excess saliva is shown in Figure 24. Sample labels indicate biofilms selected

which was based on high (Hi) median (Med) and Low (Low) pH range and each sample

consisted of 6, 9 and 8 genera respectively. A KWT compared the group median and showed

no significant differences within the group (P = 0.79). A Mann-Whitney analysis was used to

compare individual samples and found that there was no difference between profiles (P >

0.05). Table 3 shows the relative abundances of phylotypes in the samples chosen for

HOMIM analysis.

Relative abundance for genera profiles for 5 day biofilms run in 100% (ES) FP

Relative Abundance (%)
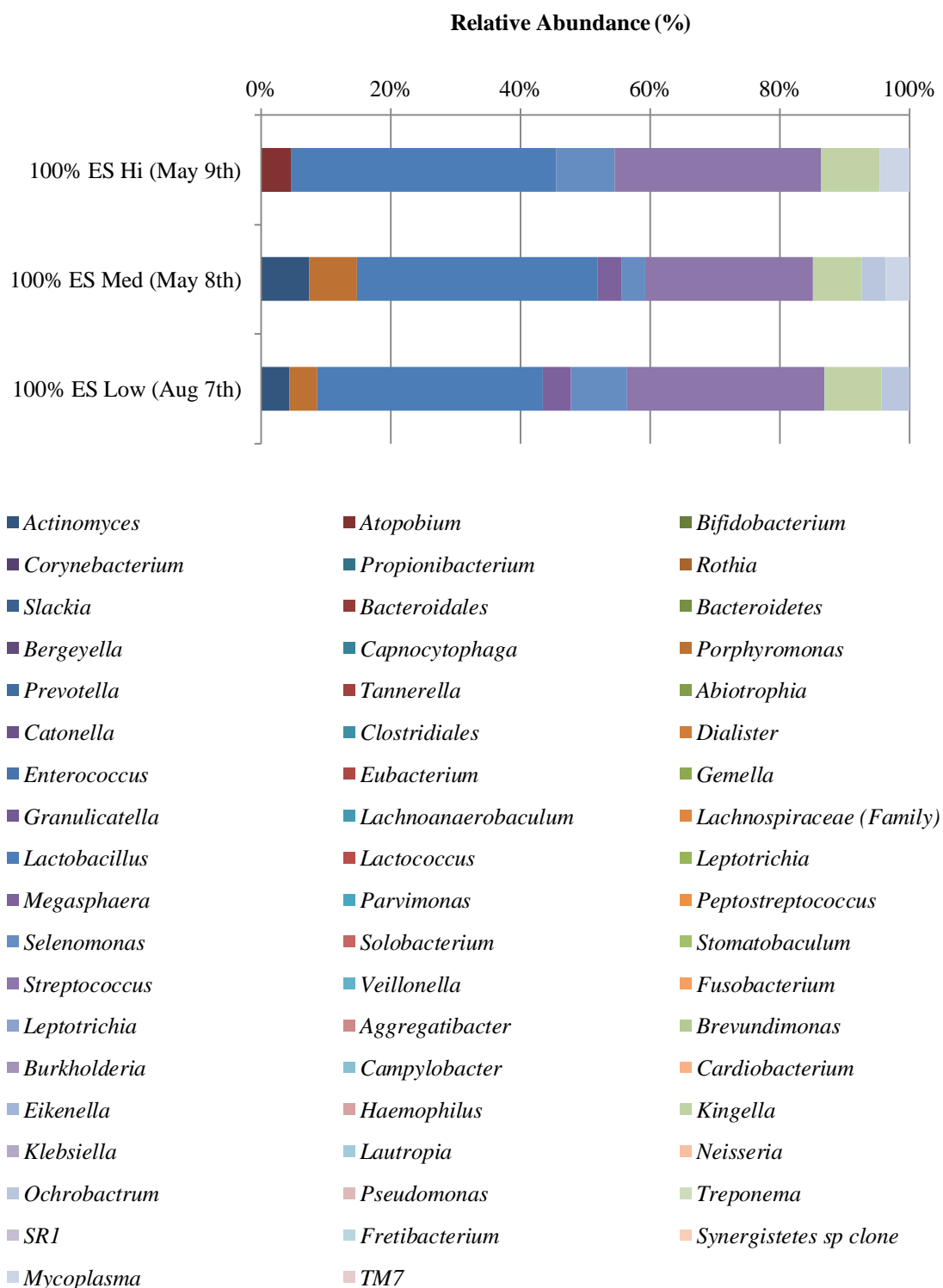
Figure 24. Relative abundance (%) of genera identified within individual 5 day old biofilms cultured after 100% FP (ES) treatment.

The relative abundances of phylotypes, detected by HOMIM for each of the PGRM treatment groups, categorised into HI, Med and Low as defined by the pH profile experiments (Table 3). In every case the profile was dominated by lactobacilli and streptococci though variations in abundance were observed and were dependant on the estimated pH of the culture. Significant differences were observed between 0% Hi and 50% Hi (P = 0.005), 0% Hi and 100% Med (P = 0.03), 0% Hi and 100% Low (P = 0.001), 0% Med and 50% Hi (P = 0.03) and 0% Med and 100% Low (P = 0.009).

Phyla	Spp.		0% Hi	0% Med	0% Low	50% Hi	50% Med	50% Low	100% Hi	100% Med	100% Low	100% ES Hi	100% ES Med	100% ES Low
			♠✠♱	♦✦		♠♠				✠	♱✦			
Firm	*Lactobacillus casei/ L. paracasei/ L. rhamnosus*	*	31.25	27.78	20.83	14.71	20.00	23.81	25.00	16.13	14.29	22.73	18.52	21.74
Firm	*Streptococcus salivarius /S. vestibularis /Streptococcus spp*	*	25.00	22.22	20.83	14.71	16.00	23.81	25.00	16.13	8.57	22.73	18.52	21.74
Firm	*Lactobacillus fermentum*	*	18.75	16.67	8.33	5.88	8.00	9.52	10.00	9.68	8.57	9.09	11.11	8.70
Firm	*Streptococcus downei*	*	12.50	11.11	8.33	5.88	8.00	9.52	10.00	6.45	2.86	9.09	7.41	8.70
Prote	*Kingella oralis*	H	0.00	11.11	8.33	5.88	8.00	9.52	10.00	6.45	5.71	9.09	7.41	8.70
Actino	*Actinomyces georgiae*	*	0.00	5.56	8.33	5.88	4.00	4.76	5.00	6.45	5.71	0.00	7.41	4.35
Firm	*Selenomonas dianae*	H	0.00	0.00	4.17	2.94	4.00	9.52	5.00	6.45	2.86	9.09	3.70	8.70
Firm	*Lactobacillus jensenii/L. salivarius*	*	0.00	0.00	4.17	5.88	8.00	0.00	0.00	6.45	5.71	9.09	7.41	4.35
Prote	*Ochrobactrum anthropi*	H	0.00	5.56	4.17	2.94	4.00	4.76	5.00	3.23	2.86	0.00	3.70	4.35
Tener	*Mycoplasma salivarium*	U	0.00	0.00	4.17	2.94	4.00	4.76	0.00	3.23	5.71	4.55	3.70	0.00
Bacterio	*Porphyromonas sp.*	H	0.00	0.00	0.00	2.94	4.00	0.00	0.00	3.23	2.86	0.00	3.70	4.35
Firm	*Megasphaera micronuciformis*	*	0.00	0.00	0.00	2.94	4.00	0.00	0.00	3.23	2.86	0.00	3.70	4.35
Bacterio	*Capnocytophaga sp.*	H	0.00	0.00	4.17	2.94	0.00	0.00	5.00	3.23	5.71	0.00	0.00	0.00
Firm	*Streptococcus oralis / Streptococcus spp.*	*H	12.50	0.00	0.00	2.94	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacterio	*Prevotella oulora*	*	0.00	0.00	0.00	2.94	4.00	0.00	0.00	3.23	2.86	0.00	0.00	0.00
Bacterio	*Porphyromonas gingivalis*	*	0.00	0.00	0.00	2.94	0.00	0.00	0.00	3.23	2.86	0.00	3.70	0.00
Actino	*Atopobium spp.*	*	0.00	0.00	0.00	2.94	0.00	0.00	0.00	0.00	2.86	4.55	0.00	0.00
Fuso	*Leptotrichiaceae spp.*	*	0.00	0.00	0.00	0.00	4.00	0.00	0.00	3.23	2.86	0.00	0.00	0.00
Prote	*Haemophilus parainfluenzae*	H	0.00	0.00	0.00	5.88	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
Firm	*Dialister invisus*	*	0.00	0.00	4.17	0.00	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
Actino	*Bifidobacterium dentium*	*	0.00	0.00	0.00	5.88	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacterio	*Bacteroidetes spp.*	H	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
Bacterio	*Capnocytophaga ochracea/Capnocytophaga spp.*	H	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
Firm	*Enterococcus saccharolyticus*	U	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.86	0.00	0.00	0.00
Number of genera/phylotypes identified			**2/5**	**5/7**	**9/12**	**14/19**	**11/14**	**7/9**	**7/9**	**12/16**	**17/22**	**6/9**	**9/13**	**8/11**

Table 3. The relative abundances (%) of phylotypes identified in PGRM biofilms.

Phylogenetic abbreviations are as follows: Firm = Firmicutes, Prote = Proteobacteria, Tener = Tenericutes, Bacterio = Bacteriodetes, Fuso = Fusobacteria.

Phylotypes were labelled for their associations with Caries (*), associations with health (H), associations with both caries and health (*H), and those that were unknown (U). Labelled profiles were statistically significant at ♠ (P = 0.005), ✠ (P = 0.03), ♱ (P = 0.001), ♦ (P = 0.03), ✦ (P = 0.009).

Biofilms were cultured for 5 days under conditions suitable for a plaque glycolysis regrowth experiment, after treatments with 0%, 50%, 100% and 100% ES, FP. Cultures were selected for bacterial composition analysis by HOMIM based on the pH profile in relation to the mean, i.e. 0% FP, High (Hi), Median (Med) and Low (Lo). The results (Figure 25) show increased phylotype richness after 50% (n=20) and 100% (n=22) treatments with FP when compared to the 0% (n=13) and 100% ES (n=14) respectively. A KWT was used to compare intra group 0%, 50%, 100% and 100% ES profiles as well as intergroup results. All results showed no significant differences ($P \leq 0.17$) with the exception of 0% vs. 100% (P = 0.048). A subsequent Mann-Whitney test was used to analyse the individual profiles and showed differences between 0% Low vs. 50% Hi (P=0.005), 0% Low vs. 100% Med (P=0.03), 0% Low vs. 100% Hi (P=0.001), 0% Med vs. 50% Hi (P=0.03), and 0% Med vs. 100% Lo (P = 0.009).
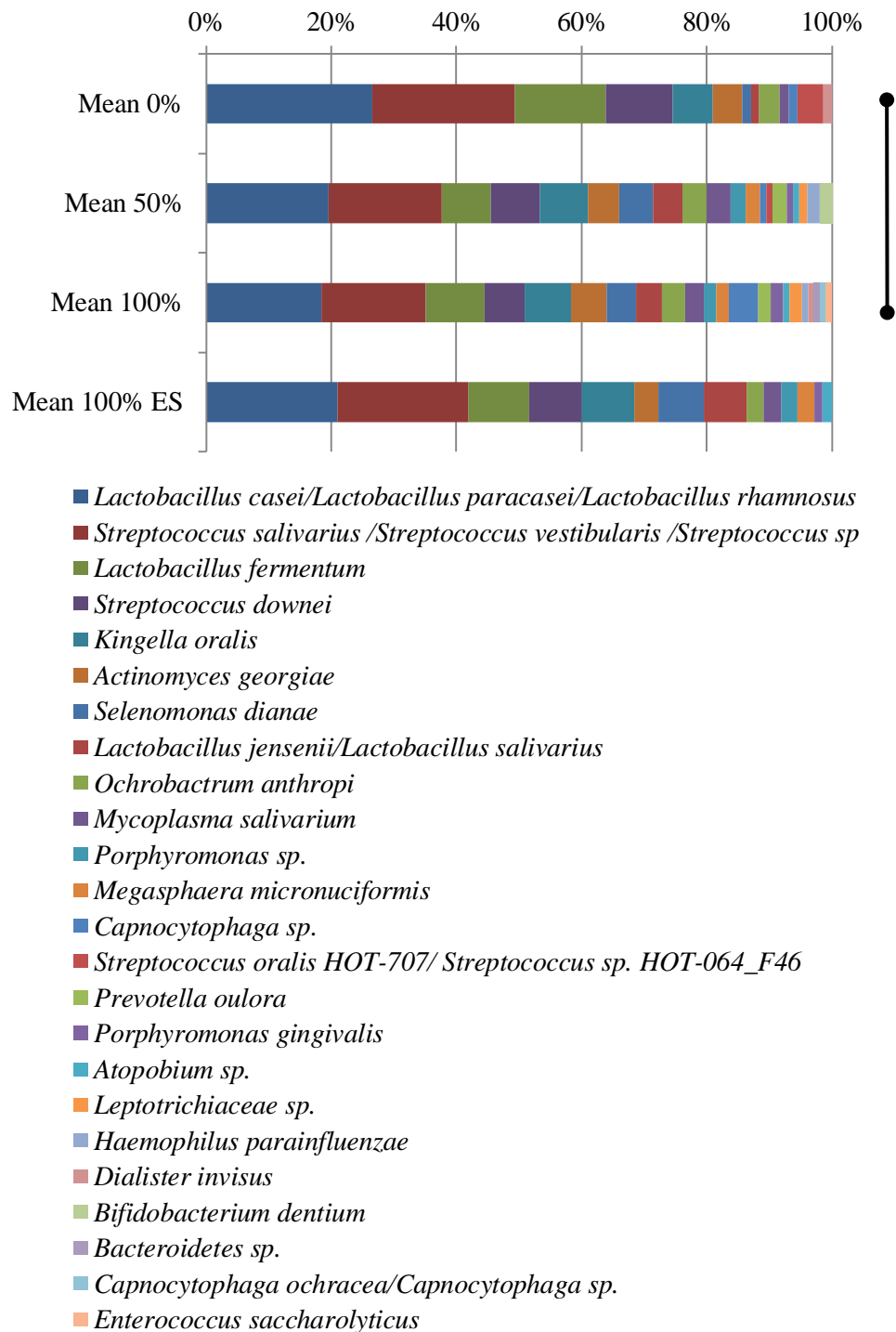
Figure 25. Mean relative abundance (%) of phylotypes identified in biofilms incubated over 0%, 50%, 100% and 100% (ES) Fluor Protector varnish as determined by HOMIM.

The adjoining vertical bar linking the mean 0% to 100% indicates a significant difference between relative abundances (P = 0.048).

4.3. Dry weights of 5 day old PGRM processed biofilms.

Dry weights of processed biofilms were determined and mean values are shown in Figure 26. On day 5 biofilms were removed from the disc using sonication and lyophilised until dry. The results show the mean values of a triplicate experiment utilising 0%, 50%, 100% and 100% ES FP. While there was an obvious difference between the 50% and 100% treatments and the 0% and the 100 ES, a Student T-Test showed no significance between weight profiles, $(P > 0.05)$.
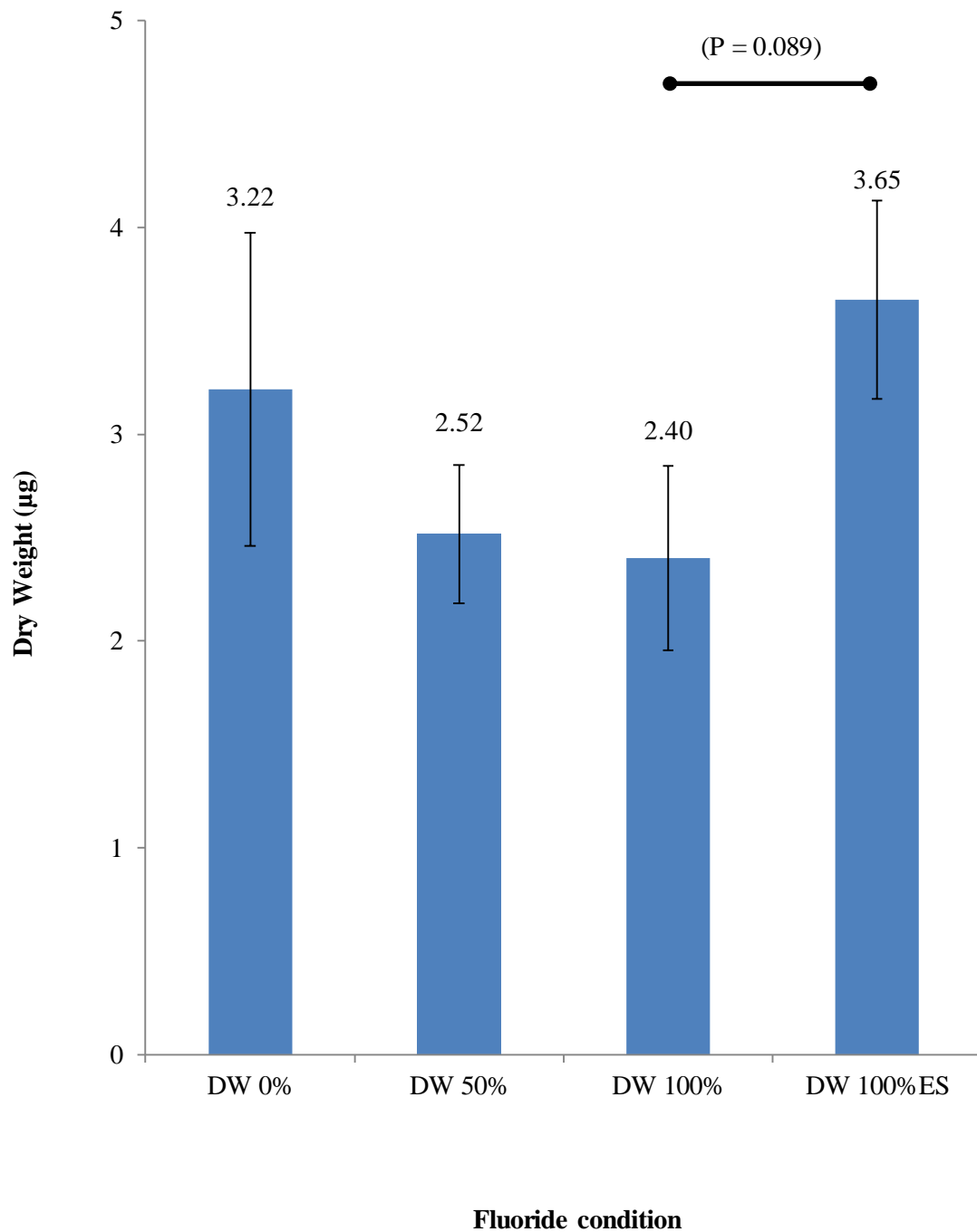
Figure 26. Biofilms were cultured on sHA in the presence of Fluor Protector at varying coverage (%).

The adjoining bar linking the mean dry weight at 100% and 100% ES indicates no significant difference (P = 0.089). Data labels indicate dry weight (μg) and error bars show standard error.

On day 5 of incubation, biofilms were removed from the disc using sonication and lyophilised until dry and weighed (Figure 27). An analysis of the inter-FP categories for May and August variations after 5 days incubation with 0%, 50%, 100% and 100% ES FP showed no significant differences ($P > 0.05$). However, an intra-analysis showed significant differences were only observed between May 100% FP and August 100% ES ($P = 0.04$) indicating that, despite there being a greater degree of biomass production in the May experiments, there was little difference in mean dry weights regardless of FP treatments.
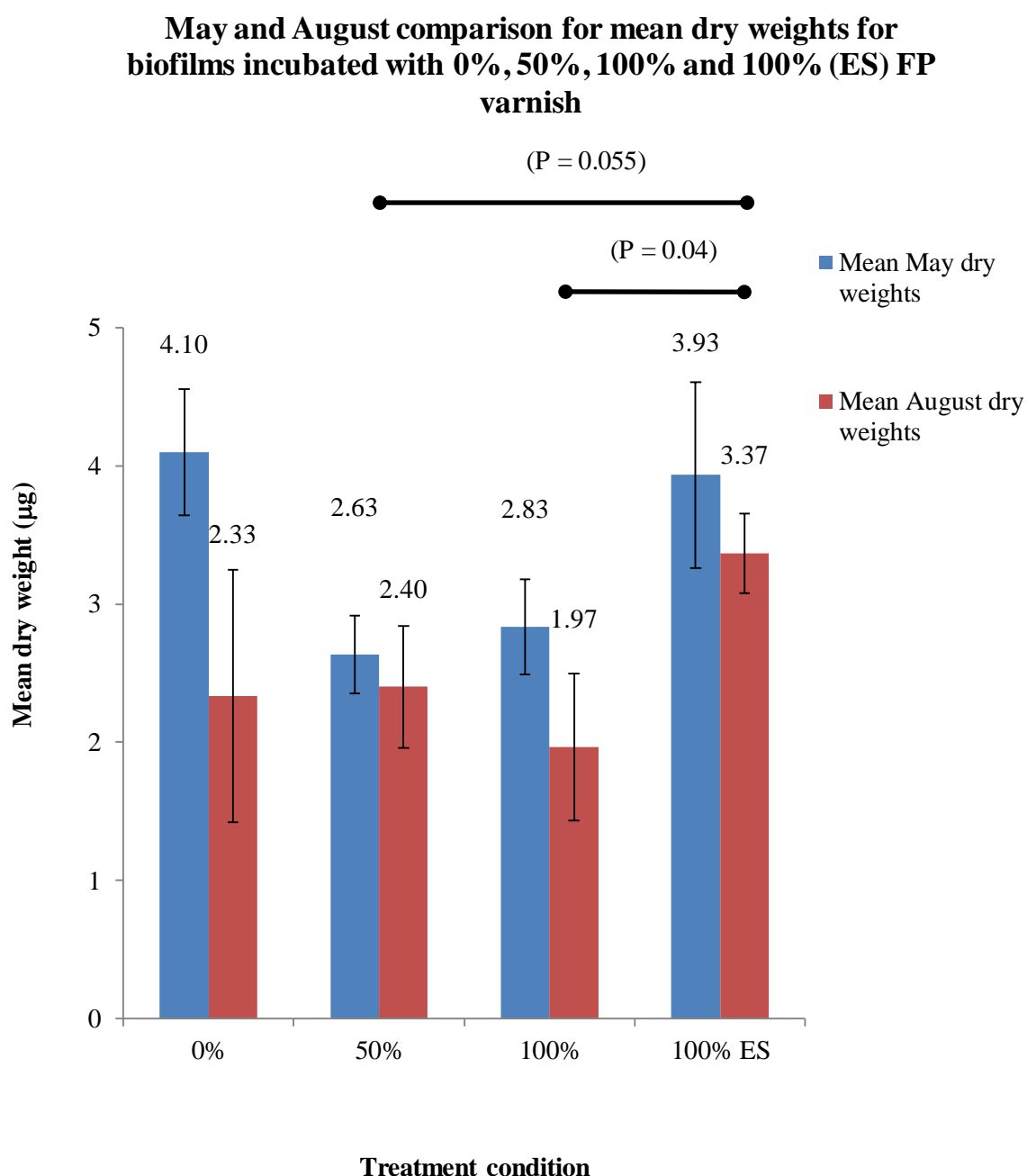
Figure 27. Variations in May and August biofilm dry weight after 5 d cultured in the presence of FP at varying coverage (%). Data points were included for ease of interpretation.

The adjoining horizontal bars linking the mean dry weights at 50% (May) and 100% ES (August) and 100% (May) and 100% ES (August) indicate that results were statistically similar (P = 0.055) and significantly different (P = 0.04) respectively. Error bars show standard error.

4.4. Discussion.

The aims of this study were to observe the effect of varying treatments of FP varnish, designed to mimic field-quality applications on the development of biofilms and the production of acids after 5 days incubation.

A comparison of the mean pH profiles for the four treatment conditions (0%, 50%, 100% and 100% ES) indicated no differences between acidogenicity ($P = 0.96$). However, a closer look at the initial 10 minutes of the reaction showed that that 0% and 100% and 0% and 100% ES differed significantly ($P < 0.02$) and variations observed between May and August pH profiles indicated significant differences between almost every direct comparison.

The analysis of the bacterial composition of biofilms converted from the HOMIM data found no significant differences between mean profiles of phyla and genera. However, the intra-analysis of sample profiles comparing the distribution of genera between May and August showed that the 100%, 100% ES and 0% control all differed significantly with at least one direct comparison. The exception to this was the 50% treatment which did not differ between May and August samples ($P > 0.05$).

The analysis of mean phylotypes identified by HOMIM indicated an increase in the richness of 50% and 100% FP biofilms when compared to the 0% and 100% ES profiles which produced similar results; further indicating a significant difference between 0% to 100% profiles ($P = 0.048$).

Trends in the richness of individual biofilms showed that, between Hi, Med and Low profiles (as determined by their estimated pH) all profiles were dominated by high abundances of lactobacilli and streptococci which, in the case of 0% and 100% FP, dropped with the pH of the biofilm. In the case of 50% profile, lactobacilli and streptococci abundances increased

with a drop in pH while the 100% ES biofilms showed stability across the three pH conditions. The overall richness of biofilms across the three pH conditions resulted in an increase at 0% and 100% FP from Hi to Low, and reduction at 50% (from Hi to Low) and an increase in relative abundance until the approximate mean (Med) and then dropped again, remaining approximately stable at Hi and Low readings for the 100% ES cultures. There were significant differences observed between 0% Med and 100% Med, 0% Med and 100% Low and 0% Low and 100% Low.

Despite the disparity observed in the pH variations and bacterial distributions between treated and untreated groups, the only differences observed between the dry weights of treated biofilms were observed between 100% (May) and 100% ES (Aug, P = 0.048). Mean values showed no significant differences between treatment concentrations (P > 0.05).

This study was devised to compare the development of biofilms and potential effects of sucrose-induced acid production with the results of the Fluoride study, carried out as part of the Bright Smiles trial. However, while this product may contribute to reduced incidences of symptomatic caries (perhaps due to the development of a calcium-fluoride reservoir at the enamel surface) the antimicrobial effects are still unclear (Baygin *et al.*, 2013; Marquis *et al.*, 2003; Pinar Erdem *et al.*, 2012). In addition it was readily apparent at the beginning of the study that the treatment could not be carried out as described by the manufacturer and so it was deemed vital to understand what, if any, were the effects of this product on the development of the biofilm and the subsequent production of acids after a sugar challenge.

In order to achieve these aims a number of tests were carried out. Firstly pooled human biofilms were grown in the presence and absence of FP and positive and negative treatment controls. These biofilms were then challenged with sugar and the pH was measured over 90 minutes. To further this study, tests were repeated after five days of incubation in partial treatments of the varnish. This would provide information on its effects after a good but

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partial application. Finally, five day biofilms were grown in the presence of the varnish after being applied over the top of a salivary pellicle. This was the most likely outcome of the application in the field as children were too young to undergo the required methodology.

Next, in order to test for the effects on the developmental processes of the biofilm, the dry weight of accumulated biomass was calculated and biofilms were despatched for HOMIM analysis to outline bacterial composition. In this case biofilms that were destined for HOMIM analysis were not tested via the PGRM. This method relies upon direct contact with the biofilm and results in an irreversible loss of biomass. Therefore, cultures were produced in batches from a pooled source, and divided into experiments (i.e. PGRM or Dry weight and bacterial composition analysis) as required. In addition, in order to appreciate the bacterial composition in relation to the pH, three biofilms were selected from each treatment batch (i.e. 0%, 50% 100% and 100% ES, FP) based on the acidity of its tested counterpart, during the PGRM.

The first experiment carried out was the pH profiling of 5 day mixed biofilm which were sourced from a pooled and enriched set of samples and run between May (7th, 8th and 9th) and August (6th, 7th and 8th) in the same year. The pH of all four mean profiles dropped below the standardised demineralisation threshold (pH 5.5) within 10 minutes and proceeded to fall for the remaining 80 minutes to a pH of ≤ 4.75; an ANOVA with GLM found there was no significant difference between mean profiles at this point (P = 0.96).

Throughout the process it was evident that there was a lot of variation in the individual profiles and so they were analysed separately for differences. At 0% there were significant differences (P < 0.02) within the May group and between May the August groups however, there were no differences between profiles within the August group. The 50% treatment was similar in that May 7 was significantly different from every other profile (P < 0.012), as was the case for May 8th (with the exception of a pairwise comparison with May 9th, P = 0.87), P

< 0.01 and May 9th and the remaining profiles (P < 0.01). However, when the August groups were compared, August 6th was significantly different from August 7th (P < 0.05) but the remaining profiles were not significantly different. At 100% FP and 100% ES the results changed in that the August readings were predominantly different (P < 0.01) with the exception of August 6th vs. August 7th at 100% and August 7th vs. August 8th at 100% ES. These data appeared to present variations between May and August experimental runs that, until the later stages of analysis, had not been detected. During the warmer months, August particularly, the CO_2 incubator used for the production of biofilms *in vitro* had regular breakdowns due to overheating. Other than the temperature of the incubator, which regularly increased beyond the required 37°C, destabilisation also resulted in the failure to maintain the correct concentrations of CO_2 (5%) resulting in numerous experimental losses. Of additional interest was that, without exception, all the May profiles were above the mean and slightly less acidic than the majority of the August profiles (except August 8th, 100% FP and August 6th, 100% FP) which were below the mean and slightly more acidic, demonstrating a culture variable that had not been accounted for. However, as the correct temperature and CO_2 concentrations could not be monitored continually, the link between mechanical malfunctions and variations in results could not be explained.

The next phase of analysis was the comparison of bacterial composition of biofilms submitted for HOMIM analysis. The results showed the mean phyla and genera identified by HOMIM for the four treatment conditions were relatively similar throughout (P = 0.98 and 0.79 respectively).

Due to the apparent differences between spring and summer pH results, bacterial profiles were also analysed for similar trends. Unfortunately variations between May and August results were not identified until after the biofilms were submitted for composition analysis so the spread of spring and summer results is uneven. The genera identified in 0%, 50%, 100%

and 100% ES treatments, subcategorised into 'Hi' (above the mean) 'Med' (median, around the mean) and 'Lo' (below the mean) were determined. At 0% the number of genera identified was minimal, 2, 5 and 9 (mean = 5.3), for the Hi, Med and Lo profiles respectively, with a significant difference between Hi and Lo (P = 0.04).

Interestingly for the 50% profiles the number of genera identified was opposite, falling from 14, 11 and to 7 (mean = 10.6) for Hi, Med and Lo profiles respectively. This was again reversed within the 100% FP profiles from 7, 12 and to 17 (mean = 12, Hi, Med and Lo profiles respectively) and appearing to stabilise with the 100% ES biofilms, 6, 9 and 8 genera (mean = 7.7) through Hi, Med and Lo profiles respectively. In each of the FP treatment groups, no significant difference was observed between the median ranks, suggesting an element of stability within these groups. Closer analysis of the bacterial profiles, specifically phylotypes indicated that of the 24 phylotypes identified, 13 were found to be caries-associated, 8 were health associated, 1 was isolated from healthy and diseased sites and the remaining 2 were unknown (Aas *et al.*, 2008; Belstrøm *et al.*, 2014; Brailsford *et al.*, 1999; Drucker *et al.*, 1984; Gross *et al.*, 2010; Kianoush *et al.*, 2014; Nakajo *et al.*, 2010; Peterson *et al.*, 2013; Piwat *et al.*, 2010; Preza *et al.*, 2008; Romero Gómez *et al.*, 2004; Tanner *et al.*, 2011; Warren *et al.*, 2013; Yang *et al.*, 2012) . In every case, profiles were dominated by cariogenic lactobacilli and streptococci. Streptococci are a highly abundant member of the dental and oral flora contributing to symptomatic caries via a species-dependant range of aciduric tolerance. Lactobacilli are also part of the oral microbiome but typically exist in much lower numbers and tend to be more associated with decay (Badet & Thebaud, 2008; Loesche *et al.*, 1984). For example, a study by Lif Holgerson *et al.* (2015) showed that, at age 3 only 3% (N = 155) of caries-free subjects were identified as having lactobacilli in the saliva compared to 17% of children found to be caries-active (P < 0.001). So the observations made here are significant as lactobacilli have been shown to demonstrate some resistance to the antimicrobial effects of fluoride providing it with a tactical advantage and room for

proliferation within the community (Yoshihara *et al.*, 2001). Some workers have observed an increase in lactobacilli one week after a treatment with FP *in vivo* (Baygin *et al.*, 2013). In each of the cases shown here, as the abundance of streptococci and lactobacilli increased, the richness of species within the profile decreased demonstrating an earlier point regarding the positive selection of aciduric species, reducing diversity and leading to prolonged periods of acidogenicity. An important caveat with the present model is that the species were not replenished after the initial inoculation meaning that while there may have been some diurnal shifts in species dominance, based on increasingly limited carbon source, whichever species became dominant during early biofilm development were likely to remain dominant as long as either the carbon source was made available or an interaction with the chemical treatment group prevented metabolic dominance.

The 50% FP treatment differed in that there was an area of the substrate which remained untreated and in such may have acted as a refuge for more sensitive species, redefining the profile distribution. This depended upon the assumption that the fluoride did not precipitate into the surrounding media,

This part of the experiment was designed to observe two possibilities. Firstly, whether the partial treatment altered the acidogenicity of the biofilm, and second, whether the partially coated surface promoted a change bacterial diversity.

A change in the bacterial composition of the biofilm would be comparable to either the 0% or the 100% in that, if the fluoride was ineffective as an antimicrobial, only affecting those cultures attached to the treated side if the disc then the biofilm would look more like the 0% control as the untreated side of the substrate would promote greater diversity. If the culture was more closely comparable to the 100% treatment it would suggest that fluoride was providing some inhibitory effect which was linked to the loss and resuspension of fluoride from the treated surface.

This latter point has a secondary implication; the length of time the varnish would provide a cariostatic benefit to the patient with particular emphasis on single treatments once every 6 months and the rate of fluoride loss. Fluor Protector was manufactured to provide a fluoride reservoir at the surface of the enamel (Todd & Fischer, 2010) but the antimicrobial effects *in vivo* are unclear (Baygin *et al.*, 2013; Pinar Erdem *et al.*, 2012).

The 0% and 50% 'Hi' profiles had a pH ranges of 6.1 – 5.1 and 6 – 5.3 (respectively) over 90 min but despite being within the same pH range, the 50% profile indicated a clear difference with 12 more genera ($P = 0.02$) and 14 more phylotypes ($P = 0.005$) than that of the 0% profiles. In both cases the predominant cariogenic species were the lactobacilli and streptococci though the abundances were much greater at the 0%. The 50% profile also had 7 of the 9 species which were found to be associated more with dental health whereas the 0% only had one. Additionally, as the pH fell (Med to Lo) the diversity of the 0% profile increased whereas the 50% profile decreased. In an enclosed system this may be an indicator that the FP is promoting the proliferation of health associated species, under acidic conditions which, when conditions became too acidic are lost as aciduric species were being positively selected for. This being the case it would seem that the fluoride did have some effect on the development of biofilms in the system. Firstly, the dominance of lactobacilli over streptococci is highly unusual but the reduction in streptococci in parallel with the falling pH in every case but the 50% profile, and confirmed by relative stability in the 0% control, suggests that pH dependant inhibition may be a factor in this system. This same result also indicates that fluoride precipitating out of the product may not be the dominant mechanism by which this product imposes an antimicrobial effect. Had that been the case the profile would have looked a lot more like the 100% treatment, top-heavy with lactobacilli, with streptococcal abundances diminishing as the pH fell. The similarities described here are represented again in the 100% profile, in that at 'Hi' pH the biofilm was dominated by lactobacilli and streptococci the latter of which declined rapidly as the pH fell, which was reciprocated with

the highest phenotype richness in the experiment; 17 genera and 22 phylotypes, 8 of which have been found to be associated with health (Aas *et al.*, 2008; Gross *et al.*, 2010; Peterson *et al.*, 2013; Romero Gómez *et al.*, 2004; Tanner *et al.*, 2011; Yang *et al.*, 2012).

The final test group of PGRM experiments was devised to focus on the potential antimicrobial (cariostatic) benefits of FP as it might have been applied in the field. Fluor Protector was manufactured to be applied over a cleaned, dried tooth, flossed in to include the proximal surfaces and permitted 60 sec to dry (Anon, N.D.). In the field such applications were not possible, specifically with young children and so the varnish was applied directly onto uncleaned teeth. In order to replicate this *in vitro* the varnish was applied over an artificially applied salivary pellicle, hence 'excess saliva'. The pH profiles were not dissimilar from that of the 0%, 50% and 100% treatments, final pH readings being 5.6, 4.8 and 3.8 for Hi, Med and Lo respectively. However the bacterial profiles appeared to be a combination of the results presented in the treatment and control groups. Again dominated by lactobacilli and streptococci which showed no change in abundance from Hi to Lo profiles, as observed in the 0% treatment, the phylotype richness peaked in the Median profile which was also observed in the 50% treatment. It would appear that while the presence of fluoride may have had some influence on the cultures developed here, the more extreme indicators such as the decline of streptococci in a pH-dependant manor or spikes in richness suggest a closer similarity to the 0% profile. To answer this problem, statistical analyses were carried out on the phylotype profiles which compared treatment groups as a single cluster and then combined multiple groups to test for median rank analysis. Results showed the mean species profiles for the four treatment groups, those treated with fluoride are clearly more diverse while the 100% ES profile is reminiscent of the 0% profile, suggesting that the lack of proper application will have a reduced effect on biofilm development due perhaps to a lack of proper binding to the enamel.

Tests found no significant difference between intra-group mean profiles (P > 0.2) while combined observation between 0%, 50% and 100% ES were also found to be similar (P > 0.2). The exception was found in the comparison between 0% and 100% (P < 0.05) and subsequent analyses found that 0% Med differed from 100% Med and 100% Lo (P = 0.02, 0.009 respectively) and 0% Lo and 100% Lo (P = 0.001). In all three cases the main difference in richness observed was so pronounced that it is difficult to find an alternative explanation other than an interaction between the FP varnish and the biofilm.

In order to observe the effects of FP on the production of biomass in the biofilms, the dry weight of biofilms were measured before submission to HOMIM. There was an apparent difference between mean FP treated and untreated biofilms but no statistically significant difference was observed (P > 0.05). This is likely to be a result of extreme variations observed and indicated by the standard error.

In order to compare with previous results, the dry weights were also examined for fluctuations between May and August readings and while there were differences between treated and untreated profiles a significant difference was only observed between the May 100% and August 100% ES (P < 0.04) with an arguable difference between the May 50% reading and August 100% ES (P = 0.055). All intra-pH profiles between May and August were significantly different (P < 0.003) and in every case the August result was the most acidic. This more extreme environment may have resulted in the reduction in the relative abundance of S*treptococci* observed (mean May 0% control 41.7%, mean Aug 0% control 29.17%; see Appendix XIII – 'Microbiology data - PGRM' > 'By Genera'') and subsequent glucan production, leading to the reduction in dry weight observed here. Overall, these two results (dry weight and pH profiles) may be interconnected, pairing higher pH with more stable conditions and lower pH with more extreme conditions. Furthermore, streptococci are the

dominant genus within dental biofilms, acting as the early colonisers and major producers of the glucans which encapsulate and protect the biofilm from extremes in environmental conditions. The mean abundance of streptococci, specifically, *S. salivarius*, *S. downei*, and *S. oralis* across 0%, 50%, 100% and 100% ES was 38%, 27%, 23% and 30% which, in terms of distribution, follows the mean dry weight, closely. In this case, the 100% ES mean dry weight was greater than the control which results in an imperfect match with the abundance distribution of streptococci. However, factor in the degrees of standard error and the measure of estimation used to parallel PGRM pH profiles with upper, median and lower biofilms and while the patterns were similar, it follows that whatever defined the changes in distribution in phylotypes also played a part in restricting glucan development.

The PGRM study aimed to elucidate the potential benefits or drawbacks to treating teeth with Fluor Protector varnish with special emphasis on the effects of biofilm development and the production of organic acids. The results here show that, after 5 d of incubation under conditions which promote caries at an experimental level, the varnish does not appear to impair acid production. The PGRM phylotype profiles, specifically the 50% tests indicated that this may be due to the limited release of fluoride from the treated surface. Fluoride needs to cross the cell wall to inhibit glycolysis (Hamilton, 1990; Marquis *et al.*, 2003) and so if fluoride did dissolve and precipitate from the treated layer in sub-bacteriostatic concentrations such that it made little observable difference to the pH profile, but in its absence, the bacterial profile becomes redefined (as seen at 0% FP) and then it leaves an interesting question regarding the mechanism of protection afforded to the biofilm by its presence.

The PGRM was used to highlight the potential effects that the FP varnish had on acidogenic biofilms and the results showed that the presence of fluoride provided a pH dependant antimicrobial effect which is in keeping with the literature (Marquis *et al.*, 2003), however the specifics of the inhibition in this case were undetermined. The major action of fluoride as an

antimicrobial agent in oral biofilms is thought to be in the inhibition of glycolytic enzyme 'enolase' (Bunick & Kashket, 1981; Guha-Chowdhury *et al.*, 1997; Levine, 2011; Marquis *et al.*, 2003). It was thought this may also be the primary action of FP varnish here, which may have been the case. However, the presence of fluoride-insensitive, cariogenic species such as the lactobacilli are likely to have clouded the result with uninhibited acid production.

A review of the human samples taken shows that lactobacilli were identified in very small concentrations (range 0.1 – 0.6%) across the whole Bright Smiles study (chapter 5) indicating that the results observed in the PGRM are biased, possibly by a non-replenishing system,

This was one of the major issues with this experiment and as such, provided only a model trend for comparisons with the human study. Biofilm culture conditions for the PGRM experiments were done so under conditions which promote caries. This is due, firstly, because cultures were treated with sucrose and secondly, because the bacterial composition was not regularly replenished, as it would be in the mouth. This ensured that any shift towards cariogenesis would not naturally recover from a fall in bacterial diversity. It may have been that samples collected from human subjects who had not brushed their teeth since the previous evening were of a cariogenic composition and certainly the process of culture enrichment probably brought about a bacterial profile not dissimilar from that observed in the results. However, due to an oversight in the protocol, the pooled stock material from which each PGRM was sourced was not assessed for bacterial composition and due to this lack of baseline data it is not possible to discuss the suitability of the culture preparation methods at this time.

Another issue associated with the PGRM protocol was methods of pH measurement which required direct contact with the biofilm during the 90 minute sucrose challenge. During early optimisation it was found that measuring pH changes in the media rather than via direct contact with the biofilm did not always work. Early trouble shooting arrived at the possibility

that this was due the ratio between culture size and the volume of surrounding media, diluting

out pH fluctuations but in hindsight this could also have been attributed to poorly matured

biofilms associated with methods development. It was considered that the only way to control

for a combination of these two potential errors was to make direct contact with the biofilm.

Firstly, in every case direct contact with the biofilm resulted in an immediate drop in pH and

was probably attributed to a release of acids from within the biofilm (Xiao *et al.*, 2012) but

also served as a control for the presence of a culture and turned out to be an effective method

of controlling for culture failure. The method utilised here was highly sensitive and regularly

resulted in loss of biomass and for this reason, closer inspection of the culture to ensure

success prior to testing was not possible. While being a vital part of the process it also posed

another significant problem. Direct contact with the biofilm meant that the biofilm was no

longer in its native state. This was also an issue once testing had been completed such that

biofilm material could be seen suspended in the media. This ruled out further analysis of the

culture and resulted in bulk produced biofilm cultures being selected for HOMIM analysis by

'day of culture' based on the response of the PGRM-processed counterpart. While this

method of analysis proved useful it also carries an important caveat associated with the

comparisons made.

In an effort to further investigate this, a line of research was carried out into the

redevelopment of new method of biofilm production using the Modified Robbins Device

(MRDe). The MRDe (McCoy *et al.*, 1981) is a closed laminar flow chamber designed to

investigate biofilm removal techniques in enclosed, inaccessible environments such as water

pipes (Bomo *et al.*, 2004; Carter *et al.*, 2000; Kerr *et al.*, 1998; Silvestry-Rodriguez *et al.*,

2008). However, more recently the device has been used to investigate the complexities of

biofilm formation, maturation, adhesion and antibacterial and antibiotic resistance (Coenye &

Nelis, 2010; Hoyle *et al.*, 1993; Linton *et al.*, 1999; Millar *et al.*, 2001; Nickel *et al.*, 1985a;

Ramage *et al.*, 2008; Shah *et al.*, 2002; Vorachit *et al.*, 1993), viral infection of biofilms

(Doolittle *et al.*, 1995) and oral and dental health (Chin *et al.*, 2006; Coenye *et al.*, 2007; Honraet & Nelis, 2006; Silva-Lovato *et al.*, 2010; Yabune *et al.*, 2008; Yamamoto *et al.*, 2011). The MRDe is a rectangular chamber which can be made from a variety of different materials including acrylic, polysulfone, polyvinyl chloride (PVC) and stainless steel, providing resistance to a range of pressures and temperatures (TylerResearch, 2012). The device comes with 12 – 24 studs which are used to mount test materials (e.g. polyethylene or hydroxyapatite disks) which are colonised by test bacterial cultures. These studs penetrate the device via the dorsal face, such that the mounted disks run parallel with the inner wall of the laminar chamber. The device is then inoculated with the required media-suspended bacteria via inlet and outlet portals at opposing ends, allowing the mounted disks to be colonised by suspended bacteria. Upon reaching maturity, biofilms may be exposed to a variety of test materials which are dissolved or suspended in media which is pumped through the device. Culturing biofilms in this way promotes uniformity meaning that throughout testing one or all of 12 studs may be removed providing the user with a representative cross section of the whole for analysis.

One of the major hurdles encountered by researchers in the fields of dental health is the inability to reproduce the oral environment *in vitro*. The human mouth is composed of numerous tissue groups including muscle and mucosal membranes (Berkovitz *et al.*, 2009; Marsh, 2000) which make up the major and minor salivary glands and adjacent tissues (including the tongue, palate, cheeks and lips), each of which secrete fluids and numerous proteins and carbohydrates throughout the day (Avery *et al.*, 2002). Slight variations in temperature and pH are not only determined by diet, hydration and physical health but they also fluctuate throughout the day, from person to person and at different regions of the mouth (Airoldi *et al.*, 1997; Basavanthappa, 2003; Cole, 1993; Fejerskov & Kidd, 2008; Kelly, 2007; Newman & Martin, 2001; Rabinowitz *et al.*, 1996; Surdacka *et al.*, 2007). With these considerations, it can be understood that *in vitro* testing has major limitations and that

advancements in the understanding of oral health may only be reached in the development of model systems with more accurate environmental replication.

The MRDe designed with the aim of combining multi-biofilm uniformity with the standardised maturation and glycolytic testing (*in vitro*) of the PGRM. The new design was built around that idea that current PGRM involves small amounts of matured biofilm (approximately 1-2µg) being tested in large volumes of media (3ml) which is changed daily for approximately one week. On completion of the incubation process acid production is measured by pressing a pH probe against the disc, disrupting the biofilm and rendering it useless for future investigations.

The current study design was readapted to include docking chamber for micro pH probe which sits behind the internal culture platform, millimetres for the base of the biofilm (Appendix XII). Early experiments carried out included the development of porous disc prepared from polystyrene in the first instance and then hydroxyapatite coated porous stainless steel filters (0.2 µm). Unfortunately however, the method failed and the reason for this, while unproven is that it was believed the pH chamber would need to be inverted in order to properly circulate the media which connected the biofilm with the pH probe which was buffered using potassium chloride which, when inverted moves away from the reference junction preventing pH reading from being taken.

Due to time and financial constraints, this line of investigation was discontinued, and the lack of major results mean that it was not included as part on the main study. However, with more work could result in an improved method for the analysis of biofilms *in situ* while permitting pH readings from beneath the substrate and ensuring reduced damaged to the culture.

5. Comparison of oral biofilm composition in Control, Behavioural and Fluoride varnish treated children.

5.1 Introduction.

The Bright Smiles project was devised to review and compare two preventive dental services and their associated benefits to child dental health and cost to the NHS. The present study became associated with the Bright Smiles trial in order to observe the development and composition of oral biofilms during the first two years after tooth eruption and due to the implementation of said preventative services. Complete methods are described in full in sections 2.6.1 and 2.6.2.

Prior to the outset of this investigation it was realised that, to avoid conflicts of interest with the Bright Smiles study, which aimed to improve the oral health of the study volunteers, it was not deemed appropriate to request the avoidance of normal oral hygiene routines prior to biofilm sampling. Therefore, it was considered reasonable that volunteers could have brushed their teeth at any given point prior to session attendance which would have a major impact on the results and presented a variable which could not be controlled within this study. In order to account for this, parents were asked to estimate the last time the child had brushed their teeth and so additional intra- and inter-category analyses could be carried out to identify trends between brushing periods. In this case samples were subcategorised into four time slots, 0 – 2 h, 2 – 4 h, 4 – 6 h and 6+ h since brushing their teeth and attending the sampling session. The following data illustrates the results of those analyses and the complete set of data is displayed in Table 4.

5.2. Experimental controls analysed between 2011 and 2014 via HOMIM.

As described previously (section 2.6.1) throughout the Bright Smiles study, split-sample controls were added to the human samples and analysed together by HOMIM. To prevent biases in the analyses the Forsyth Institute were not alerted to the control system; the results are shown in Figure 28. The Number of genera identified were (presented 'sample number/number of genera') 47A, 10; 47B, 14; X1, 15; X2, 7; Y1, 1;, Y2, 7; SE1, 4; SE2, 4. A Kruskal-Wallis test was used to analyse the collective data, and indicated that there were differences between profiles though they were not significant ($P = 0.057$). Due to the P values approximation to the 95% alpha level a subsequent Mann Whitney test was carried out to analyse each sample couplet which also indicated significant differences ($P > 0.05$).

Relative abundance (%)

Bar chart showing composition of genera for Control samples 47A, 47B, X1, X2, Y1, Y2, SE1, and SE2 (%AB), with x-axis from 0% to 100%.

Legend:
- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 28. Composition of genera identified in experimental 'split-sample' controls submitted with BS samples, designed to determine the effects of the splitting of samples.

Sample codes indicate the two halves of each sample (e.g. 47A and 47B).

Control Group

Baseline		Round 1		Round 2	
Sample ID	Hrs	Sample ID	Hrs	Sample ID	Hrs
2	5	4	0.5	6	6.0
8	1	6	1.35	39	6.4
10	2	14	1		
12	3	32	0.35		
14	1.5	51	1.1		
16	5.5	2	3.45		
18	1.5	10	5.15		
32	8	18	6		
36	8	39	4.35		
39	6.5				
43	6.5				
45	1				
65	4				
69	9				
73	4				

Behavioural Group

Baseline		Round 1		Round 2	
Sample ID	Hrs	Sample ID	Hrs	Sample ID	Hrs
28	2	28	UK	28	8.3
30	6.5	67	2	55	4.3
55	5			57	4.1
57	6			67	0.5
67	2				

Fluoride Group

Baseline		Round 1		Round 2	
Sample ID	Hrs	Sample ID	Hrs	Sample ID	Hrs
22	1.5	20	9	20	5.2
41	0.5	41	0.5	22	6.3
49	3.5	53	7	49	2.1
59	6	61	2	53	4.3
61	6	63	UK	59	1.2
63	7	22	UK	61	4.3
71	2	49	UK	63	UK
75	5			71	1.5
77	2				

Table 4. Total samples collected over the 2 year study period.

Data indicates, for each study group (Control, Behavioural, Fluoride), the trial-assigned sample number (Sample ID) and hours between brushing and sampling (Hrs). Samples where the time between brushing and plaque collection could not be determined were marked 'unknown' (UK).

5.3. The mean abundance (%) of oral phyla collected from participants at Baseline.

Oral swabs were collected from the upper incisors and lateral incisors for 30 sec and analysed for bacterial composition via HOMIM analysis; data was converted into relative abundances (%) and the results are shown in Figure 29. From 29 samples (Control x 15; Behavioural x 5; Fluoride x 9), analyses identified 9 phyla across the combined set (Control x 8; Behavioural x 6; Fluoride x 8) which included 45 genera (Control x 41; Behavioural x 24; Fluoride x 31) and 133 phylotypes (Control x 124; Behavioural x 58; Fluoride x 79) of relative proportions throughout the data set. Standard error bars indicate some variability on the relative abundances, however, a Mann-Whitney test, used to analyse the distribution of phyla between the three groups and found no significant differences (P = 0.62) indicating phylogenetic consistency throughout the set.
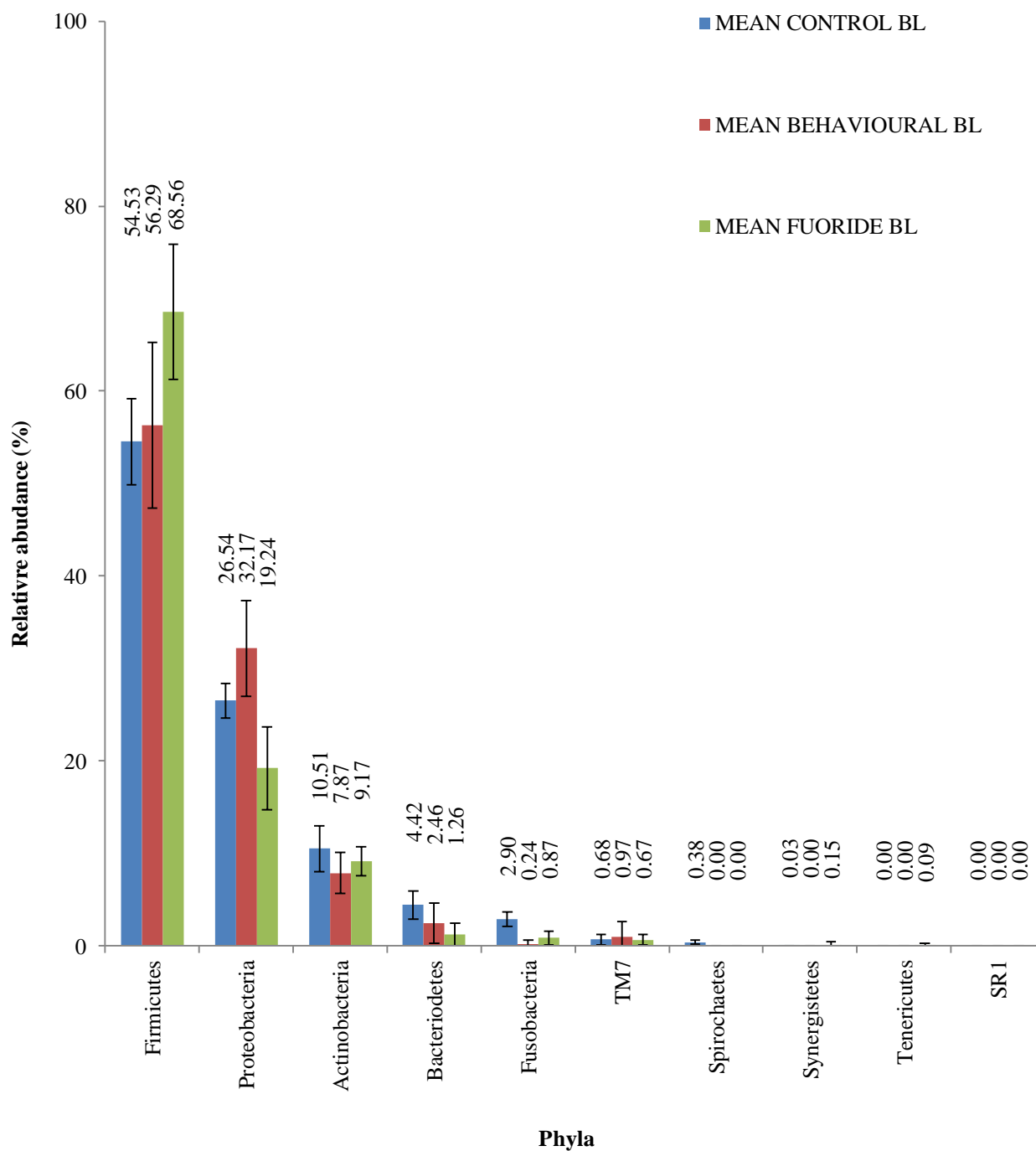
Figure 29. The mean relative abundance of phyla in Baseline samples of plaque, as analysed by HOMIM.

Error bars indicate the standard error and data labels have been included for ease of interpretation.

5.4 The abundance (%) of human oral samples collected from BS volunteers at Round 1.

Oral swabs were collected from the upper incisors and lateral incisors for participants after approximately 1 year (Round 1) for 30 sec and analysed for bacterial composition via HOMIM analysis; data was converted into relative abundances (%) and analysed for trends (Figure 30). From 18 samples (Control x 9; Behavioural x 2; Fluoride x 7), analyses identified a compiled total of 9 phyla (Control x 8; Behavioural x 7; Fluoride x 6), which included 41 genera (Control x 39; Behavioural x 26; Fluoride x 21) and 131 phylotypes (Control x 108; Behavioural x 55; Fluoride x 68) in relative proportions throughout the data set. Standard error bars indicated some variability however, a Mann-Whitney test analysis of the distribution of phyla between the three groups and showed no significant differences (P = 0.67).
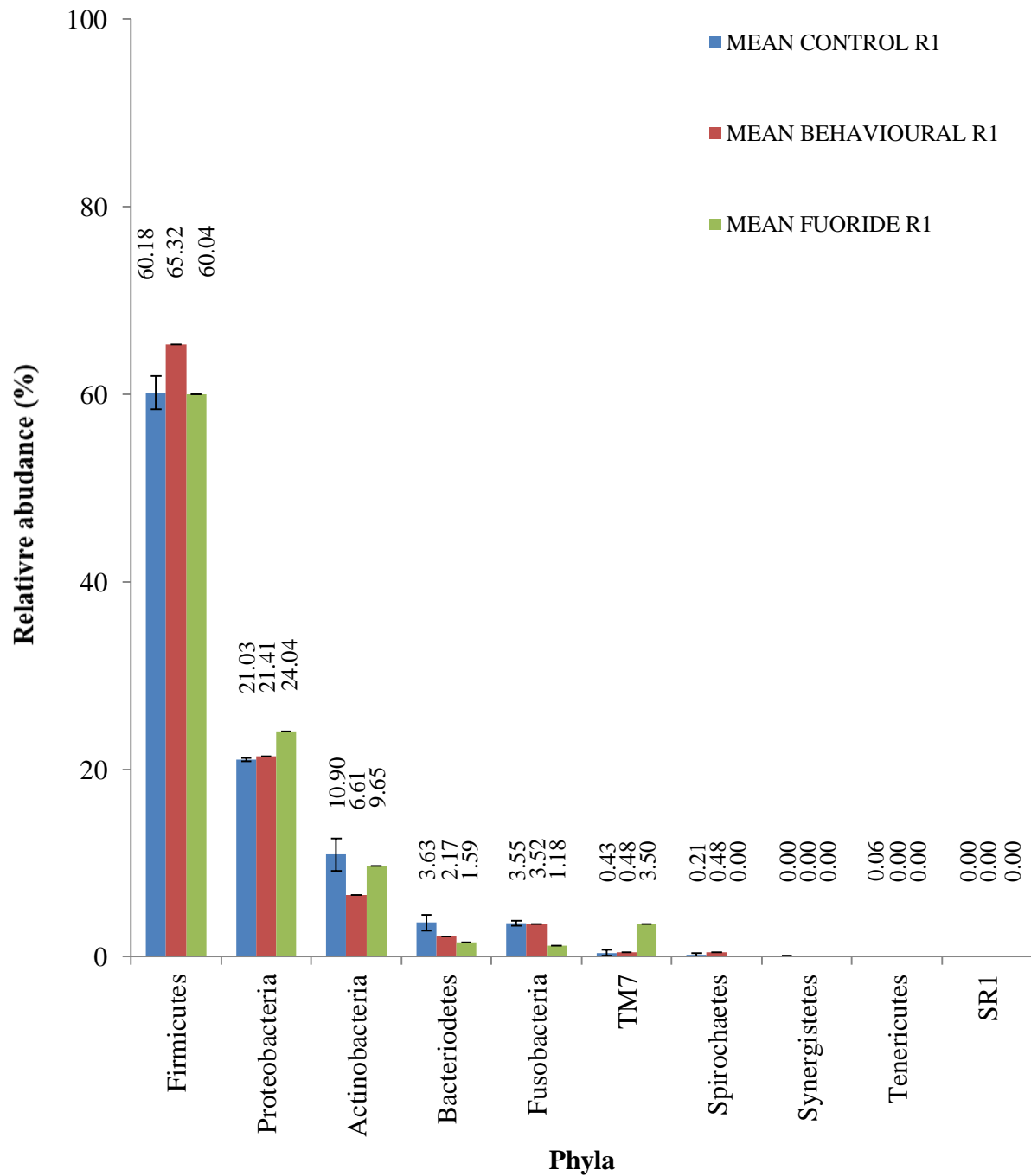
Figure 30. The mean relative abundance of phylotypes identified in Round 1 plaque samples, as analysed by HOMIM.

Error bars indicate the standard error and data labels have been included for ease of interpretation.

5.5 The Abundance (%) of human oral samples collected from BS volunteers at Round 2.

Oral swabs were collected from the upper incisors and lateral incisors for 30 sec after approximately 2 years (Round 2) and analysed for bacterial composition via HOMIM analysis; data was converted into relative abundances (%) and analysed for trends (Figure 31). From 14 samples (Control x 2; Behavioural x 4; Fluoride x 8), analyses identified a compiled total of 7 phyla (Control x 3; Behavioural x 7; Fluoride x 5), 33 genera (Control x 9; Behavioural x 31; Fluoride x 19), and 88 phylotypes (Control x 17; Behavioural x 78; Fluoride x 48) in relative proportions throughout the dataset. At Round two HOMIM identified only 3 phyla in the Control group compared to 7 and 5 in the Behavioural and Fluoride groups (respectively) as well as an 8% increase in *Firmicutes* over the Behavioural and Fluoride groups which appear to have remained stable. Despite the observable differences, the Mann-Whitney test analysed the distribution of phyla between the three groups and found no significant differences (P = 0.26).
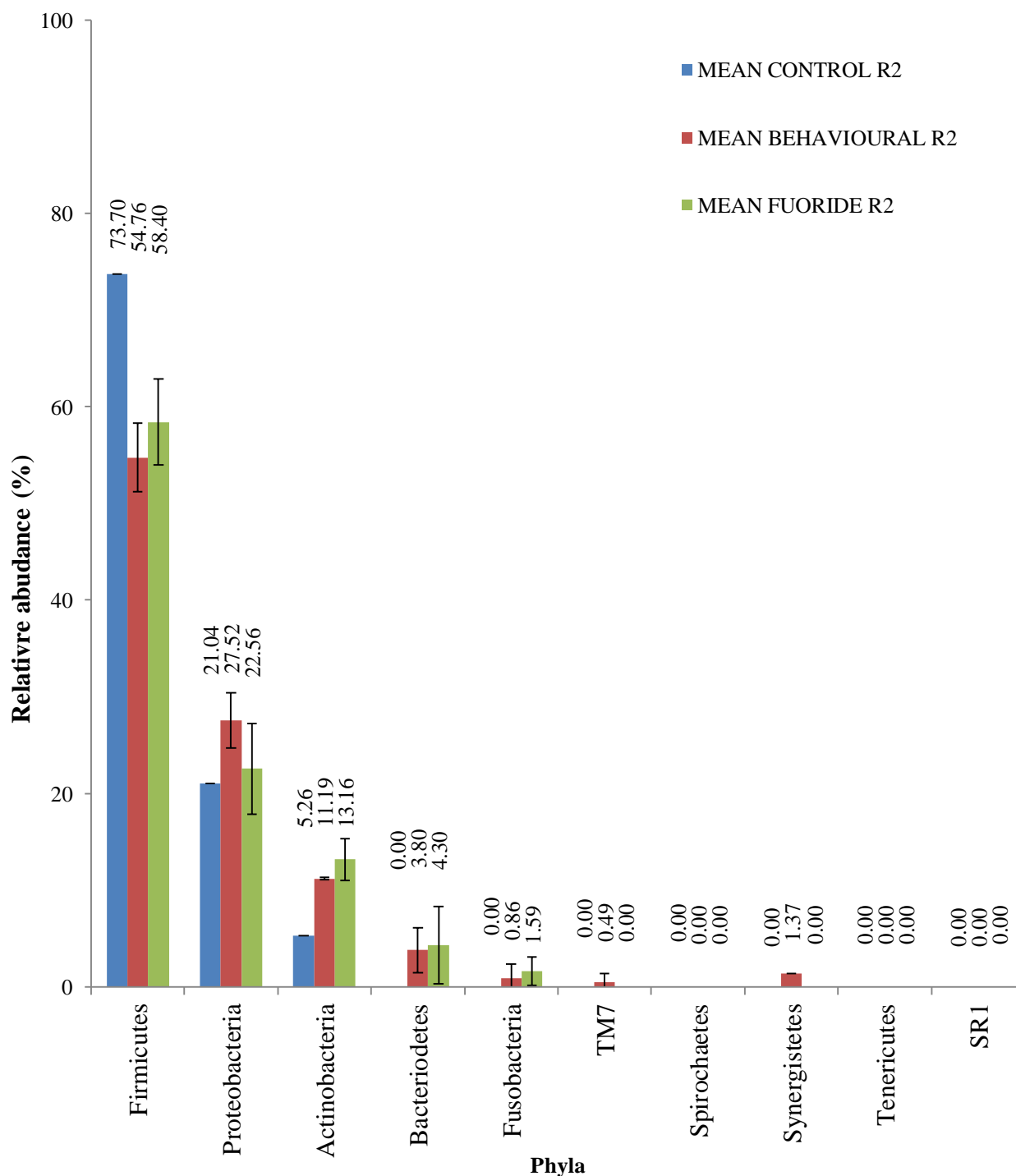
Figure 31. The mean relative abundance of phylotypes identified in Round 2 samples, as analysed by HOMIM.

Error bars indicate the standard error and data labels have been included for ease of interpretation.

5.6. Genera identified from human oral samples collected from BS participants at Baseline.

HOMIM data for Baseline samples was converted into the mean relative abundance (%) by genera for the Control, Behavioural and Fluoride groups which totalled 41, 24 and 31 respectively (Figure 32). The Kruskal Wallis test was used to compare the distribution of genera between the combined profiles and no statistical difference was observed ($P = 0.23$); a Mann Whitney U test then compared individual profiles and gave the same result ($P > 0.05$).

Relative abundance (%)

Legend:
- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 32. The mean relative abundance (%) of genera identified by HOMIM analysis, for Baseline samples in the three treatment groups.

Data indicates the results for Baseline Control (BL CON, n x 15 samples), Baseline Behavioural (BL BEH, n x 5 samples) and Baseline Fluoride (BL FLU, n x 9 samples).

5.7. Genera identified from human oral samples collected from BS participants at Round 1.

For each study group, Round 1 Control (R1 CON, n x 9 samples), Round 1 Behavioural (R1 BEH, n x 2 samples) and Round 1 Fluoride (R1 FLU, n x 7 samples), HOMIM analysis of Round 1 samples was converted into the mean relative abundance (%) by genera; the mean number of genera identified for the Control, Behavioural and Fluoride groups was 39, 26 and 21 respectively (Figure 33). The Kruskal Wallis test was used to compare the distribution of genera between the combined profiles and no statistical difference was identified (P = 0.55). Furthermore, a Mann Whitney U test then compared individual profiles and calculated a significant difference between Mean R1 CON and Mean R1 FLU (P < 0.034).
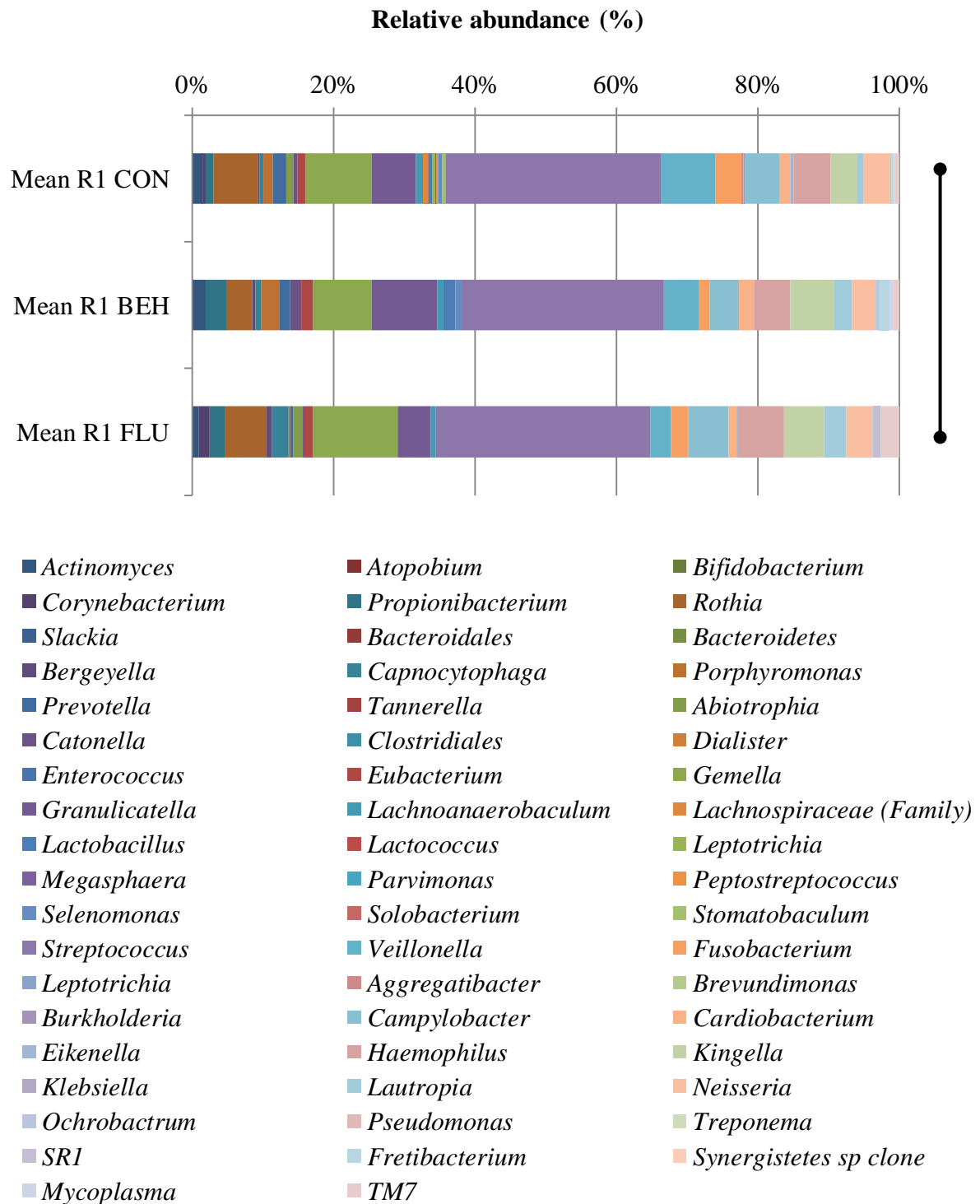
Figure 33. Relative abundance (%) of genera identified within plaque samples collected from Round 1 samples, across the three treatment groups as analysed by HOMIM.

The vertical bar, to the right of the figure and linking the Mean R1 CON and Mean R1 FLU indicates a significant difference ($P < 0.034$) between the profile distributions.

5.8. Genera identified from human oral samples collected from BS volunteers at Round 2.

For each study group, Round 2 Control (R2 CON, n x 2 samples), Round 2 Behavioural (R2 BEH, n x 7 samples) and Round 2 Fluoride (R2 FLU, n x 8 samples), the HOMIM analysis of Round 2 samples was converted into the mean relative abundance (%) by genera (Figure 34). The mean number of genera identified for the Control, Behavioural and Fluoride groups was 9, 31 and 19 respectively. The Kruskal Wallis test was used to compare the distribution of genera between the combined profiles and calculated a statistical difference between combined profiles (P = 0.02); a subsequent Mann Whitney U test calculated a highly significant difference between Mean R2 CON and Mean R2 BEH (P < 0.01).

Relative abundance (%)

Legend:
- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 34. Comparison of % abundance of plaque analysed by HOMIM analysis for Round 2 samples in the three treatment groups.

The vertical bar, to the right of the figure and linking the Mean R2 CON and Mean R2 BEH indicates a significant difference ($P < 0.01$) between the profile distributions.

5.9 Genera comparison of mean Control group samples through Baseline to Round 2.

HOMIM data was divided to Baseline Control (BL CON, n x 15 samples), Round 1 Control (R1 CON, n x 9 samples) and Round 2 Control (R2 CON, n x 2 samples) and the relative abundances (%) by genera for the intra-group analysis were compared. There was marked reduction in bacterial composition of Control samples from Baseline to Rounds 1 and 2 which totalled 41, 39 and 9 genera and 124, 108 and 17 phylotypes, respectively (Figure 35). The Kruskal Wallis analysis found a highly significant difference between complied profiles over the three sampling periods ($P < 0.01$) and a subsequent Mann-Whitney analysis was used to compare individual mean profiles and found highly significant differences between BL and R2 and R1 and R2 ($P < 0.01$).

Relative abundance (%)

Legend:

- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 35. The relative abundance (%) of mean phylotypes identified by HOMIM within the Control group, Rounds 1 - 3.

The vertical bar, to the right of the figure and linking the Mean BL CON and Mean R2 CON and Mean R1 CON and Mean R2 Con indicates highly significant differences (P < 0.01) between the profile distributions.

5.10. Genera comparison of mean Behavioural group samples through Baseline to Round 2.

HOMIM data was converted into the relative abundance (%) by genera for an intra-group, annual Round analysis for the Control study, specifically Baseline Behavioural (BL BEH, n x 5 samples), Round 1 Behavioural (R1 BEH, n x 2 samples) and Round 2 Behavioural (R2 CON, n x 4 samples; Figure 36). Within each mean sample set the bacterial composition from Baseline to Rounds 1 and 2 remained relatively stable, including 24, 26 and 31 genera and 58, 55 and 78 phylotypes, (respectively) and the Kruskal Wallis and the Mann-Whitney U comparisons showed no significant differences between profiles over the period (P > 0.05).

Relative abundance (%)

Legend:
- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 36. The relative abundance (%) of mean phylotypes identified by HOMIM within the Behavioural group, Rounds 1 - 3.

5.11. Genera comparison of mean Fluoride group samples through Baseline to Round 2

HOMIM data was converted into the relative abundance (%) by genera for an intra-group, annual Round analysis for the Control study, specifically Baseline Fluoride (BL FLU), Round 1 Fluoride (R1 FLU) and Round 2 Fluoride (R2 FLU) and the results are shown in Figure 37. Within each mean sample set the bacterial composition from Baseline to Rounds 1 and 2 indicated a trend of loss in biodiversity over the three year period which included 31, 21 and 19 genera and 79, 68 and 48 phylotypes, (respectively). However, a Kruskal Wallis and Mann-Whitney U comparisons showed no significant differences between profiles over the period ($P > 0.05$).

Relative abundance (%)
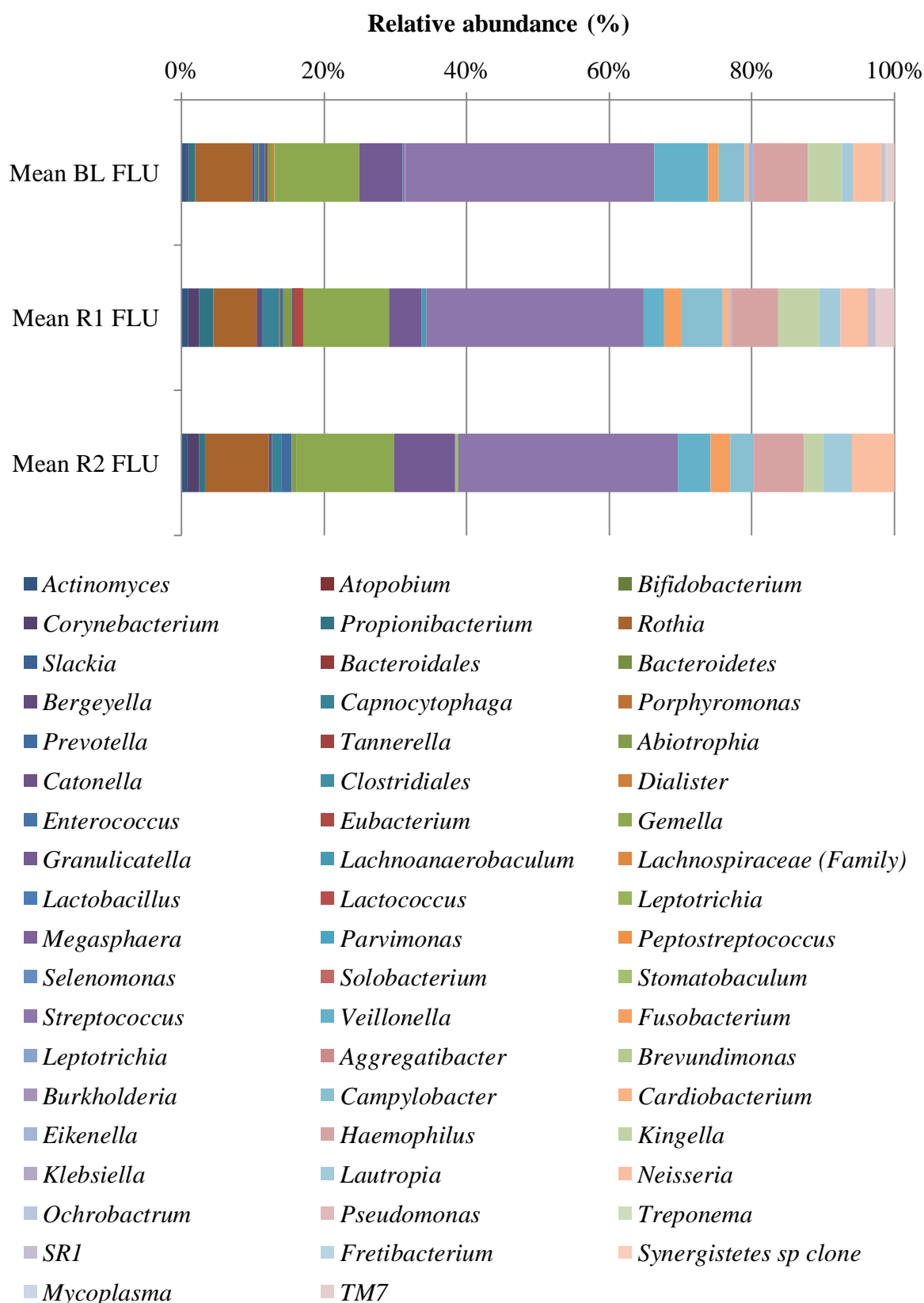
Figure 37. The relative abundance (%) of mean phylotypes identified by HOMIM within the Fluoride group, Rounds 1 - 3.

5.12 Genera comparison of mean Control, Behavioural and Fluoride inter-study and inter-round groups.

Previous data highlighting the distribution of genera between treatments and sample rounds was compared for statistical differences across the total set (Figure 38). A Mann Whitney U analysis was carried out between mean Baseline Control (BL CON), Round 1 Control (R1 CON) and Round 2 Control (R2 CON), Baseline Behavioural (BL BEH), Round 1 Behavioural (R1 BEH) and Round 2 Behavioural (R2 BEH) and Baseline Fluoride (BL FLU), Round 1 Fluoride (R1 FLU) and Round 2 Fluoride (R2 FLU). Statistical differences between specific mean profiles are indicated on the right the graph panel; P values (L-R, $P \leq 0.03$) and indicate a disparity between the Control study and treatment groups.

Relative abundance (%)

Legend:
- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 38. Relative abundance (%) of genera in mean Control, Behavioural and Fluoride inter-study and inter-round groups.

Significant differences are highlighted by vertical linking bars on the right of the figure (P ≤ 0.03).

5.13. Genera comparison of mean Control, Behavioural and Fluoride inter-study and inter-round samples categorised by 'brushing hours before sampling'.

Total Control profiles for genera were then analysed for differences due to the period of time between brushing the teeth and sample collection (Figure 39). For convenience, profiles were split into 0-2 h, 2-4 h, 4-6 h, and 6+ h time slots; empty slots indicate no samples falling into this group. Intra-time slot analyses found that there was one difference at baseline (0-2, P<0.03), Six at Round 1 (0-2, P<0.034, 2-4, P<0.03; 4-6, P<0.03) and none in Round 3. The number of genera per mean sample set ranged from 0 (represented by the blank spaces) then 9 to 38. A Mann-Whitney analysis calculated 9 significant differences within the complete set which are illustrated by linking bars on the right of the figure (P ≤ 0.02). Analysis indicates differences that span all three annual sampling periods.

Relative abundance (%)

Legend:

- Actinomyces
- Atopobium
- Bifidobacterium
- Corynebacterium
- Propionibacterium
- Rothia
- Slackia
- Bacteroidales
- Bacteroidetes
- Bergeyella
- Capnocytophaga
- Porphyromonas
- Prevotella
- Tannerella
- Abiotrophia
- Catonella
- Clostridiales
- Dialister
- Enterococcus
- Eubacterium
- Gemella
- Granulicatella
- Lachnoanaerobaculum
- Lachnospiraceae (Family)
- Lactobacillus
- Lactococcus
- Leptotrichia
- Megasphaera
- Parvimonas
- Peptostreptococcus
- Selenomonas
- Solobacterium
- Stomatobaculum
- Streptococcus
- Veillonella
- Fusobacterium
- Leptotrichia
- Aggregatibacter
- Brevundimonas
- Burkholderia
- Campylobacter
- Cardiobacterium
- Eikenella
- Haemophilus
- Kingella
- Klebsiella
- Lautropia
- Neisseria
- Ochrobactrum
- Pseudomonas
- Treponema
- SR1
- Fretibacterium
- Synergistetes sp clone
- Mycoplasma
- TM7

Figure 39. The mean genera profiles for Control group samples across the entire study, categorised by 'brushing hours before sampling'

Significant differences between individual mean profiles are highlighted by vertical linking bars on the right of the figure ($P \leq 0.02$).

Total Behavioural profiles for genera were then analysed for differences between the period of time between brushing the teeth and sample collection. For convenience, profiles were split into 0-2 h, 2-4 h, 4-6 h, and 6+ h time slots; empty slots indicate no samples falling into this time period group. Intra-time slot analyses found that there were no differences at Baseline or Round 1 however there was one difference in Round 2 (4-6, P<0.01). In the Behavioural study the number of genera per mean sample set ranged from 0 (represented by the blank spaces) then 8 to 27 (Figure 40). A Mann-Whitney analysis calculated 5 significant differences within the complete set which are illustrated by linking bars on the right of the figure. Statistical analysis of this sample cluster spans all three annual sampling periods (P $\leq$ 0.02).
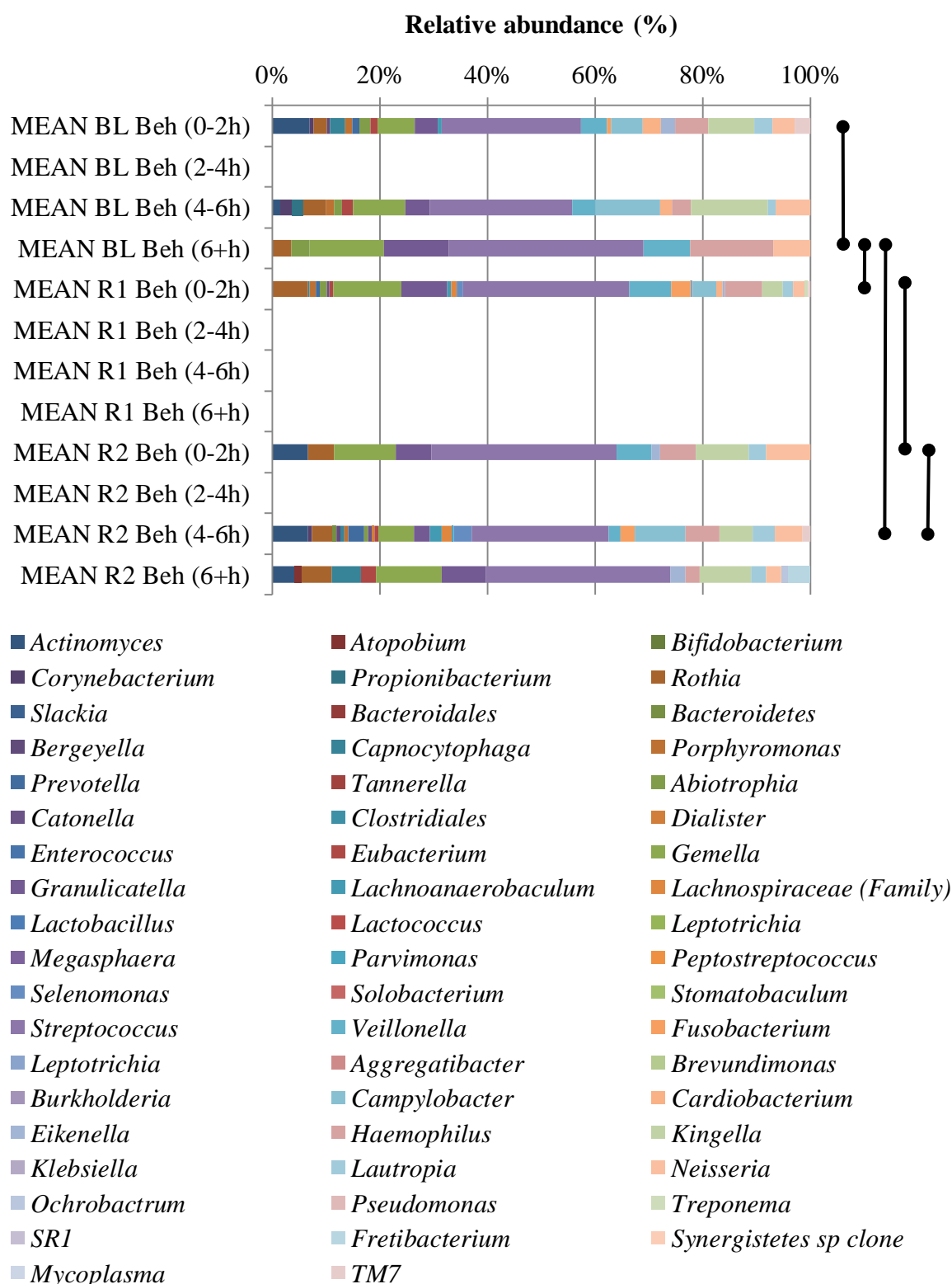
Figure 40. Mean genera profiles for Behavioural group samples across the entire study, categorised into by 'brushing hours before sampling'.

Significant differences are highlighted by vertical linking bars on the right of the figure (P ≤ 0.02).

Next, the total Fluoride profiles were analysed for differences due to the period of time between brushing the teeth and sample collection. For convenience, profiles were split into 0-2 h, 2-4 h, 4-6 h, and 6+ h time slots; empty slots indicate no samples falling into this group. Intra-time slot analyses found one differences at Baseline (4-6, P<0.03) and none in rounds 1 or 2. In the Fluoride group the number of genera per mean sample set ranged from 0 (represented by the blank spaces) then 4 to 26 (Figure 41). A Mann-Whitney analysis calculated 13 significant differences within the complete set which are illustrated by linking bars on the right of the figure. Statistical significances of this sample cluster spans all three annual sampling periods (P ≤ 0.05) and all differences linked the pre-treatment Baseline profile to post-treatment profiles. No differences were observed within or between Round 1 and 2.

Relative abundance (%)

	0%	50%	100%
MEAN BL Flu (0-2h)			
MEAN BL Flu (2-4h)			
MEAN BL Flu (4-6h)			
MEAN BL Flu (6+h)			
MEAN R1 Flu (0-2h)			
MEAN R1 Flu (2-4h)			
MEAN R1 Flu (4-6h)			
MEAN R1 Flu (6+h)			
MEAN R2 Flu (0-2h)			
MEAN R2 Flu (2-4h)			
MEAN R2 Flu (4-6h)			
MEAN R2 Flu (6+h)			

Legend:

- *Actinomyces*
- *Atopobium*
- *Bifidobacterium*
- *Corynebacterium*
- *Propionibacterium*
- *Rothia*
- *Slackia*
- *Bacteroidales*
- *Bacteroidetes*
- *Bergeyella*
- *Capnocytophaga*
- *Porphyromonas*
- *Prevotella*
- *Tannerella*
- *Abiotrophia*
- *Catonella*
- *Clostridiales*
- *Dialister*
- *Enterococcus*
- *Eubacterium*
- *Gemella*
- *Granulicatella*
- *Lachnoanaerobaculum*
- *Lachnospiraceae (Family)*
- *Lactobacillus*
- *Lactococcus*
- *Leptotrichia*
- *Megasphaera*
- *Parvimonas*
- *Peptostreptococcus*
- *Selenomonas*
- *Solobacterium*
- *Stomatobaculum*
- *Streptococcus*
- *Veillonella*
- *Fusobacterium*
- *Leptotrichia*
- *Aggregatibacter*
- *Brevundimonas*
- *Burkholderia*
- *Campylobacter*
- *Cardiobacterium*
- *Eikenella*
- *Haemophilus*
- *Kingella*
- *Klebsiella*
- *Lautropia*
- *Neisseria*
- *Ochrobactrum*
- *Pseudomonas*
- *Treponema*
- *SR1*
- *Fretibacterium*
- *Synergistetes sp clone*
- *Mycoplasma*
- *TM7*

Figure 41. Mean relative abundance (%) genera profiles for Fluoride group samples measured by 'brushing hours before sampling'.

Significant differences are highlighted by vertical linking bars on the right of the figure (P ≤ 0.05).

5.14. Shannon Weiner index comparison of mean Control, Behavioural and Fluoride inter-study and inter-round samples categorised by 'brushing hours before sampling'.

In addition to the analyses described, Shannon Weiner indices were calculated following Gafan (2005) and Jost (2006) in order to observe changes in the mean bacterial diversity between brushing and sample collection (Figure 42). All samples that were collected were assigned to one of either '0 -2 h, 2 - 4 h, 4 - 6 h, or 6+ hours since brushing' test groups. Data shows the mean Shannon indices for Baseline samples collected at each time period. Standard error bars are included and gaps in the data indicate that no samples were collected in this time slot. The Baseline study indicated a trend of loss in all three study groups, analysis of genera between Control group, 0-2 h and 6+ h indicated a significant difference ($P=0.02$); a Mann-Whitney analysis of the Shannon Weiner distribution confirmed this finding ($P=0.012$). Similarly the genus distribution in the Behavioural study 0-2 h and 6+ h ($P=0.01$) showed significant differences however, this was represented by a single Shannon distribution index and could not be further analysed with the Mann Whitney. In the Fluoride group there was a large drop in diversity between 0-2 h and 2–4 h ($P<0.001$) and differences were also observed between 0-2 h and 6+ ($P<0.001$), 2 - 4 h and 4 - 6 h ($P=0.001$) and 4 - 6 h and 6+ h ($P=0.004$). An analysis between 0-2 and 4-6 showed a P-value of 0.67 which was confirmed by the Mann-Whitney ($P = 0.85$) as not being significantly different, however, individual indices could not be analysed and so no further tests could be carried out. The Shannon Wiener data is summarised in Table 5

Mean Shannon-Wiener Indices for the Basline distibution of genera in the hours between brushing and sample collection.
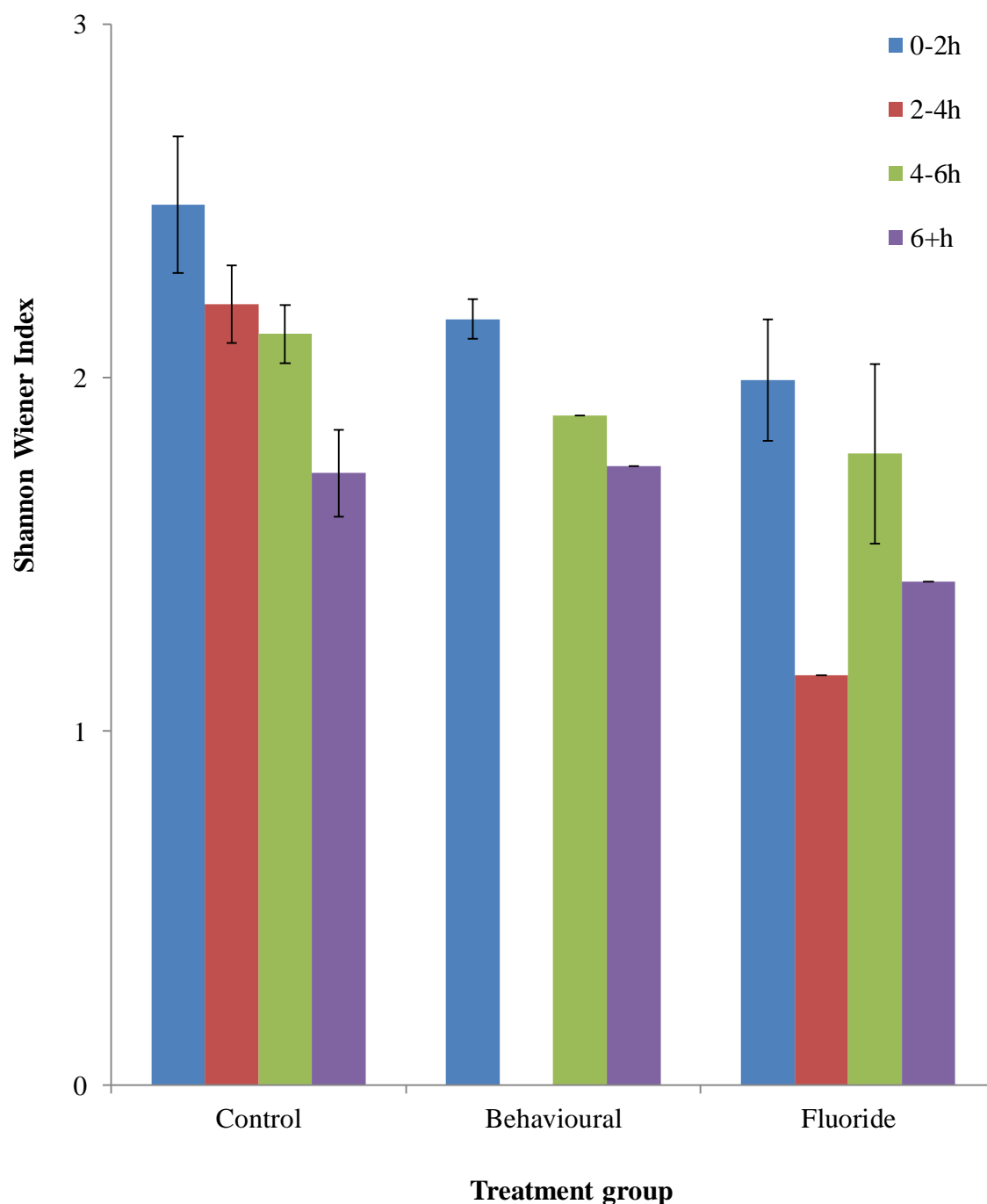
Figure 42. Mean Shannon-Wiener indices for the Baseline genera categorised by hours between brushing and sampling.

Standard error bars are included and gaps in the data indicate that no samples were collected in this time slot.

Next, the results of the mean Shannon Weiner index for Round 1 samples collected at each of the allotted time periods are shown in Figure 43. Standard error bars are included and gaps in the data indicate that no samples were collected in this time slot. Unfortunately there were a number of representatives missing from this study and so direct comparisons were difficult. The results of the Control study were missing the 6+ h reading however, 0 - 2 to 4 – 6 readings are similar to those at Baseline and indicate a trend of diversity loss over time and a significant difference between, 0 - 2 to 2 - 4 (P=0.014). The Behavioural 0 – 2 h reading showed an increase in diversity when compared to the Baseline however, like the 6+ h reading 12 months earlier there was only one sample at this time slot and so remarks would be speculative. The Fluoride 6+ h results showed an increase on the 0 – 2 h reading and while both Fluoride readings are made up of only two samples each, there was no significant differences calculated between bacterial profiles at the genera level ($P \leq 0.05$). Multiple samples within time slots for the Shannon Weiner analysis were only observed in Control 0 – 2 h and 4 – 6 h and Fluoride 0 – 2 h and 6+ h, both of which were not significantly different in both the genus abundances (P = 0.35 and 0.25 respectively) and the Mann-Whitney test for Shannon distribution (P = 0.55 and 0.69 respectively). Shannon Wiener data are summarised in Table 5.
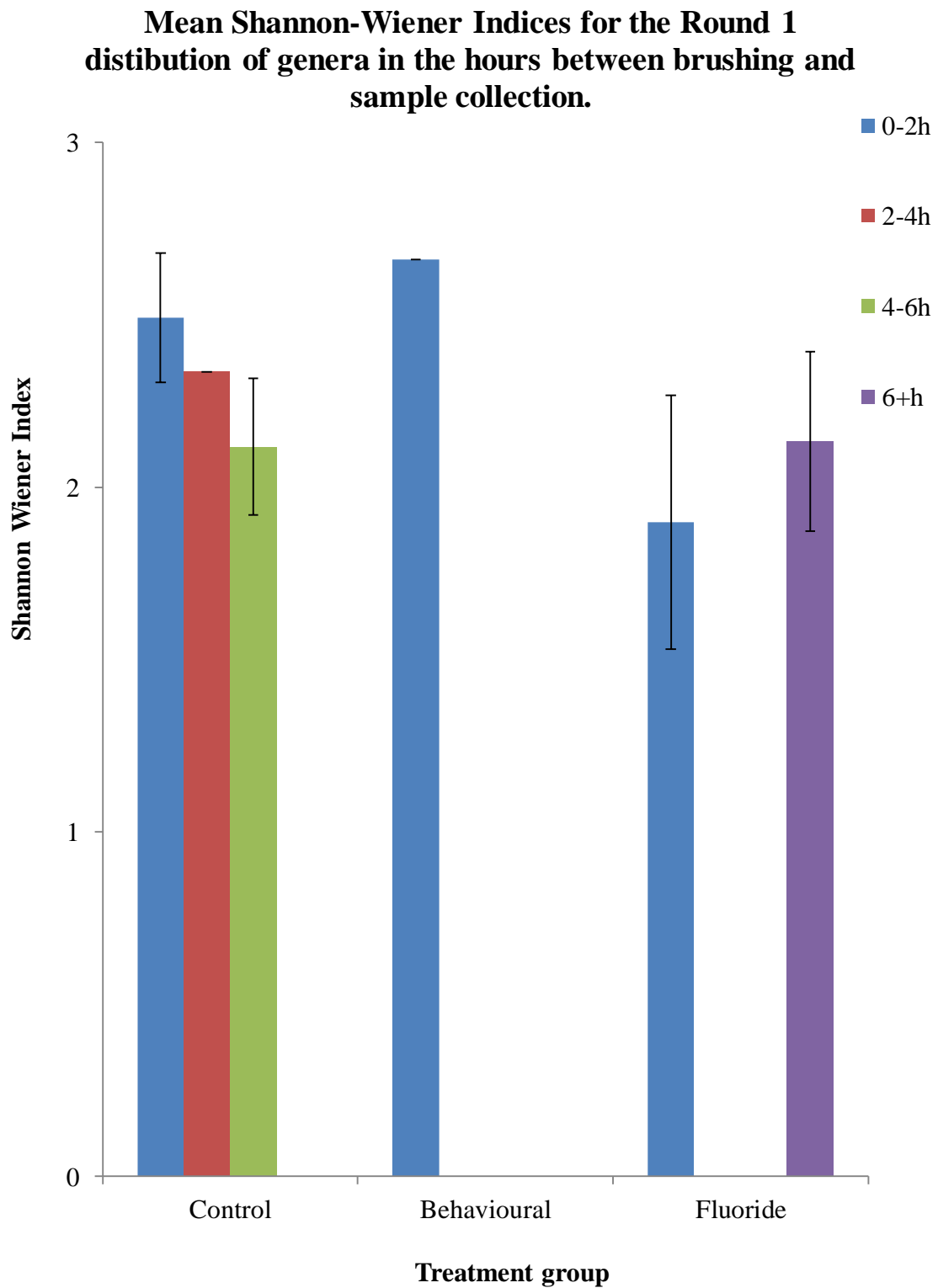
Figure 43. Mean Shannon-Wiener indices for the Round 1, genera categorised by hours between brushing and sampling.

Standard error bars are included and gaps in the data indicate that no samples were collected in this time slot.

The mean Shannon indices for Round 2 samples collected at each of the allotted time periods are shown in Figure 44. Again there were a readings missing, particularly from the Control study but the Behavioural study indicates an increase in diversity between $0 - 2$ h and $4 - 6$ h was significantly different at the genus level (P=0.015), however, individual readings meant that this figure could not be confirmed with the Mann-Whitney analysis. The third full set of readings (0-2 to 6+), in this case for Fluoride study, follows the same trend as the Control and Fluoride set in the Baseline study and the partial Control study in Round 1, indicating a loss of diversity between 0 and 6+ hours though the data was not found to differ significantly (P<0.05). This was confirmed with a Mann-Whitney analysis of Fluoride group Shannon indices between $0 - 2$ h and $4 - 6$ h (P=100) though single samples per time slot meant that no further Mann-Whitney analyses could be carried out. Shannon Wiener data is summarised in Table 5.

Figure 44. Mean Shannon-Wiener indices for the Round 2 genera categorised into 'hours before brushing'.

Standard error bars are included and gaps in the data indicate that no samples were collected in this time slot.

	Time slot	Shannon indices for each time slot					Mean	SD	SE	Sig
Baseline	**0-2h**	2.89	2.92	2.54	2.08	2.01	2.49	0.43	0.20	◆
	2-4h	2.26	2.26	2.10			2.21	0.10	0.06	
	4-6h	2.30	1.95				2.12	0.24	0.17	
	6+h	1.84	1.24	1.87	1.93	1.77	1.73	0.28	0.12	◆
	0-2h	2.28	2.06				2.17	0.16	0.11	
	4-6h	1.99	1.79				1.89	0.14	0.10	
	6+h	1.75					1.75	-	-	
	0-2h	2.10	1.97	1.77	2.13		1.99	0.16	0.08	
	2-4h	1.16					1.16	-	-	
	4-6h	1.93	2.13	1.29			1.79	0.44	0.25	
	6+h	1.42					1.42	-	-	
Round 1	**0-2h**	2.93	2.69	2.13	2.74	1.97	2.49	0.42	0.19	
	2-4h	2.33					2.33	-	-	
	4-6h	2.78	2.05	1.52			2.12	0.64	0.37	
	0-2h	2.66					2.66	-	-	
	0-2h	1.70	2.10				1.90	0.28	0.20	
	6+h	1.87	2.39				2.13	0.37	0.26	
Round 2	**6+h**	1.40	1.75				1.57	0.25	0.18	
	0-2h	2.04					2.04	-	-	
	4-6h	2.51	1.99				2.25	0.37	0.26	
	6+h	2.12					2.12	-	-	
	0-2h	1.90	2.14				2.02	0.17	0.12	
	2-4h	2.14					2.14	-	-	
	4-6h	2.07	2.06	1.96			2.03	0.06	0.03	
	6+h	1.81					1.81	-	-	

Table 5. Summary of the Shannon indices identified for the three study groups, Control, Behavioural and Fluoride across the full study period.

Data include the Shannon indices for each time slot (0 -2 h, 2 - 4 h, 4 - 6 h, and 6+ h) between brushing and sample collection across all three treatment groups, the Control (in BLUE), the Behavioural group (in GREEN) and the Fluoride group (in YELLOW). Table includes the standard deviation (SD), the standard error of the mean (SE) and the significant of differences (Sig) in Shannon Weiner indices at the genus level, analysed using the Mann-Whitney U test. Significant P values are indicated with the following symbol: ◆ P≤0.02.

5.15. Discussion.

The aim of this study was to elucidate trends in the development of the oral microbial community as result of the described interventions for a period of zero to two years after primary tooth eruption.

The results showed that the phylogenetic profiles across the entire study and genus profiles at baseline did not differ statistically, both within treatment groups and between them, a trend that has been observed previously (Bik et al., 2010; Diaz et al., 2006). However from Round 1 to Round 2 differences in the distribution of genus profiles appeared between the Control and Fluoride ($P < 0.03$) and Control and Behavioural ($P < 0.01$) groups respectively. Internal analyses of phylotypes within treatment groups showed that there was a decline in the number of species identified (i.e. species richness) in both the Control (124 to 17 phylotypes, $P < 0.01$) and Fluoride (79 to 48 phylotypes, $P = 0.12$) groups, between Baseline and Round 2 whereas the Behavioural study showed an increase in the number of phylotypes identified over the period (58 to 78, $P = 0.2$). Finally, a comparison of both genera and phylotype across the mean values between the Baselines to Round 2 studies showed a significant difference between the Control group and treatment groups only ($P \leq 0.03$ and 0.04 respectively).

Initially, all HOMIM data entries were assigned to their relevant phyla and in order to accurately compare all data, all samples associated with the present study (including the PGRM analysis) were subcategorised into phyla (and genus) together. This was done to ensure that, while one or more phyla (or genus or phylotype) may have been present in only one of the studies, all studies were quantified against the maximum number of taxa identified; this was done for ease and accuracy of analysis. It is noteworthy that not all samples collected were analysed in every study and exclusion from certain aspects of the analysis was dependent upon the requirements of the analysis at hand. For example, samples collected within 12 weeks of antibiotic treatment were excluded in all analyses. This is in keeping with

the literature (Ly *et al.*, 2006; Pellegrini *et al.*, 2009) and is because the use of antibiotics may or may not supress specific species within oral microbiome and because it presented an unknown parameter samples were excluded. In addition some samples were excluded from one set of analyses and included in others. An example of this could be observed in the analysis of time periods between brushing and sampling where the parent could not recall the time of brushing. Because the timeframe analyses were included as a representative of intra-group factors which could not be controlled at a clinical or experimental level it was important to present some analysis on the issue. However time periods between brushing and sample collection were not thought to diminish the value of parallel investigations (such as the analysis of genera distribution, dependent upon the intervention used) which contribute to the overall result and so they were included in those studies.

An additional factor of the methodological process was the protection of samples which were analysed commercially, i.e. HOMIM, which required the use of international courier services. To control for sample losses or anomalies in the sampling results which may have required a second analysis, all Bright Smiles samples were split in two, one half was sent for HOMIM analysis and the second half was stored at Salford. This presented an obvious issue with regards to the sample quality and the potential to miss genera or species which are typically present in quantities close to the detection limit or the overrepresentation of species in one half of the sample rather than another, due to handling error. For this reason every batch of samples sent to HOMIM included internal controls which were single samples split in two which were later compared for consistency of species distribution. The analysis of internal controls showed no significant difference between split couplets ($P > 0.05$) indicating reduced biases in the archiving process.

Sample quality was also quantified against similar studies the literature (Crielaard *et al.*, 2011) as studies have shown that oral samples present similarly at the phylum and genus level

while presenting greater disparity at the species level. To demonstrate this Bik and colleagues (2010) carried out a survey of the oral bacterial diversity of ten healthy individuals and found that 9 phyla were present in all samples. These included *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Fusobacteria, TM7, Spirochaetes Synergistes* and (*OD2*, < 1%. Bik *et al*. 2010) and all but the latter were isolated in the present study. Similarly, Bik *et al*. identified the genera present in 51 – 100% of their samples and included (bracketed data indicates the number of samples from which the genus was identified in the present study) *Streptococcus* (100 %), *Gemella* (98%), *Haemophilus* (98%), *Rothia* (90%), *Granulicatella* (89%), *Veillonella* (80%), *Neisseria* (80%), *Kingella* (70%), *Campylobacter* (67%), *Lautropia*, (62%), *Fusobacterium* (54%) indicating a measure of uniformity with the literature at this taxonomic level. However, of the 160 phylotypes identified by HOMIM in BS study only one (*S. oralis*) was present in all 61 samples. The next six most abundant were *Gemella haemolysins/Gemella sanguinis/G. haemolysans* (98% of samples), *Haemophilus parainfluenzae* (98% of samples), *Streptococcus australis* (91% of samples), *Rothia dentocariosa /R. mucilaginosa* (90% of samples), *Granulicatella elegans* (85% of samples), *Streptococcus constellatus/S. intermedius* (82% of samples). After this, approximately 10 phylotypes were present in 50 – 70% of samples, 27 phylotypes were present in 20 – 57% of samples and 115 phylotypes were present in 2 – 20% of samples indicating the potential for variability in individual plaque samples.

The mean relative abundance of the nine phyla isolated at Baseline from 29 samples and from all three study groups was *Firmicutes* (59.79), *Proteobacteria* (25.98%), *Bacteriodetes* (2.71%), *Actinobacteria* (9.18%) , *Fusobacteria* (1.34%) , *Synergistetes* (0.06%), *Spirochetes* (0.13%), *Tenericutes* (0.03%) and candidate divisions *TM7* (0.77%) and *SR1* (0.00%) all of which are consistent with the literature (Brinig *et al.*, 2003; Sampaio-Maia & Monteiro-Silva, 2014; Xu *et al.*, 2014) indicating smaple quality. The phyla showed little deviation from the mean values with the possible exception of the *Actinobacteria* and *Fusobacteria* clusters

however, reports have suggested that these, as well as other phylo-clusters can differ greatly from sample to sample depending upon the number and quality of erupted teeth (Bik *et al.*, 2010; Shi *et al.*, 2016). For example, in the present study the groups were composed of approximately 9.18% and 1.34% for *Actinobacteria* and *Fusobactera* respectively whereas literature reports have described concentrations of 1 – 21% and 3 – 39% respectively (Shi *et al.*, 2016; Xu *et al.*, 2014). However, the phylogenetic distribution of mean sample profiles at Baseline showed no significant differences ($P < 0.62$); a factor which may expected at Baseline levels and prior to the onset of treatments, indicating that the variations observed are consistent.

Approximately 12 months later at age 2 years (Round 1) most volunteers were likely to have most of their deciduous teeth, with the possible exception of their second upper and lower molars (ADA, 2005) and phylogenetic resulted showed an even distribution between samples ($P > 0.67$). At age three (Round 2) greater differences in the phylogenetic distribution became more apparent, for example, the *Firmicutes* identified within the mean Control profiles were approximately 25% greater than that observed in either of the treatment groups. It must be noted, however, that due to a decline in volunteer numbers there were only two samples submitted for Control group analysis at this stage which resulted in relative abundances of 81% and 65% (Round 1, *Firmicutes* abundances were 58% - 63%) and was likely due to biases brought about by small sample sets (S.E. 5.8). It must also be noted that, although appearing extreme, *Firmicutes* composing 73% of the biofilm's bacterial composition is not out of the ranges observed in the literature (Xu *et al.*, 2014).

When genus profiles were considered at Baseline the Control, Behavioural and Fluoride, studies identified mean profiles of 41, 24 and 31 genera (respectively). Like phylogenetic analyses, statistical analysis showed that the baseline distribution of genera, at both mean and at individual comparisons were not statistically significant ($P > 0.05$) which may have been

expected at Baseline and prior to treatments. However by ages 2 and 3 (Round 1 and 2 respectively), differences in the genera profiles were beginning to emerge. It has been recorded that throughout childhood and due to the numerous developmental and dietary changes that go with it, oral bacterial load increases whereas diversity decreases (Sampaio-Maia & Monteiro-Silva, 2014), which appeared to be the case here. For each of the Round 1 Control, Behavioural and Fluoride mean study groups, the number genera identified numbered 39, 26 and 21 which was a reduction of 5% and 32% for the Control and Fluoride groups ($P < 0.03$) but an increase of approximately 8% for the Behavioural study. By age 3 (Round 2) the difference between the number of genera identified between the Control and Behavioural studies had widened; the Control study falling to 9 genera whereas the Behavioural study remained approximately stable with and 31 genera which was found to be highly significant ($P < 0.01$). The Fluoride study had also fallen but only by approximately 10%, to 19 genera. However, it must be noted that by Round 2, the number of volunteers and subsequently, viable samples which entered into the group analysis had fallen. At this stage, the sample numbers for the Control, Behavioural and Fluoride studies were 2, 4 and 8 (respectively) suggesting that the disparity in calculated means, with specific reference to that of the Control group, may have been skewed by potentially extreme individual profiles.

In an effort to answer this, treatment groups were analysed internally for the distribution of genera throughout the three annual sets. It was considered that deviations from a mean may be more pronounced between treatments than within a treatment group and the analysis of the median distribution of genera in both the Behavioural and Fluoride treatment groups found no statistical differences between the Baseline, Round 1 and Round 2 mean values ($P > 0.05$), despite variations in the readings. However, the same analysis of the Control group showed a highly significant reduction in genera ($P < 0.01$) by Round 2, which differed from both Baseline and Round 1. This does not answer the question as to whether there was an unrepresentative distribution of genera in the Round 2 Control study, and as there were no

other samples there was no way to resolve this issue. However the two samples in question, both of which belonged to the Control group and sampled 6 hours after brushing yet the bacterial profiles were not found to be statistically different (P = 0.59). These observations indicate that the bacterial profiles for treatment groups appear to stabilise over the study period. Observed collectively the mean genera identified across the three study groups showed that, with the exception of Baseline samples, all statistical analyses separated the Control from the treatment groups (P ≤ 0.03) and no differences were observed between or within the Behavioural or Fluoride treatment groups (P > 0.05) indicating treatment-associated changes, possibly due to the interventions described.

A major factor of the present study and subsequent analyses was the necessity to subcategorise samples into hours between brushing and sampling. This was because there was no realistic method for controlling the volunteer's daily routines and requesting that participants refrained from brushing before attending a sampling session was considered to be in conflict with the aims of the Bright Smiles study. This meant that sampling two volunteers within the same treatment group, with hours of difference between brushing and sampling could have dramatically skewed results if not properly addressed. Here samples were assigned to time slots which ranged from 0 – 2 h, 2 – 4 h, 4 – 6 h or 6+ h and compared for equality and measured for an approximate measure of diversity. In order to assure consistency throughout the data set, Mann Whitney analyses were carried out on intra-time slot readings at the genus level. In the Control group, one difference was observed in the Baseline, 0-2 slot and six differences in the Round 1, 0-2 (x3), 2-4 (x1) and 4-6 (x2) slots. In the Behavioural study only one difference was observed in the Round 2, 4-6 slot and the Fluoride study had three differences at Baseline 2-4 and 6+ slots. The numerous observations in the Control group may indicate a trend of instability in individual microbiomes which would fit with the narrative of this study. However, it must be noted that no further lines of enquiry were carried out to this end and so conclusive remarks would be speculative. It is also difficult to say

whether the readings described indicate consistence across the time slots. For example, the onset of FP treatment with the potential for inconsistencies in application or in the Behavioural group where the reliance upon the parental understanding and employment of behavioural instructions may have led to variations in bacterial activity, as previously described. In this case it is probably best to reflect upon the baseline readings which indicated only two significant differences (P<0.03) across the twelve time slots and three study groups, indicating a good measure of accuracy within time slots.

At the inter-slot level, in almost every case, the division and analysis of time slots produced a significant difference. Within the Control study, Baseline to Round 2 analyses (15, 9 and 2 samples respectively) showed 9 statistical differences across the three sampling periods. However as there were 4 readings missing from between Round 1, 6+ h to Round 2, 4 – 6 h it is difficult to draw any clear conclusions regarding variations in bacterial distributions. At Baseline there was a single difference observed between distribution analyses 0 – 2 h and 6+ h (P = 0.02) with the remaining differences spanning the Baseline to Round 2 (P ≤ 0.02). The Baseline Control study is one of three (totalling 12) study periods where all four time slots are present and the Shannon H statistic for the 0 -2 h, 2 - 4 h, 4 - 6 h, and 6+ h ranges from 2.18, 2.21, 2.12 and 1.73 (respectively). This indicated a reduction in diversity over time and a statistically significant difference between 0 -2 h and 6+ h (P=0.02) which confirms the genus distribution analyses for this time slot. The same is true for the Round 1 Control study where the number of genera identified fell to 39 and again to 9 at Round 2 and while the Shannon indices for these readings remain consistent with the Baseline, there is still a trend for diversity loss over time.

Like the Control study, the Behavioural study was missing 5 time periods (Baseline, 2 – 4 h, Round 1, 2 – 4 to 6+ h and Round 2, 2 – 4 h) which broke up all three annual subgroups and so any inferences made would be speculative. However, of interest is the significant

difference between Baseline readings 0 – 2 h and 6+ h (P = 0.009) which reflects the findings from the same time point in the Control study. In this case the 2 – 4 h reading was missing however, the Shannon Weiner indices for this cluster of results was 2.17, 1.89 and 1.75 for 0 - 2 h, 4 - 6 h, and 6+ h, again indicating a tendency for diversity loss over time. Individual analyses of Shannon-Weiner indices was only possible at Baseline 0- 2 to 4 – 6 which were not significantly different (P=0.25) but confirms the genus distribution result (P=0.35) and thus, the quality of the data presented. Round 1 has only one time category which is the 0 – 2 reading and is the result of a single sample and could not be compared with at the intra-group level , however the Shannon index for this profile is comparable to both Baseline readings at the same time point. Round 2 also shows significant differences between profiles and the Shannon statistic presents the first instance of stability. Unfortunately the number of samples for the three time slots (0 - 2 h, 4 - 6 h, and 6+ h) was 1, 2 and 1 respectively and so it is possible that these data are subject to the effects of an outlier. Interestingly the two samples which comprised the 4 - 6 h reading were found to be significantly different (P = 0.01) which may go to explain the slight deviation from the group trend.

In the Fluoride study a similar trend of diversity loss over time was observed. At baseline, Shannon Weiner indices for 0 -2 h, 2 - 4 h, 4 - 6 h were 1.99, 1.16, 1.79 and 1.42 (respectively), and like the Control and Behavioural studies, this group had a number of significant differences in genus distribution which separated 0 -2 h and 2 - 4 h (P < 0.01), 0 -2 h and 6+ h (P < 0.001) and 4 - 6 h, and 6+ h (P < 0.004), though none could be confirmed by analysing the Shannon indices for differences due to there being too few samples per time slot. Additional analyses of this data was only possible in the case of 0 -2 h and 4 - 6 h where genus distribution was not significantly different (P=0.67) which was confirmed using the Mann-Whitney U test between the same periods (P=0.85) and could be used as a benchmark for the quality of genus distribution analyses. Although there are two readings missing from the Round 1 study neither in this round or in the Round 2 results was there a statistical

difference calculated at an intra-round level (P > 0.05) yet the Shannon Weiner indices for these readings still replicate the trend diversity loss.

To explain the elevation in the bacterial load immediately after brushing and the subsequent statistical differences requires some speculation as only two references from the literature were identified as observing similar trends yet neither made an attempt to explain them (Okpalugo *et al.*, 2009; Oyarekua *et al.*, 2015). To articulate the process of brushing, it is better to say that biofilms are 'disrupted' rather than removed and so while the vast majority of the oral biofilm-associated cells may be cleared with the toothpaste slurry and saliva the process is not one of sterilisation and so it follows that a proportion of the biofilms bacterial composition become re-suspended in the saliva, and are available for reattachment and biofilm formation. This was considered a plausible reason for the trends observed here as the sampling mechanism is absorbent as well as corrugated and so the samples collected are likely to be a combination of both saliva and plaque. In Rounds 1 and 2 of the Control group there were too few samples to make any accurate deductions however the Fluoride study showed no significant differences between these time points, a factor which was not observed in the Behavioural study.

This study set out to discover the potential benefits of fluoride on the developmental processes of the dental biofilm throughout tooth eruption. Caries is the symptomatic (observable) terminus of the gradual demineralisation and loss of dental enamel and is fuelled by the production of organic acids, notably lactate (Walsh, 2006) from the fermentation of dietary sugars. Acid production can continue as long as sugar is in excess and leads to a rapid and prolonged decline in pH and subsequently the loss of diversity from the local microbiome (Belstrøm et al., 2014; Loesche, 1986).

The data presented here shows that in spite of some variation over the period there was no significant differences between phylogenetic profiles throughout the study. However, when

analysed at the genus level, profile equality was limited to the Baseline pre-treated samples which may have been an expected outcome. This is not only because the treatments carried out were expected to produce a developmental deference but at 12 months the participants will have had roughly the same number of teeth and a similar diet. Twelve months later the mean Fluoride profile was found to be significantly different from the both the Behavioural and Control study and by the second round it was the Behavioural study which differed. Perhaps this was because the processes implemented in the Behavioural study, specifically, methods of accurately brushing the teeth were ineffective (e.g. child cooperation) in combination with the beginnings of diet variations between participants, whereas the presence of fluoride brought about a specific chemical interaction. By Round 2 (age three) behavioural routines, as directed by the Bright Smiles trial may have been fully developed; a benefit that may not have been observed to the parents and guardians of the Control and Fluoride participants. This is not to suggest that the cleaning routines of the latter two groups were ineffective, however, for a lasting difference associated with the Fluoride group, the product used would require an antimicrobial property that was effective for six months or more (2 x biannual applications) which is unlikely (Pinar Erdem *et al.*, 2012). This not being the case the results observed here would be the product of routines specific to the parent(s), which was not assumed to be the case in the Behavioural group. However, Figure 38 presents an interesting challenge for the future of this field as, regardless of the differences which separate the Fluoride and Behavioural studies, specifically the benefits of a chemical intervention over health–associated methodology and education, both treatments presented similar profiles (P > 0.05) but differed statistically from the Control study (P < 0.05). The Shannon indices showed a clear reduction in genera over the space of a day yet over the space of a year both treatment groups showed stability in the number of genera identified. Even the analysis of mean phylotypes per study round, which are often different at this taxonomic level (Bik *et al.*, 2010), highlighted significant differences separating the Control from the study groups. This

suggests that improved dental routines and the incorporation of fluoride has an effect on the developmental process within the oral biofilm, further implying that the lack of such interventions and subsequent decline in diversity may have been evidence of early cariogenic conditioning (Belstrøm *et al.*, 2014).

6. General discussion.

In order to meet the requirements of the study, a protocol needed to be devised for the collection of dental biofilms utilising a method which would need to be scientifically reproducible, ethical and safe.

A number of methods were researched and were primarily focussed on the collection of 'live' plaque samples using standard apparatus as seen at a dental clinic. However, this was soon discarded as an option due to the lack of suitably trained personnel (e.g. dentists, hygienist) attached to either of the studies who were qualified to collect such samples. Another parameter of the BS trial was the variability of sampling venues. Meeting participants at Sure Start centres and at times of their choosing meant that the sampling process would need to be flexible. Once discovered, the protocol required optimisation to ensure reproducibility. It needed to be simplified and comprehensible to ensure accurate communication to those taking the sample (i.e. the volunteering public) and it needed to be suitable for collaboration with a subsequent methods of downstream analysis. Finally, and perhaps most importantly, the method needed to be ethically viable. Only at this stage, once all requirements had been met could the trial be presented to the BS cohort, a process that took approximately 13 months and was ready for volunteer recruitment on February 16th 2012.

By December 2012, approximately 400 children had been enrolled onto the BS cohort and in the original plan it had been assumed that the microbiological study would include most or the entire cohort, sampling every three months for two years. This led to a number of logistical problems, specifically how the participants of the Control group, who only attended study appointments once annually, and the remaining intervention groups who met biannually would respond to a new directive which required an additional 1-3 meetings per year. It was considered that the extension of the original agreement may discourage appointment attendance and lead to participant withdrawals. It also became apparent that processing,

analysing and interpreting 1,200+ samples would have been difficult. Then there were the financial constraints; in 2012 the approximate charge for HOMIM analysis was £80 per sample and with an estimated 1,200 samples per year, the cost was also unrealistic. Therefore sampling was reduced to once annually which were collected from approximately 40 children (at baseline) during scheduled BS meetings. Vitally, exposure to the ethical implications of a clinical study and methods associated with protocol optimisation provided a greater understanding of the necessity for adherence to the associated legal guidelines. However, from an experimental perspective, the loss of 12 months to protocol optimisation for ethical approval was unfortunate and likely to have resulted in fewer samples for the microbiological study; a major consideration were the tests to be repeated.

While being convenient for both the participant and the sampler, the method of combining sampling sessions with scheduled BS appointments also led to a sampling issue which would remain unresolved for the duration of the study due a conflict of interests. The BS study was conceived as a method of improving the dental health of children in Salford and Greater Manchester and as such, took steps to rigorously promote regular brushing and high standards of oral hygiene. This had implications for the microbiological study as it meant that participants tended to brush their teeth prior to attending the appointment and to discourage brushing for any reason was considered as being counterproductive. In addition, the participants involved in the Fluoride study had the FP varnish applied during the allocated sessions and so brushing before attendance and application was encouraged (Anon, N.D.). At Baseline 34% of participants attended the session after brushing their teeth within two hours. That figure rose to 57% in Round 1 and then dropped down to 23% by Round 2. This meant that subsequent analyses would need to address the potential for biases in the samples due to inconsistencies in the sample pool. In spite of this issue, these additional analyses indicated that bacterial diversity was highest in the first hours after brushing and then fell throughout that day; a trend that has been identified in previous studies (Okpalugo *et al.*, 2009; Oyarekua

et al., 2015) and has yet to be explained. It is possible that this may be due to the temporary resuspension of cells immediately after brushing which subsequently adhere into biofilms. It has also been shown that there are diurnal factors which influence oral bacterial diversity and that indices are generally higher in the morning and fall thought the day. This may have been applicable here however; only 7.5% of samples were collected before 10am, 42% before 12 noon and 57% after 12.01pm. However this does not account for periods of sleep prior to the appointment, which at 12 months old is likely to have been a factor and typically results in a reduced rate of saliva production and subsequently, a proliferation of the bacterial diversity (Walsh, 2007). It was also considered that this trend of decreasing diversity over time may have been due to a broader sampling set at the $0 - 2$ h slot, increasing the potential for greater observations in bacterial diversity. However, numbers of participants in different brushing periods were not evenly spread. As an example, the Baseline Control group's $0 - 2$ h slot had the same number of samples as the $6 +$ h yet the Shannon Weiner index calculated for $0 - 2$ was 0.76 greater which was the equivalent of 26 genera or 61 phylotypes.

The results of the BS trial showed that the fluoride varnish (FP) and behavioural interventions provided no clear benefit to the dental health of children aged 3 years. In each of the study groups the incidences of caries were 9.1%, 9.2% and 10.5% for Control, Behavioural and Fluoride studies (respectively) and no significant difference was observed between Control and Behavioural ($P = 1.00$) and Control and Fluoride ($P = 0.96$) groups (Burnside *et al.*, 2014).

In the PGRM study it was observed that FP varnish did not alter the acidogenicity of the biofilm however, these were isolated, non-replenished bacterial cultures incubated with sucrose which may have led to the positive selection of fluoride-insensitive lactobacilli that have been positively associated with FP applications *in vivo* (Baygin *et al.*, 2013; Yoshihara *et al.*, 2001). Such *in vitro* studies, including the PGRM, cannot be directly compared to *in*

vivo conditions and only act as a model example and although it is clear that under trial conditions the use of FP or indeed Behavioural interventions did not prevent the development of caries; the PGRM study indicated a low probability that FP would inhibit acidogenesis. Furthermore, the mean incidence of caries observed by the BS study was approximately 10%; this is a much lower figure than previous works have shown (Davies *et al.*, 2013; Davies *et al.*, 2001). Davies (2001) and Davies (2013) showed that in the North West approximately 35% of children of between 3 and 5 years had caries experience, compared to approximately 25% in England yet the disparity between the BS results and that of the cited reports may reflect a consequence of clinical observations combined with variations in social commitment to such studies, rather than any antimicrobial intervention. In a chapter by Ravi Thadhani (2006) which discussed the limitations of formal clinical trials, specific points of inaccuracy were described and included the 'principle' (i.e. the nature of the method) and the 'procedure', (i.e. the way in which the trial is conducted). In the case described the procedural limitations included a disparity between non-trial observations made in hospitals and those made during associated clinical trials. He described how, under trial conditions mortality rates were lower than non-trial (e.g. hospitalised) observations and was linked to the age of the patient who tended to be younger in the trial as opposed to the older patients from observations made within a care environment. In the subsequent BS report by Burnside *et al.* (2014) an investigation into the recruitment and retention of trial volunteers found that a major motivation for parent enrolment was the fear of their child having poor oral health. It is possible, therefore, that the observations made by Davies *et al.* (2013) and Davies *et al.* (2001) differed from that observed in the Burnside study because that latter was composed largely of volunteers who were proactive to the importance of dental health whereas the former was made by observations across the whole spectrum, including those who may be less aware of the importance of dental health and those who were.

Caries has been associated with poverty, socioeconomic deprivation and low education (Chi *et al.*, 2013; Davies *et al.*, 2013; Dugmore & Rock, 2005; Dye *et al.*, 2010; Freire *et al.*, 1996; Riley *et al.*, 1999; Satcher, 2000) and Manchester's caries status in children and association with social deprivation was the target of the BS study. However, in spite of efforts to target the poorer areas of Salford, only 43% participants came from homes in the most deprived areas. This may account for the low incidences of caries observed but it was also considered that placing the parent in a position where their child would be observed throughout the first years of their life posed an interesting question as to whether brushing routines would improve on the strength of simply being observed. No significant difference was found between the Control group and the Behavioural group (P = 1.00, Burnside *et al* (2014)) which may indicate that they did.

One possible explanation is the Hawthorne effect, though widely debated in its generalisations and implications in human-associated observational studies, relates to changes in performance or behaviour due to being observed (Macefield, 2007). The theory was developed through repeated observations of factory workers under numerous conditions but was applied to the present study in that it was considered likely that a parent, under the conditions associated with the clinical trial would attempt to improve on 'normal' routines due to regular observation from a health official. This may demonstrate a degree of unwarranted cynicism but whether the Hawthorne effect (generalised for the purposes of this argument) did play a part in the present studies can only be speculated yet the theory may be applicable given the low incidences of caries observed here. An alternative theory is that because caries has been associated with low education, it is possible that the low incidences observed may be linked to a lack of understanding, not only of the causes and effects of tooth decay but also the importance of research into such matters. Therefore, simply making the parents aware of the problem of tooth decay in children may have altered their behaviour. There is no evidence provided here to suggest that this was the case however, it follows that if caries in the North

West was associated with social deprivation and low education as it has been observed elsewhere, the lack of symptomatic caries observed in this study may also be a product of these same parameters. Another possibility for the low incidences observed is that there has been a community wide improvement in recent years. A recent study has shown that while the North West still has the highest rates of tooth decay in England, the age group most affected was 5 – 9 years (RCS, 2015) and not the 0 – 4 year age group who participated in the BS study. This could explain the lack of major differences in the bacterial profiles between the different study groups.

Of particular interest was the statistical separation of Control and treatments studies in the microbiological analysis of the BS samples. This may link with the PGRM investigation which indicated that while FP varnish did not interfere with the production of acids from cariogenic biofilms, it did appear to provide a measure of homeostasis within the biofilm. Also, while FP use appeared to promote lactobacilli proliferation, the study indicated that after proper applications the abundance of streptococci declined in a pH dependant manor (Belli *et al.*, 1995). Given that lactobacilli are typically isolated in low numbers (Loesche, 1996) and that the oral microbiome is in a continual state of microbial and conditional flux it is reasonable to speculate that the antimicrobial effects of the FP varnish, with regards to the pH dependant inhibition of streptococci would still be applicable in conditions more closely related to the oral cavity.

In addition, results showed that when applied incorrectly (PGRM, 100% ES) FP has little effect on the biomass after 5 days which is in keeping with the literature (Baygin *et al.*, 2013). However, the mean biomass results for the 100% ES experiments were greater than that of the 0%, and when combined with the positive selection of lactobacilli it may provide reasoning for the slightly elevated rates of caries observed in the Fluoride study (10.5%) when compared to the Control (9.1%). Although there was no significant difference observed between the

rates of caries in the BS study, observing the highest incidence of caries in the Fluoride group may have been the opposite of what was expected (Marquis *et al.*, 2003). This may be explained by factoring in the potential for an increase in fluoride-tolerant lactobacilli (Hamilton et al., 1985; Yoshihara et al., 2001) and an increase in visible plaque which has been associated with FP varnish previously ($P < 0.02$, Baygin et al., 2013). Here it was demonstrated that in excess saliva, the condition considered more closely associated with 'real life' FP treatment (section 4.3. and Figure 26), the mean biofilm biomass increased by 12% when compared to the 0% control treatment. Therefore, any changes in brushing behaviour, possibly due to a decline in due diligence because individuals assumed that their child's teeth were protected from decay by topical fluoride varnish, may have promoted conditions that, if exposed to excess sucrose and a lapsed in regular or effective brushing, lead to caries conditioning. In contrast, as the untreated control presented the smallest incidence of caries it may be due to an increase in caries awareness which in turn may have resulted in changes in brushing behaviour or a combination of both, subsequently resulting in an unconscious improvement in oral hygiene standards. Marsh's (1991) ecological hypothesis suggests that the onset of caries is due to a multitude of factors combining the bacterial composition of the biofilm/oral microbiome, diurnal influences, the health of the individual and their diet. Perhaps the differing routes of caries development may also be based upon the differing methods of oral hygiene. It has been demonstrated that the MS group are considered to be the major culprit of caries. However, *S. mutans* are not isolated in high numbers (Loesche, 1996), indeed over three years *S. mutans* was not identified once but there are a multitude of species which are not considered to be directly cariogenic, including *S. sanguis, S. oralis, S. gordonii, S. anginosus, S. mitis* and *S. salivarius* but have been positively associated with cariogenic 'conditioning' due to a capacity to survive at low pH (Harper & Loesche, 1984; Svensäter *et al.*, 2003; Walsh, 2006). This, combined with the estimated 19,000 phylotypes associated with the oral cavity (Keijser *et al.*, 2008) and the data presented here, which demonstrated

variations in biofilm development in association with fluoride, may indicate that 'improved' or altered dental hygiene routines may produce unlimited variations in the oral microbiome which are both specific to the individual and in their conditioning of caries development.

The BS study demonstrated that behavioural and chemical interventions did not change the cariogenic outcome of patients by age 3. However, the results of the microbiological study suggest that, despite the failure to control specific experimental parameters, the interventions did appear to have an effect on biofilm development in that it improved the chances for the survival of less aciduric species, maintaining bacterial diversity and so providing the colony with a greater chance of recovery from the acidic conditions. Any specific changes made by the interventions used were likely clouded by the wide diversity of microbiota, many of which are specific to the individual (Diaz *et al.*, 2006).

6.1. Conclusions.

Although improved behavioural techniques, which included tooth brushing and the incorporation of fluoride into the dental routines did not reduce the incidence of caries in clinical subjects, the results presented here suggest that fluoride imposes a homeostatic effect which promotes increased diversity in oral biofilms, a feature which has been shown to be associated with oral health.

Chapter 7: References.

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Appendix I - DNA collection and isolation protocol v1.0, 24th October 2011 - Study number 31168

DNA Isolation Protocols

Experimental Aims

To isolate bacterial DNA from human dental plaque in order to assess the antimicrobial effects of fluoride.

Materials

ISOHELIX DNA collection Swabs plus Isolation kit #DDK50/SK2 (see figure 1 below), vortexer; Microcentrifuge (13K rpm); waterbath (60˚C).

Figure 1. (Above) *Swab optimised DNA isolation swab'* – Cell Projects Ltd # DDK-50/SK2. A) Detachable, derivatised cellulose head; B) Handle; C) Specialised Failure Point.

Methods

- Remove the swab from its sterile paper packaging (taking not care to touch the swab head with hands, clothing or other equipment) immediately before the sample is ready to be taken.
- Holding the swab by the handle and using reasonable, firm circular motion, swab the inner and outer surfaces of the incisors and lateral incisors (covering tooth surfaces, gingival margins, interdental papilla, and periodontal pocket); see figure 2, (below) for 1 min ± 30 sec.

Figure 2 (above) showing the process of plaque removal. **1.** Lift up the upper lip exposing the four front teeth. **2.** Remove the swab from its wrapping and using firm, circular motion, swab the outer surfaces of the four front teeth. **3.** Remember to include the inner surfaces of the same front teeth for a total of no less than 30 seconds. If the top teeth are missing swab the bottom four front teeth

- Immediately after sample extraction, taking care not to touch the swab head with hands, clothing or other equipment, place the swab (head first) into the storage tube containing 520µl lysis solution and detach head thus:

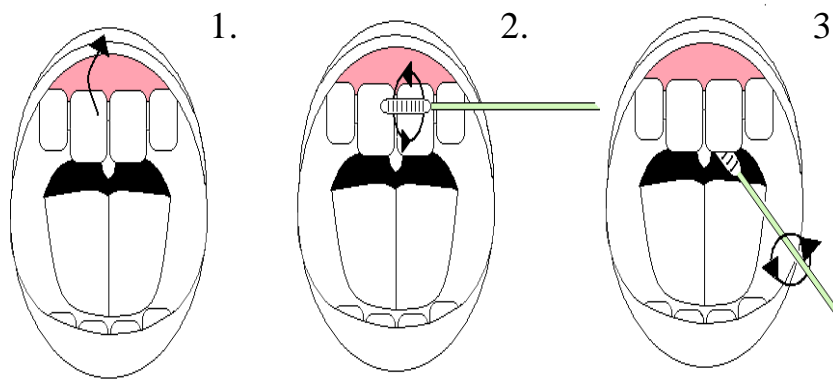   Hold the swab handle Failure Point [Fig. 1-C] to the side of the reaction tube, break the upper handle sideways. Handle will break away and the swab-head will fall into the tube. The tube is to be capped, mixed by inversion and stored upright (ensuring the swab head is submerged) at room temperature in the storage box provided.

- At this stage cells within the samples have been lysed and the DNA released has been stabilised. Samples may be stored at room temperature for at least 2 years.
- The sample should then be marked with the date, the study group number (1, 2 or 3) and a unique serial number which must indicate the time of sampling, subject's age and time period since last brush.

    E.g. Time (17. 45) – Age (3yr, 11mnth) - Time lapse (12hrs) $\therefore$ 1745-3.11-12

- Sample analysis will be carried out with emphasis on the strict protection of subject anonymity. However it is necessary for biological researchers to understand the nature of the subject's oral hygiene procedures, medical history and geographic residence in order to track anomalies in the data. Therefore the following details, highlighted in the 'Volunteers Data Sheet' (below) must be included with each sample.

Volunteers Data Sheet						
Sample Serial Number	………………………………………………………………………… ………………………………					ppm (if Known)
Method(s) of last clean (please tick as many as required)	• Brushing	☐	Fluoridated?	Yes ☐	No ☐	……………
	• Flossing	☐	Fluoridated?	Yes ☐	No ☐	……………
	• Mouth Rinse	☐	Fluoridated?	Yes ☐	No ☐	……………
	• Other - Please specify (*Product type? Brand?*)					
			Fluoridated?	Yes ☐	No ☐	……………
Antibiotics used	Brand/Name		Dosage		Approx. Usage Period	
Prior Addresses (post code)	(Up to 12 months prior)					

- On returning to the lab, sample DNA will be isolated using the following methods as described in the isohelix user manual. Note that this is a commercial kit therefore the exact components of the solutions used are not available.

Step	**Process**
1	Place the tube containing the swab, LS solution and PK solution in a 60°C water bath for 1 hour. Vortex briefly.
2	Transfer the liquid in the tube (approx. 400μl) into a 1.5ml centrifuge tube using a sterile pipette tip.
3	Spin the SK-2 tube containing the swab head briefly and using a sterile pipette tip add the recovered supernatant to the 400μl collected previously.
4	Add 400μl CT solution to the tube. Vortex briefly
5	Place the tube in a microcentrifuge (see *tip* below) and spin at approx. 13K rpm for 7 minutes to pellet the DNA.
★	*Place the tube with hinge positioned outwards so the liquid can be removed from the opposite side*
★	*The pellet may not be visible*
6	Remove all the supernatant carefully with a pipette tip taking care not to disturb the DNA pellet
7	Re-spin the tube briefly and remove any remaining liquid
★	*It is important to remove all of the liquid*
8	Add 150μl TE solution to the tube. This volume may be decreased to as little as 30μl if a higher

concentration of DNA is required.

9   Leave for at least 5 minutes at room temperature for the DNA to re-hydrate, longer if a reduced volume of TE has been used. Vortex briefly.

★   **Store the DNA sample at 4°C for short term storage or -20°C for long term storage.**
     The expected yield from a buccal swab is 2 to 10µg DNA (10 to 70ng/µl).

Appendix II - PIL (micro) v1.0, 24th October 2011 - Study number 31168 page 1.

You will not receive payment, or any treatment which is different from that, normally available to other people. It will not affect your access to any other dental or medical services.

We will only be examining bacteria from the samples. No human DNA or cells that are collected in the swabs will be used in any way for research purposes.

All the information and plaque samples you give us will be collected, stored, archived, analysed and disposed of according to the Human Tissue Act 2004 and the Data Protection Act 1998.

Who should I contact?
Should have any questions about the study, please feel free to contact

1. **Rosy Armstrong** (Clinical Trial Coordinator) 0161 295 5095
r.armstrong@salford.ac.uk
College of Health & Social Care,
Allerton Building,
University of Salford,
Salford,
M5 6PU.

2. **David Greenwood** (Trial Microbiologist) 0161 295 2652
Lab 209, Cockcroft Building
University of Salford
Salford
M5 4WT
D.greenwood3@edu.salford.ac.uk

The Chief Investigator for this study is Professor C. Pine, Honorary Consultant in Dental Public Health, Salford Royal Foundation Trust.

This study is sponsored by The University of Salford and a recognised Research Ethics committee has reviewed and approved this phase of the study.

If you have a complaint about the study please use the contact numbers above.

PIL(micro) v1.1, 27th January 2012

University of Salford MANCHESTER

Salford Bright Smiles Baby Study

Salford Bright Smiles

Bacteria that grow on baby teeth

Dear [parent]

Invitation to take part in research
We are inviting you to take part in a second phase of the **Bright Smiles Baby Study.** As with the first phase, taking part is entirely voluntary and you may withdraw at any time without having to give a reason.

Why have I been invited?
You have already agreed to take part in the Salford Bright Smiles Baby Study. Over the next 18 months, your baby will be growing new teeth. We all have bacteria on our teeth and it is a normal part of life. We would like to understand about how bacteria grow as your baby's teeth grow.

What will I have to do?
Every 6 months of the Bright Smiles study, we will ask you to attend an appointment to collect a sample of the sticky, creamy coating (dental plaque) on the front of yours and your baby's teeth. We will do this in a very quick, simple and painless way with no known risks (explained further on pg 2).

Wherever possible we will make sure that the appointments are at the same time as your normal Bright Smiles appointments/ check ups. When this is not possible we will make sure that the appointment is at a time and place convenient for you.

How many samples will we be collecting?
We would like to collect at least 5 samples over the next two years from you and your baby. About 40 families from each of the 3 study groups will be asked to take part (120 families in total).

What will happen to me at the appointment?

At each appointment we will ask you to rub a swab (like a cotton bud) on your baby's top and/or bottom front teeth (inside and outside surfaces).
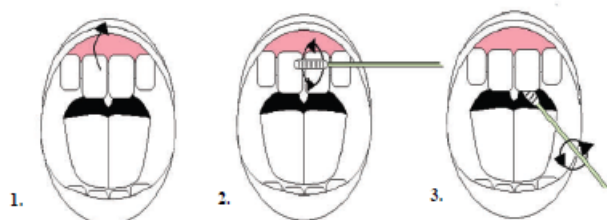
We will ask you to do the same for your own teeth. If your own natural top teeth are missing, then you will rub it over the bottom teeth instead. The swab is then placed into a solution which has been designed to store all the bacteria's DNA. This will then be taken to the lab for analysis.

We will also ask you to tell us what toothpaste/mouthwash you have been using, specifically, brand and fluoride content. We will also need to know if you have taken any antibiotics in the last 12 weeks, and if you need to do so **during** the study. This is because they have an effect on bacteria and give different results.

Taking a Swab Sample

Someone from the study team will be there to show you how to the use the swab. We will help with timing, and altogether it takes up to 1 minute. There are three simple steps, all shown below

1. Lift up the top lip to show the four front teeth

2. Take the swab out of its wrapping and using firm, round movements; swab the outside part of the four front teeth.

3. Using the same swab, now rub it on the inside part of the same front teeth.

What we hope to achieve through this research.

This phase will run hand-in-hand with the Bright Smiles Baby Study and aims to understand the bacteria that live in dental plaque and those that can cause tooth decay.

With the use of cutting edge scientific methods, we hope to understand the biology of these bacteria and how to help reduce tooth decay.

What will happen when the research stops?

Your usual family dental care arrangements will continue as normal after the study finishes and we will make the results known to you in a newsletter to all families who have taken part.

Any results that are made public will not contain any information that can identify you personally.

How you will be affected by the research/ What if anything happens to me while I am on the study?

It is very unlikely that you will be affected in any way, however we are legally required to tell you that if your child's health and well being is made worse by being in the study, the Sponsor (University of Salford) will compensate you without legal commitment to proving fault. This means that the Sponsor will compensate you for any injury resulting from procedures carried out within this study protocol. Your right in law to claim for compensation for injury where you can prove negligence is not affected.

Will I gain or lose anything by being in the study?

There is no direct benefit taking part in this research. However, as a token of our appreciation, families who take part in testing out this new method of observing bacteria will be entered in a 6-monthly prize draw for £50 *Love2Shop* vouchers for each study group.

PLUS an additional £100 voucher draw for all parent and child pairs that complete all plaque sample appointments!

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Bacteria that grow on baby teeth

Please make sure that you read the information sheet carefully before making a decision about whether you would like to take part in the second phase of the Bright Smiles Baby Study. This is entirely voluntary and you therefore do not have to take part. You can also choose to take part and then decide to withdraw at any time.

If you would like to take part in the study about **Bacteria that grow on baby teeth** please complete the section below and return this form to us in the pre-paid envelope provided.

- ✓ I have had the opportunity to ask further questions and time to reflect on the information provided.

- ✓ I have read and understood the Participant Information Sheet version 1.1, dated 27 January 2012. I understand that this is a research study, that it is voluntary and that I am free to withdraw at any time without providing a reason.

- ✓ I confirm that I have parental responsibility for the child enrolled on this form and that I am consenting to donate plaque samples for myself and my child for research purposes into how the normal bacteria on babies' teeth changes as the new teeth grow and mature.

Please complete this section:

Your Full Name:...

Full name of your child: ....

Child's date of birth: ....

Your address: ....

....

Telephone Number: ....

Your signature: .... **Today's date:** ....

Thank you we will contact you again nearer the time.

IC (micro) v1.1, 27th January 2012 study number 31168

Appendix IV - Sub-amendment Form 6, 24th Oct 2011.

NHS

National Research Ethics Service

NRES Committee North West - Greater Manchester East

3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Tel: 0161 625 7830

01 November 2011

Professor Cynthia Pine
Professor in Public Health and Honorary Consultant in Dental Public Health
University of Salford/ Salford Primary Care Trust
Allerton Building
University of Salford
Greater Manchester
M6 6PU

Dear Professor Pine

Study title: A comparison of community based preventive services to improve child dental health
REC reference: 10/H1013/8
Amendment number: 06
Amendment date: 24 October 2011

Overview of Amendment

- Addition of PhD student - David Greenwood, change to principal inclusion criteria, change of details for co-investigator Dr Lindsey Dugdill to be replaced by Professor Pauline Adair, revision of start and end dates to study - end date 04/04/2014, change to A79 Level of commercial participation in this project changed from 'none' to 'industry funding, but not industry sponsored'. Various changes to the protocol
- Change to principal exclusion criteria
- Change to concomitant medications and procedures
- Additional molecular microbiology phase to investigate changes in the oral microflora. An additional Participant Information Leaflet, Informed Consent and DNA collection and isolation protocol are attached to this amendment

The above amendment was reviewed on 01 November 2011 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Consent Form	1.0	24 October 2011

Participant Information Sheet	1.0	24 October 2011
Protocol	1.0	24 October 2011
Notice of Substantial Amendment (non-CTIMPs)	06	24 October 2011

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

10/H1013/8:	**Please quote this number on all correspondence**

Yours sincerely

Burgess

PP **Mr Francis Chan**
Chair

E-mail: Nicola.burgess@northwest.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mrs Rosemary Armstrong*
Professor Warne, University of Salford

This Research Ethics Committee is an advisory committee to the North West Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within
the National Patient Safety Agency and Research Ethics Committees in England

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NHS
National Research Ethics Service

Attendance at Sub-Committee of the REC meeting on 01 November 2011

Name	Profession	Capacity
Mr Francis Chan	Consultant Orthopaedic Surgeon	Expert
Dr Michael Hollingsworth	Retired Senior Lecturer in Pharmacology	Lay
Professor Janet Marsden	Professor of Ophthalmology and Emergency Care	Expert

Appendix V - Good Clinical Practice attendance Certificate.

NHS
National Institute for Health Research

Clinical Research Network
Coordinating Centre

Certificate of Attendance

David Greenwood

attended

Introduction to Good Clinical Practice (GCP):
A practical guide to ethical and scientific
quality standards in clinical research

on 07/12/2011

Sessions include:
1. The Value of Clinical Research and the role of the NIHR CRN
2. GCP: the standards and why we have them
3. Study set up: responsibilities, approvals and essential documents
4. The process of informed consent
5. Case report form, source data and data entry completion
6. Safety reporting in clinical trials

Paul Maher
NIHR CRN GCP Training Manager

Appendix VI - Amendment to REP10/32 – Approval

University of Salford
MANCHESTER

19 December 2011

Dear Rosy,

RE: AMENDMENT TO REP10/032 – A comparison of community based preventive services to improve child dental health

Based on the information you provided requesting some amendments to your original ethics application REP10/032, I am pleased to confirm that this has been approved by the College Research Ethics Panel.

If there are any further changes to the project and/ or its methodology please inform the Panel as soon as possible.

Yours sincerely,

Rachel Shuttleworth

Rachel Shuttleworth
College Support Officer (R&I)

Appendix VII - ELS School Ethics Application

College Ethics Panel

Ethical Approval Form for Post-Graduates

Ethical approval must be obtained by all postgraduate research students (PGR) <u>prior</u> to starting research with human subjects, animals or human tissue**.**

A PGR is defined as anyone undertaking a Research rather than a Taught masters degree, and includes for example MSc by Research, MRes by Research, MPhil and PhD. The student must discuss the content of the form with their dissertation supervisor who will advise them about revisions. A final copy of the summary will then be agreed and the student and supervisor will 'sign it off'.

The signed Ethical Approval Form and application checklist must be forwarded to your College Support Office and also an electronic copy MUST be e-mailed to the contacts below at your College Support Office;

CASS: Tracie Davies –   t.davies@salford.ac.uk

CHSC: Jill Potter -   j.potter@salford.ac.uk
            Rachel Shuttleworth -   r.shuttleworth@salford.ac.uk

CST: Nathalie Audren-Howarth – n.audren@salford.ac.uk

The forms are processed online therefore without the electronic version, the application cannot progress. Please note that the form must be signed by **both the student and supervisor**.

Please ensure that the electronic version of this form only contains your name and your supervisor's name on <u>this</u> page, where it has been requested.

All other references to you or anyone else involved in the project must be removed from the electronic version as the form has to be anonymised before the panel considers it.

Where you have removed your name, you can replace with a suitable marker such as [.….] Or [Xyz], [Yyz] and so on for other names you have removed too.

You should retain names and contact details on the hardcopies as these will be kept in a separate file for potential audit purposes.

Please refer to the 'Notes for Guidance' if there is doubt whether ethical approval is required

The form can be completed electronically; the sections can be expanded to the size required.

Name of Student: David Greenwood

Name of Supervisor: Professor Howard A. Foster (University of Salford); Professor Cynthia Pine (University of Salford); Dr. David Bradshaw (GSK); Dr. Richard Lynch (GSK).

School: Environment and Life Sciences

Course of study: PhD

Name of Research Council or other funding organisation (if applicable): GlaxoSmithKline

1a.   Title of proposed research project

The Antimicrobial Effects of Fluoride (NRES **Research Ethics Committee reference number 10/H1013/8**)

1b.   Is this Project Purely literature based?

  NO (delete as appropriate)

2. Project focus

To investigate the effects of fluoride interventions on bacterial communities within dental plaque obtained from babies (aged 1-3) and their parents in the Salford area.

3. Project objectives

- To develop a protocol for the safe extraction, transport and storage of biological materials, specifically oral bacteria DNA/whole fluid plaque
- To analyse said material using molecular-microbiological techniques against a baseline sample prior to fluoride treatment and in comparison with repeat samples taken at six-monthly intervals post and throughout fluoride treatment.
- To determine the effects of fluoride at varying concentrations on the bacterial hierarchy within the oral cavity with special emphasis on species associated with the development and continuation of tooth decay.(caries)

4. Research strategy

(For example, outline of research methodology, what information/data collection strategies will you use, where will you recruit participants and what approach you intend to take to the analysis of information / data generated)

Project Collaboration

The Bright Smiles project ('*A comparison of community based preventive services to improve child dental health*'. Protocol v1.2, 21 Dec 2010 plus amendments, Professor [XXXXX], School of Health and Social Care, University of Salford – Ethics Reference: REP10/032) was developed to compare the costs to the NHS and benefits to child dental health and aimed to review three new preventive dental services (outlined below) on parents and children. This required the recruitment of parents/carers of children approaching 12 months living within the wards of Salford.

The microbiological protocols used in this survey were sub-amended to the Bright Smiles project via the Integrated Research Application System (see *sub amendment Form 6 Oct 2011* attached) and granted approval by the NRES Research ethics committee (see *REC Nov 2011* attached). Protocols have since been submitted to the Research and Development Office for ethical approval, results pending.

We intend to contact these recruited volunteers with a participant information leaflet (PIL attached) to request their further involvement in a periodic plaque sampling program which will take place at six-monthly intervals over the remaining two years.

The aim of this program is to collect an oral plaque DNA sample and a whole fluid plaque sample (targeting plaque bacterial communities only) from a nominated parent and their child. Samples will be taken at a baseline (prior to fluoride treatment) and at six-monthly intervals post and throughout the application of fluoride interventions for the projects duration. Sample analysis aims to investigate the effect that specific fluoride applications have on the oral biosphere; to facilitate sample collection the Isohelix 'Swab Optimised DNA isolation Kit' (DDK50-SK1) will be used for DNA extraction, transport and storage (See *DNA collection and Isolation Protocol* attached). Fluid plaque is to be extracted by a registered dental professional using current technique and transported and stored using a tryptic soy broth-glycerol transport solution as described by Soderling *et al*. 2000.

All raw samples will be subjected to molecular-microbiological analysis (including but not limited to Polymerase Chain Reaction [PCR], hybridisation, microarray analysis and general and selective culture growth) and subsequent data's will be compiled and are expected to highlight changes in the bacterial hierarchy due to the introduction of fluoride interventions. All sampling appointments will coincide with those required for the Bright Smiles survey or at the convenience of the volunteer.

This study will observe any significant changes in the bacterial community, specifically those associated with the inhibition of colonisation, growth and acid production. Through meticulous molecular-microbiological analysis, we aim to describe the significance of fluoride in these reactions with the overall aim of reducing the development of caries-associated bacterial biofilms.

The following text outlines the Bright Smiles Project and specifically the initial recruitment protocol and participant responsibility. This information was considered relevant as the microbiological survey will run parallel with this study.

Research Design (Bright Smiles Project – Ethics Reference: REP10/032)
The Bright Smiles Project was designed as a randomised clinical trial to compare the costs to the NHS and benefits to child dental health of three new preventive dental services.

The Population
The population is defined as all children approaching 12 months of age at the end of January 2010 living in areas of Salford served by Sure Start Children's Centres and other nursery care providers.

Health Economic Analysis
From an NHS perspective (Edwards *et al.* 2008, Glick *et al.* 2007, Drummond *et al.* 2005, MRC 2000), we will: cost the control and test groups 1 and 2 service programmes; record trial participant primary and secondary care dental and dental related health service use (using an interviewer administered Client Service Receipt Inventory (CSRI), costed using National unit costs); conduct a primary cost effectiveness analysis (using the trial primary end point i.e. dental caries experience at age 3 years as our measure of effectiveness); and conduct a secondary cost-consequence analysis setting out the full range of costs and consequences to the NHS and to parents, e.g. missed attendance at nursery due to dental problems.

Interventions
Dental attendance by Control Group and both Test Groups
All participants will be offered the new standard service i.e. provision of any necessary treatment via a dental surgery linked to each Children's Centre.

Dentists near to the Children's Centre will be advised of the trial and invited by the Commissioning Team of NHS Salford to have an additional contract, becoming the dentist of the Children's Centre. Dentists with Children's Centre contracts will be represented on the Project Steering Board. They will be asked to flag the charts of all children in the trial and to keep a full note of any visits and treatment provided, and ideally time taken. Given the age of the children, few, simple visits are expected and this recording requirement will be built into the commissioning contract for the new dental service.
Hospital records of children referred for secondary care will be followed up by the trial team.

Periodic Administration of Questionnaires
The same standardised measures/ questionnaires will be used to assess all participants.
- Modified Oral Health Behaviours Questionnaire; Pine *et al.*, 2004

These measures will be used at the start of the trial, at mid-point (approximately 12 months into the trial) and at the end of the trial.

Test Group 1
The behavioural interventions will be conducted in the Children's Centres over 10 to 12 sessions from approximately age 12 to 30 months as a group session of 8 -10 parents/carers facilitated by a dental nurse and an 'expert parent' following

standardised training with a health psychologist.
At age 12 months – Brushing Routines
Visits 1 - 3 (weekly intervals)
- Discuss tooth-brushing
- Show brushing technique for baby; give brush and toothpaste
- Give brushing chart which includes section to note any problems; aim to record current brushing patterns and barriers to tooth-brushing twice a day
- Review brushing charts, identify problems, share best practice, techniques and discuss tools
- Give next brushing chart include problems/ease of brushing; aim twice daily brushing
- Reinforcement
- Review brushing charts, identify problems, share best practice, techniques and discuss tools
- Give next brushing chart include problems/ease
- Give reminder leaflet at end of session; aim twice daily brushing

Between age 13 months to 18 months
Hand in brushing charts to Centre, follow-up phone call; send reminder leaflets, brushing charts

At age 18 months – Sugar free bedtime
Visit 4 - 6 (weekly intervals)
- Reinforce brushing routines and give toothbrush and toothpaste
- Introduce sugar-free bedtime & discuss
- Give bedtime drink/snack charts: aim to record current bedtime foods/drinks and when and why given
- Reinforce brushing routines
- Review drink/snack charts, discuss sugar-free foods & drinks alternatives, display and samples to try
- Give bedtime drink/snack charts: aim none after brushing and sugar-free alternatives; if different note problem
- Reinforcement
- Review drink/snack charts, discuss sugar-free foods & drinks alternatives, display and samples to try
- Give combined bedtime drink/snack and brushing charts: aim no snacks after brushing and sugar-free alternatives; if different note problem
- Celebrate progress and success
- Give reminder leaflet at end of session

Between age 18 months to 24 months
Hand in combined drink/snack and brushing charts follow-up phone call, send reminder leaflets, and charts

At age 24 months - Reinforcement
Visit 7 - 8 (weekly intervals)
- Review brushing and sugar-free bedtime routines and give toothbrush and toothpaste
- Discuss problems, share best practice and describe tools to help behaviour

- Give combined bedtime drink/snack and brushing charts
- Reinforce brushing and bedtime routines
- Identify success/problems from charts, discuss
- Celebrate progress and success
- Reminder leaflet at end of session
- For those with problems, organise additional session (visit **11**).

Between age 24 months to 30 months
Hand in combined charts, follow-up phone call, send reminder leaflets, charts

At age 30 months - Reinforcement
Visit 9
- Review brushing and sugar-free bedtime routines and give toothbrush and toothpaste
- Discuss problems, share best practice and describe tools to help behaviour
- Give combined bedtime drink/snack and brushing charts

Visit 10 (one week later)
- Reinforce brushing and bedtime routines
- Identify success/problems from charts, discuss
- Celebrate progress and success
- Give reminder leaflet at end of session
- For those with problems, organise additional session (visit **12**).

Test Group 2
Semi Annual Varnish Applications
A trained Dental Care Professional (DCP) will arrange applications of fluoride varnish to the children's teeth at six monthly intervals from age 12 to 30 months at the Children's Centre. At each visit, parents will be advised for their child to follow a healthy diet and brush twice daily with fluoridated toothpaste.

Control Group
All families participating in the trial will be offered an opportunity for dental care with the dental practice linked to the Children's Centre. Families randomised to the control group will be offered this new service and will also be required to complete the periodic questionnaires.

Outcome measures
Dental health
A dental officer, trained in standardised techniques for the measurement of dental caries will examine all participating children at age 2 to record baseline d_1mft and the presence of plaque on index teeth. The examination will be repeated at age 3 years. The dental officer will be blind to group allocation. All children will be examined in their Children's Centres using sterilised or single use mouth mirrors, a standardised halogen lamp (2,000 lux) and cotton wool rolls as needed.

Implementation of Research Findings

Comparing these two approaches (behavioural intervention and fluoride varnishing) with opening up access to general dental services will enable commissioners to determine whether either brings additional benefit and costs incurred. If the cost-benefit of either service is significantly better than the new standard service, this will be implemented across Salford. These results will also inform the design of children's services in other disadvantaged communities across England. Research for Patient Benefit is the best NIHR programme because this research is directly linked to evaluating patient benefit, service users are involved in designing the new services, the team has the necessary range of expertise to conduct an effective evaluation and the team at the PCT and City Council are well placed to implement the findings.

It is intended that after the trial, parents participating in the behavioural intervention will be offered to opportunity to take part in a training course that will provide them with the necessary skills to provide the intervention to other parents in the Salford area. Working in conjunction with Salford City Council and PCT, opportunities will be provided to enable parents who have successfully completed the training programme to advise other families to establish twice-daily tooth-brushing and sugar-free bedtime routines with their children in local Children's Centres. This may provide an incentive to parents participating in the behavioural intervention to remain in the trial for the full duration, although it is not a condition of joining the trial. The trial can build health-promoting capacity amongst the local community.

Description of the Analyses

The analysis of the primary outcome variable will use the chi-squared method to test for differences between each intervention group and the control group, with a Bonneferroni adjustment for the two comparisons. Logistic regression analysis will be performed to investigate the effects of other potential explanatory variables such as deprivation, age and gender.

The secondary outcome variable, caries experience at age three years, will be analysed using Analysis of Variance to test for differences in d_1mft between the three groups, with pairwise comparisons between groups using Tukey adjustment for multiple comparisons. Multiple regression analyses will be performed to investigate the effects of other potential explanatory variables such as deprivation, age and gender.

Data for health economic analysis will be collected as part of the trial to provide a rigorous dataset for this population. An incremental cost effectiveness ratio (ICER) analysis will be carried out on the control group versus each test group to identify specific quality adjusted life year (QALY) benefits.

Trial Hypotheses

The primary hypotheses are that:

1. There is no difference in the proportions of children with dental caries at age 3 years between test group 1 (behavioural intervention) and the control group
2. There is no difference in the proportions of children with dental caries at 3 years between test group 2 (fluoride varnishing) and the control group

Sample Size Consideration

The primary outcome variable is presence of dental caries at age 3 years. Pilot data have been obtained from the Salford area, which gives an estimate of 40% of 3-year old children with caries. In this three group trial, the comparisons are between each

intervention group and the control group and not between the two intervention groups. Therefore, as two comparisons are being made the p-values for statistical significance will be adjusted using the Bonneferroni method to 0.025, giving an overall significance level of 0.05. Assuming 35% with caries in the control group, then the minimum clinically significant difference is set to detect a difference with 80% power, where the test groups have 17.5% with caries. Allowing for a 20% dropout rate in each group gives a final sample size of 110 per group.

5. What is the rationale which led to this project?

(For example, previous work – give references where appropriate. Any seminal works must be cited)

Caries is one of the most prevalent diseases of children worldwide and is characterised by the demineralisation of tooth enamel by organic acids from cariogenic bacteria (Arora *et al*. 2011, Bonifait and Grenier 2010). Dental biofilms are a complex polymicrobial mixture of cellular debris and food particles dominated (≥78%) by streptococci species (Nyvad and Kilian 1990, Dibdin and Shellis 1988) although to date more than 700 have been detected in the oral cavity (Bonifait and Grenier 2010). Nugent and Pitts (1997) showed that the dental health of children (5 years old) in the North West is amongst the worst in the country. A 1996 study also showed that approximately 20% of 3 year old children in Salford are at varying stages of caries developed (Hamilton and Hawley 1998).
Numerous works have indicated a trend of high caries incidence within deprived communities and vulnerable social groups (Winter *et al*. 1971; Hamilton and Hawley 1998; Satcher 2000; Nunn 2006; Tinanoff and Reisine 2009) and these same trends are clearly illustrated in Salford. It is based on these data that a comparative study has been devised to investigate the dental health of children at age 0–3 years based on the prevalence of caries and extractions after 3 years of surveillance.

The following text describes the rationale for the design of the bright Smiles Study, summarising specifically, previous and relevant Pre/clinical Studies.

Summary of Previous Pre-clinical Studies
The research plan and methodology has been informed by a pilot study (December 2008- January 2009) involving a sample of parents from the proposed population.

The pilot study was in four parts: interviews with the Oral Health Improvement Team to understand their current activities in relation to families with babies aged 0 to 3 years; focus group in a Children's Centre in Salford; dental survey of 3 year olds attending the Primrose Hill Children's Centre; questionnaires to parents about bedtime foods and drinks, use of dental services, child toothache experience and frequency of tooth-brushing. Ethical approval for the pilot study was obtained from the Ethics Committee of the University of Salford. Informed consent was sought from parents and each was given an information sheet.

Current activity of the Oral Health Improvement Team includes distributing packs containing 1450ppm (adult, family) toothpaste, toothbrush and leaflet to be given out, nominally at child's 8-month developmental checks by the health visitor. The team call into established parent and toddler groups running in the Children's Centre offering advice on tooth-brushing, healthy dietary habits and recommend regular attendance at a dental practice. In summary, the behaviours targeted in a typical single session comprise a significant number of components. To re-structure them to achieve behaviour change would need staff training and a different approach to be taken. Conducted as described, the existing activities are at best likely to enhance awareness of desired dental behaviours, increase knowledge rather than achieve the behaviour; due to the combination of complex messages, lack of reinforcement and limited support in skill acquisition.

Analysis of the focus groups identified a number of thematic issues around bedtime routines. Only one parent had any routine, with most describing chaotic bedtimes with children falling asleep and being carried to bed.

Parents demonstrated that they did know that sugary foods and drinks can cause tooth decay and that, ideally, children's teeth should be brushed twice daily, but very few translated that knowledge into the desired behaviour.

Dental screening of 3 year olds attending Primrose Hill Children's Centre was conducted and questionnaires completed by parents/guardians of 38 children. 15 children, 39%, had dental caries with 2 children having all of their 20 teeth decayed. 40% of parents of children with caries reported that their child had toothache compared to only 7% of parents of caries free children, with the latter probably linked to eruption of the last baby teeth. Almost a third of parents of children with caries had no family dentist themselves and the child had never been taken. The proportion of children eating at bedtime was the same for those with and without caries, and was around 50%, with over 90% having a drink at bedtime. However, parents of children with caries were much more likely to report that the foods and drinks were sugar-containing, such as biscuits and sweetened tea or juice.

The pilot study also looked at response and likely recruitment. The supporting organisation of the Children's Centre was excellent. They offer a range of services at the Centre and are very experienced in explaining procedures to parents and securing completion of short questionnaires. No parent refused the child dental exam and 38 out of 41, 93% of questionnaires were completed with a single distribution. Parents were asked their opinion of dental services in Salford and would like to take part in a trial of new preventive dental services.

Summary of Relevant Clinical Studies
A previous study conducted by Adair and Pine (2004) within the World Health Organisation's Global Oral Health Programme examined familial and cultural perceptions and beliefs of oral hygiene and dietary practices among ethnically and socio-economically diverse families.
The study involved over 2,800 children aged 3 to 4 years and their parents in 17 countries. Factor analysis identified 8 coherent attitudes towards tooth-brushing,

sugar snacking and childhood caries.

Attitudes were significantly different in families from deprived and non-deprived backgrounds and in families of children with and without caries.

Parents' perception of their ability to control their children's tooth-brushing and sugar snacking habits were the most significant predictors of whether or not favourable habits were reported.

A central hypothesis arising from the study was that a behavioural intervention for parents designed to improve parental efficacy and develop key skills should lead to child behaviours that will prevent caries from an early age.

The research team noted that the parenting skills required to establish the routine of regular tooth-brushing behaviour in young children could provide a valuable template for understanding how parenting skills can be developed for other (non-dental) child behaviours.

6. **If you are going to work within a particular organisation do they have their own procedures for gaining ethical approval**

   (For example, within a hospital or health centre?)

   YES (delete as appropriate)

   If YES – what are these and how will you ensure you meet their requirements?

LREC and NHS R and D approvals are being sought through the Integrated Research Application System (IRAS).
Research passports will be obtained to enable researchers to access NHS data.
Current CRB documentation will be required for all staff.

7. **Are you going to approach individuals to be involved in your research?**

   YES (delete as appropriate)

   If YES – please think about key issues – for example, how you will recruit people? How you will deal with issues of confidentiality / anonymity? Then make notes that cover the key issues linked to your study

This work has been designed to complement the Bright Smiles project (described above) for which recruitment is expected to be completed by December 2011. Samples required for the microbiological survey will therefore be taken from existing volunteers.

In the first instance volunteers will be issued with a participant information leaflet outlining the additional microbiological study, their involvement and methods of contacting the team for further information. Potential participants will then be asked to sign a consent form (see *IC (micro) v1.0, 24th October 2011 study number 31168* attached) detailing their understanding of the project at hand, specifically our intentions and their responsibilities.

This study aims to describe the significance of fluoride within any reaction which leads to the counter-development of caries inducing biofilms. This requires the careful analysis of new data against subsequent findings as well as medical, dental and environmental factors. This will include the patients' the use of antibiotics (which significantly alter both systemic and topographical biota), any history of reduced or elevated fluoride-based dentifrice usage and/or residential location within regions where drinking water is/not fluoridated. It is essential for the accurate analysis that all such information be freely available throughout the study.

Sample collection will be carried out by the parent or dental professional (as desired by the volunteer) under the direction of the microbiologist. Samples will be anonymised solely by a serial number which will indicate the study group (1, 2 or 3), the date, and a unique serial number which must indicate the time of sampling, subject's age and time period since last brush. This serial number will be added to a permanent record which can be complied and thus compared throughout the patients' treatment.

8. More specifically, how will you ensure you gain informed consent from anyone involved in the study?

Informed Consent Process

Discussion of risks and possible benefits of participation will be provided to the participants and their families. Consent forms describing in detail the intervention(s) trial procedures and risks will be given to the participant and written documentation of informed consent is required prior to starting trial intervention. Consent forms will be REC approved and the participant will be asked to read and review the document. Upon reviewing the document, the investigator, or delegated deputy trained in GCP and taking consent for research involving children, will explain the research trial to the participant and answer any questions that may arise. The participants will sign the informed consent document prior to any procedures being done specifically for the trial. The participants should have sufficient opportunity to discuss the trial and process the information in the consent process prior to agreeing to participate. The participants may withdraw consent at any time throughout the course of the trial. A copy of the informed consent document will be given to the participants for their records. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this trial.

The informed consent form will be signed and personally dated by the participant and the person who conducted the informed consent discussion. The original signed informed consent form will be retained in the chart and a copy will be provided to the participant

9. How are you going to address any Data Protection issues?

See notes for guidance which outline minimum standards for meeting Data Protection issues

The Investigator will ensure that the subject's anonymity is maintained. Subjects will not be identified in any publicly released reports of this trial. All records will be kept confidential to the extent provided by national and local law.   The trial monitors and other authorised representatives of the Sponsor may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records. The investigator will inform the subjects that the above-named representatives will review their trial-related records without violating the confidentiality of the subjects. All evaluation forms, reports, and other records that leave the site will be identified only by a coded number in order to maintain subject confidentiality. All records will be kept locked and raw materials stored within security coded laboratories within a research facility protected by proximity locks. Clinical information will not be released without written permission of the subject, except as necessary for monitoring by REC, or the Sponsor's designee.

10. Are there any other ethical issues that need to be considered? For example - research on animals or research involving people under the age of 18.

The trial will be conducted in accordance with the design and specific provisions of this approved protocol and its amendments, in accordance with the ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with Good Clinical Practice (GCP) and the applicable regulatory requirement(s). The Chief Investigator will assure that no deviation from, or changes to; the protocol will take place without prior agreement from the sponsor and documented approval from the Research Ethics Committee (REC), except where necessary to eliminate an immediate hazard(s) to the trial participants. The Chief Investigator will promptly report to the REC and the sponsor any changes in research activity and all unanticipated problems involving risk to human subjects, or others.

Summary of Ethical Issues
1. This trial requires informed consent from parents as children included in the trial will be too young to understand/ accept randomisation.
2. This trial will use standardised questionnaires to measure several constructs including oral health behaviours/attitudes, parental self-efficacy and family routines. Help will be available for parents to assist in completion if needed. Translators/interpreters will be available to assist participants whose first language is not English or who are visually impaired.
3. Dental examinations will be conducted on all participating children. Parental consent will be gained for these examinations. This will be a standard visual epidemiological dental exam without the use of radiography and will be conducted in familiar surroundings (Children's Centres) in order to minimise distress to participating children. Parents will have the opportunity to attend.

4. Biological samples will be required for microbiological analysis. These samples will be collected from the oral cavity of both a nominated parent and their child and will be facilitated by a swab optimised system which cannot distinguish and/or separate bacterial from human cells.

11. **(a) Does the project involve the use of ionising or other type of "radiation"**

NO

(b) Is the use of radiation in this project over and above what would normally be expected (for example) in diagnostic imaging?

NO

(c) Does the project require the use of hazardous substances?

NO

(d) Does the project carry any risk of injury to the participants?

NO

(e) Does the project require participants to answer questions that may cause disquiet / or upset to them?

NO

If the answer to any of the questions 11(a)-(e) is YES, a risk assessment of the project is required and must be submitted with your application.

12. **How many subjects will be recruited/involved in the study/research? What is the rationale behind this number?**

Regarding the Bright Smiles project, the primary outcome variable is presence of dental caries at age 3 years. Pilot data have been obtained from the Salford area, which gives an estimate of 40% of 3-year old children with caries. In this three group trial,

the comparisons are between each intervention group and the control group and not between the two intervention groups. Therefore, as two comparisons are being made the p-values for statistical significance will be adjusted using the Bonneferroni method to 0.025, giving an overall significance level of 0.05. Assuming 35% with caries in the control group, then the minimum clinically significant difference is set to detect a difference with 80% power, where the test groups have 17.5% with caries. Allowing for a 20% dropout rate in each group gives a final sample size of 110 per group (n = 330).

The parameters of this study are dictated by the successful recruitment of volunteers for the Bright Smiles survey for which fluoride treatment has already begun. For this study we intend to survey all volunteers approaching 1years from the point of ethical approval. Participants for the microbiological study are expected to range between 20 and 40 babies plus one allocated parent per child (n = 40 – 80)

13. Please state which code of ethics has guided your approach (e.g. from Research Council, Professional Body etc).

Please note that in submitting this form you are confirming that you will comply with the requirements of this code. If not applicable please explain why.

NHS Research Ethics Committee.
The R&D Office

Remember that informed consent from research participants is crucial, therefore all documentation must use language that is readily understood by the target audience.

Projects that involve NHS patients, patients' records or NHS staff, will require ethical approval by the appropriate NHS Research Ethics Committee. The University College Ethics Panel will require written confirmation that such approval has been granted. Where a project forms part of a larger, already approved, project, the approving REC should be informed about, and approve, the use of an additional co-researcher.

I certify that the above information is, to the best of my knowledge, accurate and correct. I understand the need to ensure I undertake my research in a manner that reflects good principles of ethical research practice.

Signed by Student   _____

Print Name   _____

Date   _____

In signing this form I confirm that I have read this form and associated documentation.

I have discussed and agreed the contents with the student on _____
(Please insert date of meeting with student)

Signed by Supervisor _____

Print Name   _____

Date   _____

Name of Applicant:

Title of Project:

The checklist below helps you to ensure that you have all the supporting documentation submitted with your ethics application form. This information is necessary for the Panel to be able to review and approve your application. Please complete the relevant boxes to indicate whether a document is enclosed and where appropriate identifying the date and version number allocated to the specific document (*in the header / footer*), Extra boxes can be added to the list if necessary.

Document	Enclosed? (indicate appropriate response)				Date	Version No
Application Form		Mandatory		If not required please give a reason		
Risk Assessment Form		No	Not required for this project	Risk assessment questionnaire showed that no assessment is required. See page 37	22/11/11	
Participant Invitation Letter	Yes		Not required for this project		22/11/11	
Participant Information Sheet	Yes		Not required for this project		22/11/11	
Participant Consent Form	Yes		Not required for this project		22/11/11	
Participant Recruitment Material – e.g. copies of posters, newspaper adverts, website, emails	Yes		Not required for this project		22/11/11	
Organisation Management Consent / Agreement Letter	Yes		Not required for this project		24/11/11	
Research Instrument – e.g. questionnaire	Yes		Not required for this project		22/11/11	

Draft Interview Guide		No	Not required for this project	Questionnaire was self administered.	22/11/11	
National Research Ethics Committee consent	Yes		Not required for this project		22/11/11	

Note: If the appropriate documents are not submitted with the application form then the application will be returned directly to the applicant and will need to be resubmitted at a later date thus delaying the approval process

Appendix VIII - Risk assessments – amendments

Underpinning codes of ethics

After speaking with the Bright Smiles project representative at the School of Health and Social Care (University of Salford) I have been advised that the underpinning codes of ethics used in the design of this project are International Conference on Harmonisation - Good Clinical Practice (ICH-GCP) and the Medicines and Healthcare products Regulatory Agency (MHRA).

Protection of electronic data.

Due to the sensitivity of the data accumulated during this project it is of primary importance that materials pertaining to the physical and dental health of our volunteers are protected.

The protocol requires that we question each volunteer as to their dentifrice usage while also gaining knowledge of the current medial history. This means that we need to know toothpaste, mouth wash, floss brands and all fluoride concentrations therein. We also need to know whether the volunteer has been treated with antibiotic material which, when administered will affect biota accumulation in the mouth.

Such information will be transferred to PC storage (and with their hard copies) secured within a locked office, within the laboratory (Cockcroft 209), which is protected behind a key-coded door within a limited access research facility (The Centre for Parasitology and Disease Research, Cockcroft Building). All materials will be used to achieve the aims outlined by the project and stored until the relevant authorities deem that the project is completed. Upon project completion all data will be deleted/destroyed.

Protocol Risks

1. Contamination.

    a. From swab.

        The swab systems used in this protocol are intended for the collection of human DNA suitable for clinical genotyping, paternity testing, forensic and analysis. They are provided (Cell Projects Ltd), in sterile (gamma treated) condition, individually wrapped and pose no known risk of contamination.

    b. Cross contamination.

        As outlined in the protocol, sample collection will be taken by the parent. Sampling protocol will be directed and supervised by the trial microbiologist or a similarly trained project representative. The parent will first be instructed in the use of the swab, they will then un-wrap the swab, take the sample and hand the swab to the project representative present. The swab will then be transferred to a DNA stabilisation solution which will be sealed and stored as directed by the manufacturer. The swab is designed for single use only and poses no risk of oral contamination.

2. <u>Physical Harm caused by sample harvest process.</u>

The swab is made of a corrugated (mildly abrasive) derivatised cellulose head and a plastic arm which was designed such that live cells from the buccal surface may be collected for analytical purposes. It was chosen for its physical qualities, specifically for its ability to remove a 'typical' sample of plaque within seconds of exposure.

We have reduced the risks of tissue damage caused by swabbing by requesting that the parent harvests the plaque DNA.

3. Chemical poisoning.

As described above, swabs are provided sterile and are of non-toxic materials.

The chemicals used in this mixture for cell lysis and DNA stabilisation are described by the manufacturer as Harmful (R22), Harmful if swallowed (R41), and Harmful to eyes, respiratory system and skin. Irritant (R 36/37/38), irritating to eyes, respiratory system and skin (S 26; 36). First-aid measures: In case of contact with eyes, immediately flush eyes with copious amounts of water for at least 15 minutes. In case of contact with skin, immediately wash skin with soap and copious amounts of water. If inhaled, remove to fresh air and monitor breathing. If not breathing give artificial respiration. If breathing is difficult, give oxygen. If swallowed, wash out mouth with water provided person is conscious. Call a physician. For further details please refer to the material safety data sheet appended below.

However, handling of cell lysis solution is restricted to trained personnel (project representatives) only and therefore poses no threat of chemical poisoning to the project participant(s). Moreover, amounts of the chemicals in each vial are small. There is therefore only a low risk from the chemicals and no risk to the subjects.

4. Choking.

The swab (as shown in the protocol) is made up of a cellulose head attached to 14cm handle with a plastic barb, designed specifically for release into the sample collection tube and not the mouth.

The long plastic arm ensures reach with reduced possibility of accidently dropping/losing the swab in the mouth. Again, the child's parent will be trained in sample collection thus replacing an unrelated clinical professional with parental 'carer'; this final factor will reduce the possibility of physical harm resulting as part of the harvest process.

5. Allergies.

Referring to points 1 – 4, the swab is constructed of plastic and derivatised cellulose, gamma-treated (sterilised) and proposes no known threat of allergies

Appendix IX - School of Environment and Life Sciences, confirmation of project proposals.

Academic Audit and Governance Committee

College of Science and Technology Research Ethics Panel (CST)

To	**David Greenwood**
cc:	**Prof Judith Smith, Head of School of ELS**

From   **Nathalie Audren Howarth**, **College Research Support Officer**

MEMORANDUM

Date   **7th February 2012**

Subject: **Approval of your Project by CST**
Project Title: **The Antimicrobial Effects of Fluoride**
REP Reference: **CST 11/12**

Following your responses to the Panel's queries, based on the information you provided, I can confirm that they have no objections on ethical grounds to your project.

If there are any changes to the project and/or its methodology, please inform the Panel as soon as possible.

Regards,

Nathalie Audren Howarth
College Research Support Officer
For enquiries please contact:
College of Science and Technology
College Research Support Officer
The University of Salford
Maxwell building, (7th floor, room 719)
Telephone: 0161 295 5278
Email: n.audren@salford.ac.uk

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Appendix X - Clinical Trials Form CT09-10

RESEARCH INVOLVING HUMAN PARTICIPANTS
QUESTIONNAIRE
(Clinicall Trial*)

Cover is automatic and a Questionnaire is NOT needed if the research is within the UK & limited to the following activities:

i. Questionnaires, interviews, psychological activity including CBT;
ii. V e n e p u n c t u r e (withdrawal of blood);
iii. M u s c l e biopsy;
iv. M e a s u r e m e n t s or monitoring of physiological processes including scanning;
v. C o l l e c t i o n s of body secretions by non invasive methods;
vi. I n t a k e of foods or nutrients or variation of diet (other than administration of drugs).

Refer all other Research involving human participants to the Insurance Officer with the following information to arrange cover - which may incur a charge. Early submission of Questionnaire recommended.

1. Institution: University of Salford   **Department:** Environment and Life Sciences

2. Title of Research: The Antimicrobial Effects of Fluoride

3. Name(s) of sponsoring organisations: GlaxoSmithKline

4. Does the research involve –

a. Investigating or participating in methods of contraception?	No
b. Assisting with or altering the process of conception?	No
c. he use of drugs?	No
d. The use of surgery (other than biopsy)?	No
e. Genetic engineering?	No
f. Subjects under 5 years of age? (other than activities i-vi above)	YES
g. Subjects known to be pregnant? (other than activities i-vi above)	No
h. Pharmaceutical product/appliance designed or manufactured by the institution?	No
i. Work outside of the United Kingdom?	No

If 'Yes' to <u>any</u> of the questions 4a- i above, the following information will also be needed:

➢ *Protocol* (pg. 3-5)
➢ *Patient Information Sheet and Patient Consent Form* (pg. 6-9 and 10 respectively)
➢ *Employee Activity Form* (page 2)

If 'Yes' to <u>any</u> of the questions, 4a-i above, and this a follow-on phase, provide details of *SUSAR*s on a separate sheet (fatal or life threatening events).

Name: David Greenwood

Date: 11/01/2012

*NB: for the purpose of indemnity/cover **Clinical Trial** means**:** an investigation or series of investigations conducted on any person for a **Medicinal Purpose.**

Medicinal Purpose means:

a) Treating or preventing disease or diagnosing disease or
b) Ascertaining the existence degree of or extent of a physiological condition or
c) Assisting with or altering in any way the process of conception or
d) Investigating or participating in methods of contraception or
e) Inducing anaesthesia or
f) Otherwise preventing or interfering with the normal operation of a physiological function

Form CT09/10   ©U M Association Ltd August 2009

Employee Activity Form

1. Has NHS Indemnity been provided?	YES	NO
2. Will Medical Practitioners be covered by the MDU or other body?	YES	NO

3. This section aims to identify those staff involved, their employment contract and the extent of their involvement in the Research.

Name the employer and if an NHS honorary contract is held:
(In some cases it may be more appropriate to refer to a group of persons rather than individuals.) Principal Investigator:
Name: **Professor Cynthia Pine**
Employer: **University of Salford.**
NHS Honorary contract **Yes [Hon consultant in Dental Public Health Salford Royal Foundation Trust]**
Activities undertaken: **Principal Investigator/PhD supervisor for David Greenwood and Richard Freeman**

Others:
Name: **David Greenwood**   Employer: **University of Salford (PhD Student)**   NHS Honorary contract?   **No**
    Prof. Howard Foster   Employer: **GSK (PhD Supervisor)**   NHS Honorary contract?   **No**
    Dr. David Bradshaw   Employer: **GSK (PhD Supervisor)**   NHS Honorary contract?   **No**
    Dr. Richard Lynch   Employer: **GSK (PhD Supervisor)**   NHS Honorary contract?   **No**

4. Please provide any further relevant information here:
We intend to collect oral dental plaque from Salford-based children, biannually, between the ages of 12 months and 3 years. The method (see *DNA Isolation Protocols* below**)** involves the use of a derivatised cellulose swab and DNA isolation solution (supplied by *Cell Projects Ltd*) which will be used by the parent (no medical practitioners will be used for the microbiological aspect of this study) to collect the sample over no less than 30 seconds – 1 minute. Participants will be recruited from an already existing project (University of Salford, School of Health and Social Care - Study number 31168) with a specified microbiological Participant Information Leaflet (see '*Bacteria that grow on baby teeth*' below) with the aim of assessing the antimicrobial effects of Fluoride in mature dental microbiological communities. Participants will be expected to confirm that they understand the study requirements by signing and submitting the patient confirmation form (see '*IC (micro) v1.0, 24th October 2011   study number 31168'* below).
We will be targeting oral bacteria only and will not be targeting human tissue in any way. This includes any human genetic materials found in mucus, saliva or that swabbed from the teeth and gums during the harvesting process. All protocols have been submitted to the NRES and R&D office from which we have received a favorable approval and temporary approved status (respectively) based on the subsequent submission of a CV and details of a successful CRB check.

Please copy this form if necessary and continue to list all individuals or groups of staff involved with the Research

N/A

DNA Isolation Protocols

Experimental Aims

To isolate bacterial DNA from human dental plaque in order to assess the antimicrobial effects of fluoride.

Materials

ISOHELIX DNA collection Swabs plus Isolation kit #DDK50/SK2 (see figure 1 below), vortexer; Microcentrifuge (13K rpm); waterbath (60˚C).
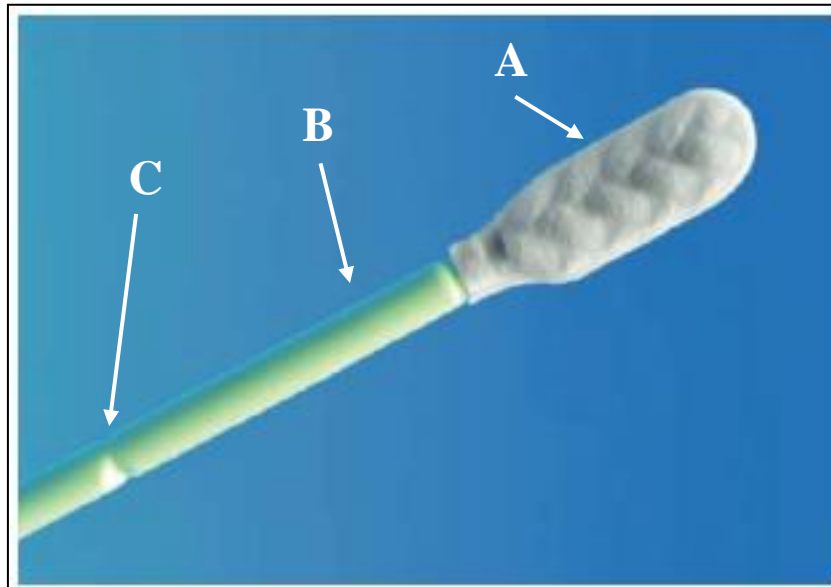

Figure 1. (Above) *Swab optimised DNA isolation swab'* – Cell Projects Ltd # DDK-50/SK2. A) Detachable, derivatised cellulose head; B) Handle; C) Specialised Failure Point.

Methods

- Remove the swab from its sterile paper packaging (taking not care to touch the swab head with hands, clothing or other equipment) immediately before the sample is ready to be taken.
- Holding the swab by the handle and using reasonable, firm circular motion, swab the inner and outer surfaces of the incisors and lateral incisors (covering tooth surfaces, gingival margins, interdental papilla, and periodontal pocket); see figure 2, (below) for 1 min ± 30 sec.
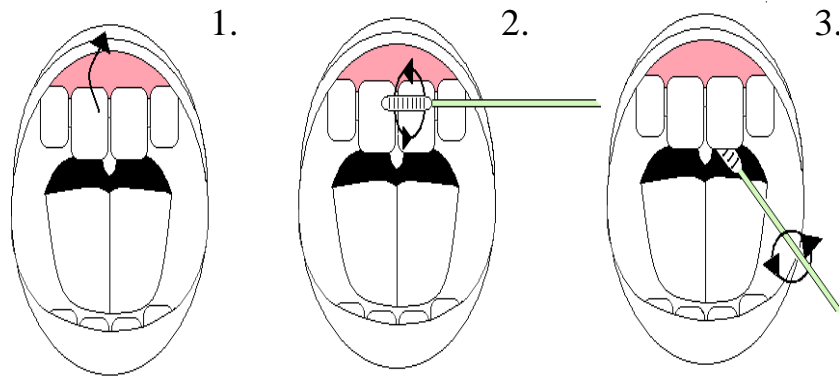
Figure 2 (above) showing the process of plaque removal. **1.** Lift up the upper lip exposing the four front teeth. **2.** Remove the swab from its wrapping and using firm, circular motion, swab the outer surfaces of the four front teeth. **3.** Remember to include the inner surfaces of the same front teeth for a total of no less than 30 seconds. If the top teeth are missing swab the bottom four front teeth

- Immediately after sample extraction, taking care not to touch the swab head with hands, clothing or other equipment, place the swab (head first) into the storage tube containing 520µl lysis solution and detach head thus:

> Hold the swab handle Failure Point [Fig. 1-C] to the side of the reaction tube, break the upper handle sideways. Handle will break away and the swab-head will fall into the tube. The tube is to be capped, mixed by inversion and stored upright (ensuring the swab head is submerged) at room temperature in the storage box provided.

- At this stage cells within the samples have been lysed and the DNA released has been stabilised. Samples may be stored at room temperature for at least 2 years.
- The sample should then be marked with the date, the study group number (1, 2 or 3) and a unique serial number which must indicate the time of sampling, subject's age and time period since last brush.

E.g. Time (17. 45) – Age (3yr, 11mnth) - Time lapse (12hrs) ∴ 1745-3.11-12

- Sample analysis will be carried out with emphasis on the strict protection of subject anonymity. However it is necessary for biological researchers to understand the nature of the subject's oral hygiene procedures, medical history and geographic residence in order to track anomalies in the data. Therefore the following details, highlighted in the 'Volunteers Data Sheet' (below) must be included with each sample.

Sample Serial Number	……………………………………………………………				ppm (if Known)
	……………………………				
Method(s) of last clean (please tick as many as required)	Brushing	☐	Fluoridated ?	Yes ☐ No ☐	…………………
	Flossing	☐	Fluoridated ?	Yes ☐ No ☐	…………………
	Mouth Rinse	☐	Fluoridated ?	Yes ☐ No ☐	…………………
	Other - Please specify (*Product type? Brand?*)				
			Fluoridated?	Yes ☐ No ☐	…………………
Antibiotics used	Brand/Name		Dosage	Approx. Usage Period	
Prior Addresses (post code)	(Up to 12 months prior)				

- On returning to the lab, sample DNA will be isolated using the following methods as described in the isohelix user manual. Note that this is a commercial kit therefore the exact components of the solutions used are not available.

Step	Process
1	Place the tube containing the swab, LS solution and PK solution in a 60°C water bath for 1 hour. Vortex briefly.
2	Transfer the liquid in the tube (approx. 400µl) into a 1.5ml centrifuge tube using a sterile pipette tip.
3	Spin the SK-2 tube containing the swab head briefly and using a sterile pipette tip add the recovered supernatant to the 400µl collected previously.
4	Add 400µl CT solution to the tube. Vortex briefly
5	Place the tube in a microcentrifuge (see *tip* below) and spin at approx. 13K rpm for 7 minutes to pellet the DNA.
★	*Place the tube with hinge positioned outwards so the liquid can be removed from the opposite side*
★	*The pellet may not be visible*
6	Remove all the supernatant carefully with a pipette tip taking care not to disturb the DNA pellet
7	Re-spin the tube briefly and remove any remaining liquid
★	*It is important to remove all of the liquid*
8	Add 150µl TE solution to the tube. This volume may be decreased to as little as 30µl if a higher concentration of DNA is required.
9	Leave for at least 5 minutes at room temperature for the DNA to re-hydrate, longer if a reduced volume of TE has been used. Vortex briefly.
★	**Store the DNA sample at 4°C for short term storage or -20°C for long term storage. The expected yield from a buccal swab is 2 to 10µg DNA (10 to 70ng/µl).**

Bacteria that grow on baby teeth

Dear [parent]

Invitation to take part in research

We are inviting you to take part in a second phase of the Bright Smiles Baby Study. As with the first phase, taking part is entirely voluntary and you may withdraw at any time without having to give a reason.

Why have I been invited?

You have already agreed to take part in the Salford Bright Smiles Baby Study. Over the next 18 months, your baby will be growing new teeth. We all have bacteria on our teeth and it is a normal part of life. We would like to understand about how bacteria grow as your baby's teeth grow.

Why are we doing this research?

From many different studies, we know that the bacteria adults grow on their teeth change depending on foods and drinks, on dental treatment, on tooth bushing and toothpaste. But, we know much less about these changes for babies and also, how growing new teeth affects which normal bacteria they have. This study will help us understand which bacteria grow on babies' teeth and gums.

What will I have to do?

Every 6 months of the Bright Smiles study, we will ask you to attend an appointment to collect a sample of the sticky, creamy coating (dental plaque) on the front teeth of yours and your baby's teeth. We will do this (explained below) in a very quick, simple and painless way with no known risks.

We will ask you to tell us what toothpaste/mouthwash you have been using, specifically, brand and fluoride content. We will also need to know if you have taken **any** antibiotics in the last 12 weeks, and if you need to do so **during** the study. This is because they have an effect on bacteria and give different results.

How many samples will we be collecting?

We would like to collect at least 5 samples over the next two years from you and your baby. We will ask some people to attend for three extra times. About 40 families from each of the 3 study groups will be asked to take part.

What will happen to me?

Swab Sample

At your first appointment we will ask you to rub a swab (like a cotton bud) on your baby's top and/or bottom front teeth (inside and outside surfaces).

We will ask you to do the same for your own teeth. If your own natural top teeth are missing, then you will rub it over the bottom teeth instead. The swab is then placed into a solution which has been designed to store all the bacteria's DNA. This will then be taken to the lab for analysis.

This is how we collect the bacteria DNA!

Someone from the study team will be there to show you how to the use the swab. We will help with timing, and altogether it takes up to 1 minute. There are three simple steps, all shown below

1. Lift up the top lip to show the four front teeth

2. Take the swab out of its wrapping and using firm, round movements; swab the outside part of the four front teeth.

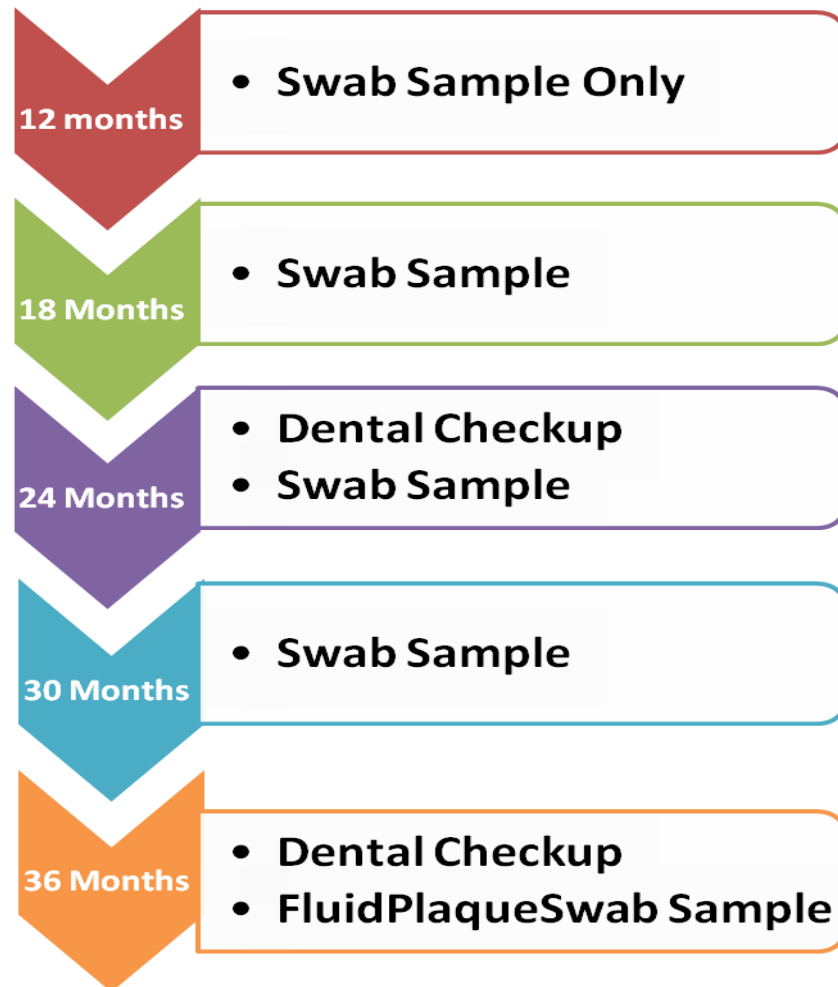3. Using the same swab, now rub it on the inside part of the same front teeth.

1.   2.   3.
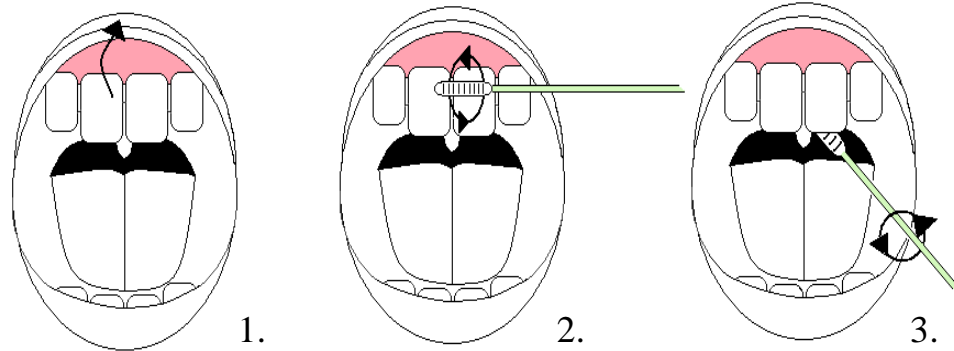
If you are due to have one of your dentist/dental nurse appointments, you may prefer for them to take the sample – it will be up to you!

IMPORTANT! Only the bacteria will be examined! No human DNA or cells that are collected in both swabs and plaque will be used in any way for research purposes.

All the information and plaque samples you give us will be collected, stored, archived, analysed and disposed of according to the Human Tissue Act 2004 and the Data Protection Act 1998.

The following flowchart describes when (baby's age) and what we need from you.

- **12 months**: Swab Sample Only
- **18 Months**: Swab Sample
- **24 Months**: Dental Checkup / Swab Sample
- **30 Months**: Swab Sample
- **36 Months**: Dental Checkup / FluidPlaqueSwab Sample

What will happen when the research stops?

Your usual family dental care arrangements will continue as normal after the study finishes and we will make the results known to you in a newsletter to all families who have taken part.
Any results that are made public will not contain any information that can identify you personally.

How you will be affected by the research/ What if anything happens to me while I am on the study?

It is very unlikely that you will be affected in any way and there is no direct benefit taking part in this research, however we are legally required to tell you that If your child's health and well being is made worse by being in the study the Sponsor (University of Salford) will compensate you without legal commitment to proving fault. This means that the Sponsor will compensate you for any injury resulting from procedures carried out within this study protocol. Your right in law to claim for compensation for injury where you can prove negligence is not affected.

Will I gain or lose anything by being in the study?

As a token of our appreciation, families who take part in testing out this new method of observing bacteria will be entered in a 6-monthly prize draw for **£50** *Love to Shop* vouchers for each study group.
PLUS an additional **£100 voucher draw** for all parent and child groups that complete all plaque sample appointments!

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You will not receive payment, or any treatment which is different from that, normally available to other people. It will not affect your access to any other dental or medical services.

<u>**What we hope to achieve through this research.**</u>

This phase will run hand-in-hand with the Bright Smiles Baby Study and aims to understand the bacteria that live in dental plaque and those that can cause tooth decay.

With the use of cutting edge scientific methods, we hope to understand the biology of these bacteria and how to help reduce tooth decay.

<u>**Who should I contact?**</u>

Should have any questions about the study, please feel free to contact
Either:

Rosy Armstrong (Clinical Trial Coordinator) 0161 295 5095
XXXXXXX@salford.ac.uk
College of Health & Social Care,
Allerton Building,
University of Salford,
Salford,
Greater Manchester,
M5 6PU.
Or
David Greenwood (Trial Microbiologist) 0161 295 2652
Lab 209, Cockcroft Building
University of Salford
M5 4WT
 XXXXXXX @edu.salford.ac.uk

The Chief Investigator is for this study is Professor C. Pine, Honorary Consultant in Dental Public Health, Salford Royal Foundation Trust.

This study is sponsored by The University of Salford and a recognised Research Ethics committee has reviewed and approved this phase of the study.

If you have a complaint about the study please use the contact numbers   above.

Bacteria that grow on baby teeth

I have had the opportunity to ask further questions and time to reflect on the information provided.

I have read and understood the Participant Information Sheet version 1, dated 24 October 2011. I understand that this is a research study, that it is voluntary and that I am free to withdraw at any time without providing a reason.

I confirm that I have parental responsibility for the child enrolled on this form and that I am consenting to donate plaque samples for myself and my child for research purposes into how the normal bacteria on babies teeth changes as the new teeth grow and mature.

Please complete this section and return in the reply paid envelope provided:
Full name of your child

Your name

Your signature   **Today's date**

Your address

_____

And a contact telephone number

Thank you we will contact you again nearer the time

Appendix XI - Salford CT Q449 – antibacterial effects of fluoride, indemnity under 5.

Hasilwood House
60 Bishopsgate
London EC2N 4AW
Tel: 020 7847 8670
Fax: 020 7847 8689

UMAL

TO WHOM IT MAY CONCERN

17th January 2012

Our Reference: **Q449**

Dear Sir/Madam

UNIVERSITY OF SALFORD AND ALL ITS SUBSIDIARY COMPANIES

Clinical Trial Coverage

We confirm that the above Institution is a Member of U.M. Association Limited and that Clinical Trials cover is in force in respect of the Trial below subject to the cover period, terms, conditions and exceptions.

Clinical Trial: The Antimicrobial Effects of Fluoride

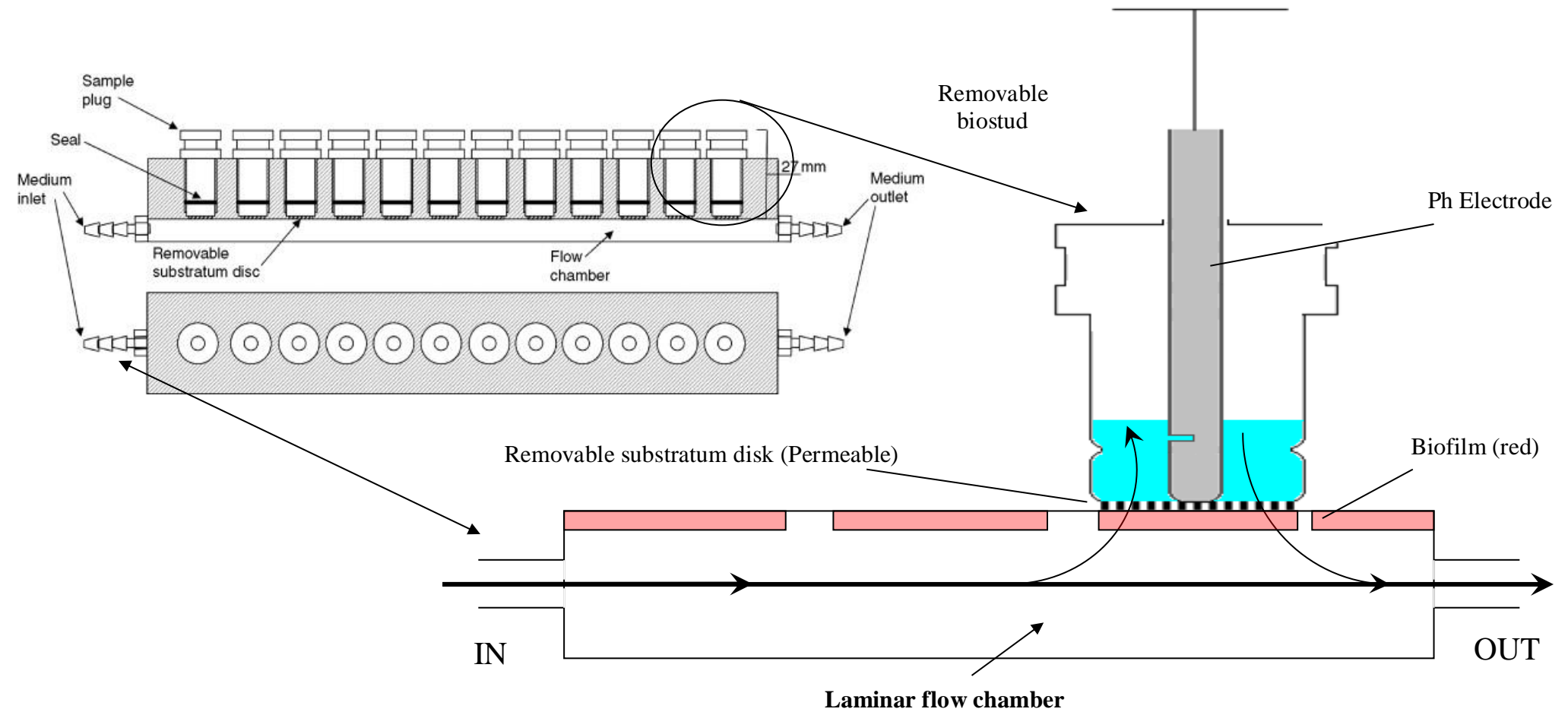|	
Certificate of Entry No.	UM047/00
Period of Indemnity	17th January 2012 to 31st July 2012
Limit of Indemnity	£10,000,000 Limit any one event and in the aggregate for any one Period of Indemnity including claims costs and expenses for all Clinical Trials undertaken by the University
Basis of Cover	Legal Liability or No Fault cover
Endorsement re Research Subjects under 5 years	Exclusion 4 to Sub-Sections 1 and 2 is inoperative (i.e. the indemnity extends to cover research involving subjects under 5 years of age)
Indemnity	To the University of Salford, its employees and its students
Cover provided by	U.M. Association Limited and Excess Cover Provider QBE Insurance (Europe) Limited

Yours faithfully

Susan Wilkinson
for U.M. Association Limited

U.M. Association Limited
Registered Office: Hasilwood House, 60 Bishopsgate, London, EC2N 4AW
Registered in England and Wales No. 2731799

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Appendix XII - The Modified Robbins Device (MRDe)

Sample plug

Seal

Medium inlet

Removable substratum disc

Flow chamber

27 mm

Medium outlet

Removable biostud

Ph Electrode

Removable substratum disk (Permeable)

Biofilm (red)

IN

OUT

Laminar flow chamber

A (Top) (McBain, 2009) shows the MRDe schematic. Image includes laminar chamber, medium inflow and outflow ports and removable sample plug (biostud). **B** (Bottom) is an enlarged schematic of the biostud, including pH electrode dock, pH electrode, removable substratum disk (Permeable) and medium in- and outflow ports. Image produced on Microsoft Office Word 2007, 02/07/12.