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Development of the cariogenic oral biofilm coincident with the evolution of immune responses in very young children

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BSc/Hons

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Abstract

Dental caries remains one of the most common chronic infectious childhood diseases and individuals remain susceptible to the disease throughout their lifetime. The disease continues to inflict a substantial economic burden. Moreover, dental caries demonstrates considerable socioeconomic disparities with the lowest socioeconomic groups suffering the greatest burden of disease. There is an unmet need to improve prevention and therapeutics and yet there remain fundamental gaps in the knowledge of the interrelationships between caries-associated risk factors, in particular how the immune system interacts with the evolving cariogenic biofilm in young children. This thesis sought to investigate the immune response to cariogenic biofilms. Three different approaches were used to achieve this. Firstly, the salivary immune response and development of the oral biofilm in very young children were investigated prior to the onset of caries, as part of a pilot longitudinal clinical study, using a dental public health program as a platform. Secondly, the initiation of adaptive immune responses to *S. mutans* exposure were investigated using a series of *In vitro* and *In vivo* studies. Thirdly, a novel *S. mutans In vitro* biofilm model was developed and optimised.

Childsmile is a dental health improvement programme for children in Scotland and provides children with specific dental health interventions depending on need, from birth and up to 16-years of age. To achieve the first and primary aim of this thesis, plaque and saliva samples were collected from children aged one-year and again at age three-years. At follow-up, dental disease scores were also measured. Additionally, the biological mechanisms underlying the socioeconomic disparities in the dental health of young children were investigated, including the measurement of salivary cortisol as a surrogate measure of stress.

Sixty-three Childsmile participants aged one-year were recruited to the study at baseline. Twenty-three children aged three-years were successfully recalled at follow-up. This work demonstrated that variables hypothesised to influence the development of carious disease can be collected and successfully quantified in children aged one- to three-years. Nonetheless, it was extremely challenging to recruit children of this age and the data were compromised by the small sample

sizes. During the study period both the intensity and incidence of *S. mutans* colonisation increased in the dental plaque of children aged one- to three-years. Coincidentally, concentrations of salivary antimicrobial proteins increased, including lactoferrin, LL37, calprotectin, the HNPs 1-3 and sIgA antibody titres specific for oral streptococci. It could not be determined from these studies whether the increased colonisation with *S. mutans* or the concentrations of salivary antimicrobial proteins influenced the prevalence of dental caries. The major limitation of this study was the low recruitment rates which resulted in low power to detect statistically significant differences. As a consequence there was insufficient evidence to identify the potential biological pathways that may underlie the socioeconomic disparities of dental caries. From this pilot study a number of valuable lessons were learned regarding the recruitment of children of this age and recommendations for future clinical studies conducted within Childsmile are made.

In children with high risk of developing dental caries effective salivary antibody responses are required to provide protection. The mechanisms leading to effective antibody responses remain unclear. Thus, the second aim of this thesis was to investigate the initiation of an adaptive immune response to *S. mutans*, in an attempt to elucidate the mechanisms that lead to effective antibody production. Using a novel system, *In vitro* evidence indicated that *S. mutans* does not elicit a robust inflammatory immune response upon colonisation of the host. Dendritic cells exposed to *S. mutans* were not functionally mature and failed to induce antigen-specific T cell proliferation. Furthermore, *In vivo*, dendritic cells failed to become activated in response to oral exposure to *S. mutans*.

An *In vitro S. mutans* sucrose-dependent biofilm model was developed and optimised. Using this model an antibody fragment known as a minibody, denoted 'SS2' was demonstrated to inhibit *S. mutans* biofilm formation. This biofilm model represents an important first step for examining the potential of therapeutic molecules to inhibit *S. mutans* biofilm formation, prior to their application in *In vivo* models of dental caries and possible subsequent use in human clinical trials.

Data described here indicate that S. *mutans* colonises the oral cavity at a time when children are immunologically immature. Increased colonisation by S. *mutans* coincides with the maturation of salivary immune responses. Moreover, *In vitro* and *In vivo* evidence suggest that S. *mutans* does not elicit a robust immune response upon colonisation of the host. Thus, early acquisition of S. *mutans* in a relatively immunologically immature host together with the absence of an inflammatory immune response likely aids the colonisation of S. *mutans* and its persistence within the oral biofilm and subsequent contribution to dental caries.

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Abbreviations

ANOVA	Analysis of variance
AMPs	Antimicrobial peptides
APC	Alcophycocyanin
BASCD	British association for the study of community dentistry
BIO	Biotin
BMDCs	Bone marrow derived dendritic cells
CD-	Cluster of differentiation
CFU	Colony forming units
DC	Dendritic cells
DLN	Draining lymph node
dmft/DMFT	Decayed, missing and filled teeth of the primary or permanent dentition, respectively
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
EU	ELISA units
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Gbp	Glucan-binding protein
Gtf	Glucosyltransferase
HK	Heat-killed
HNPs	Human neutrophil peptides
IFN-γ	Interferon-gamma
LB	Lactobacillus
MFI	Mean fluorescence intensity
MHCII	Major histocompatability complex II
MS	Mutans streptococci
NDIP	National dental inspection programme
NMES	Non-milk extrinsic sugars
OD	Optical density
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PE	Phycoerythrin
PRRs	Pattern recognition receptors
QPCR	Quantitative polymerase chain reaction
sIgA	salivary IgA antibodies
ScFv	Single-chain variable fragment
(S)ECC	Severe early childhood caries
SEM	Standard error of the mean
SIMD	Scottish index of multiple deprivation
SES	Socioeconomic status
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TLRs	Toll-like receptors
WHO	World health organisation

Chapter 1 General Introduction

1.1 Epidemiology of dental caries

Dental caries remains one of the most common chronic infectious childhood diseases and individuals remain susceptible to the disease throughout their lifetime (Edelstein and Chinn 2009; Marthaler 2004). The National Institute of Dental and Craniofacial Research (NIDCR) report that 42% of children aged two- to eleven-years have dental decay in their primary dentition and by adulthood 92% have some experience of decay in their permanent dentition (NHANES, 1994-2004). Oral health is a substantial component of general health and well being and as such dental caries has a significant impact on an individual's quality of life. Dental caries is a preventable and reversible infection that if left untreated results in severe pain, bacteraemia and subsequent tooth loss. Moreover, dental caries is associated with a substantial economic burden (Casamassimo et al. 2009). Dental caries of the primary dentition can have additional complications for young children, including impairment of growth and development due to decreased nutritional intake associated with the pain of tooth decay and speech disorders arising due to missing teeth can lead to subsequent development of psychological disorders, such as poor self esteem. The removal of teeth in young children can also pose additional risks associated with conscious sedation and general anaesthesia. In 2009/10, nearly 8000 Scottish children were admitted to hospital to undergo general anaesthesia for tooth extraction related to dental caries ((NHS:ISD) 2012).

There have been no meaningful improvements in the prevalence of dental caries among young children since the late 1980s. Improvements made up to this time are presumed to be related to the increased use of fluoride toothpaste (Pitts et al. 2005). However, with significant numbers of children still experiencing caries there is a need for more direct and innovative methods of delivering preventative care if significant improvements are to be made.

1.1.1 Aetiology

The aetiological factors of dental caries are the presence of cariogenic bacteria together with a diet containing fermentable carbohydrates. Over time this combination can lead to the development of carious lesions (Figure 1-1). Dental

caries is characterised by the localised demineralisation of tooth surfaces and is associated with the indigenous oral flora. Dental caries arises when the balance of the indigenous oral flora shifts to support the growth of potentially pathogenic microorganisms with the ability to ferment carbohydrates.

A diet high in carbohydrates, namely sucrose, supports the growth of acidogenic species that are capable of fermenting carbohydrates to produce acids, particularly lactic acid (Taubman and Nash 2006). Following sugar intake, acid metabolism by oral cariogenic species can lead to the demineralisation of tooth surfaces. Demineralisation is a process by which hydrogen ions dissolve the carbonated hydroxyapatite crystal lattice of enamel and subsequently dentin and eventually into the pulp. Demineralisation can be reversed in its early stages; however, continued demineralisation can lead to the development of cavitated lesions in the tooth surface (Featherstone 2008). Under healthy circumstances this process is balanced by remineralisation of tooth surfaces, a process by which calcium and phosphate ions dissolved in saliva diffuse back into the porous tooth surface. The cycle of demineralisation/remineralisation occurs following meals containing fermentable carbohydrates. Whether a lesion progresses, stops or reverses is determined by the balance of these two processes. Thus, a diet high in fermentable carbohydrates increases the acid metabolism of acidogenic bacteria, lowering the pH of the oral cavity and advancing the demineralisation process.

1.1.2 Risk factors

A wealth of epidemiological studies have attempted to identify biological and socioeconomic risk factors for dental caries in young children, some of which are reviewed in Table 1-1. From these studies and others, commonly risk factors for dental caries development in young children include the age of children, psychological stress, oral hygiene behaviours, previous caries experience and low socioeconomic status. Additional risk factors, not cited in the table include the misuse of nursing bottle (Robke 2008), immigrant status (Wendt et al. 1994) and diabetes (Siudikiene et al. 2008). Fewer epidemiological studies have attempted to investigate the relationship of dental caries with immunological factors that

may naturally protect against caries initiation in very young children (Naspitz et al. 1999; Parisotto et al. 2011; Parisotto et al. 2010a).

1.1.3 Socioeconomic related gradient of dental caries prevalence

Dental caries demonstrates a socioeconomic status (SES) related gradient of distribution, with those from the lowest socioeconomic groups having the highest prevalence of the disease (Table 1-1) (Radford et al. 2000; Radford et al. 2001; Shaw et al. 2009; Sisson 2007). This suggests that the position of individuals on the social gradient is an important determinant for oral health. This relationship is already apparent in children as young as three-years-old with caries prevalence increasing from 16% in the least deprived to 32% in the most deprived groups in Greater Glasgow (McMahon et al. 2010). Together with the finding that childhood dental health is predictive of adult dental health (Thomson et al. 2004) this indicates a real need to develop existing methodologies in order to target the youngest age groups if significant improvements in dental health are to be made across the socioeconomic spectrum.

1.1.3.1 Stress

There is evidence that the relationship between low SES and caries prevalence may be influenced by stress (Quinonez et al. 2001; Reisine and Litt 1993). Activation of the hypothalamic-pituitary (HPA) axis stimulates the release of neuroendocrine hormones, including noradrenaline, adrenaline and cortisol (Raison and Miller 2003). The ‘stress response’ is activated in response to physical stressors, such as infection and injury, but also in response to psychological stress, such as social stressors related to life experiences. Under stress activating conditions the immune system and neuroendocrine system communicate continuously via neurotransmitters. Thus, in chronically stressed individuals their ability to mount an adequate immune response may be diminished (Dragos and Tanasescu 2010). Over time this has high demands on the body and can lead to tissue pathology and subsequently to disease (Sabbah et al. 2007). It is now widely recognised that prolonged psychological stress can increase host susceptibility to inflammatory and infectious diseases (Bosch et al. 2002; Kiecolt-Glaser et al. 2002).

Cortisol is a major endogenous anti-inflammatory mediator and is involved in regulation of innate immune responses to bacterial and viral infection (Raison et al. 2006; Sternberg 2006; Webster Marketon and Glaser 2008). Measurement of salivary cortisol is a simple, reliable, non-invasive technique that can be used to provide information regarding HPA-axis activity under normal and stressful conditions. It can, therefore, potentially be used as a surrogate measure of stress to assess how social inequalities can impact upon the immune response. Furthermore, there is evidence to suggest that salivary cortisol levels are associated with increased carriage of cariogenic bacteria and a higher prevalence of dental caries (Table 1-1) (Boyce et al. 2010).

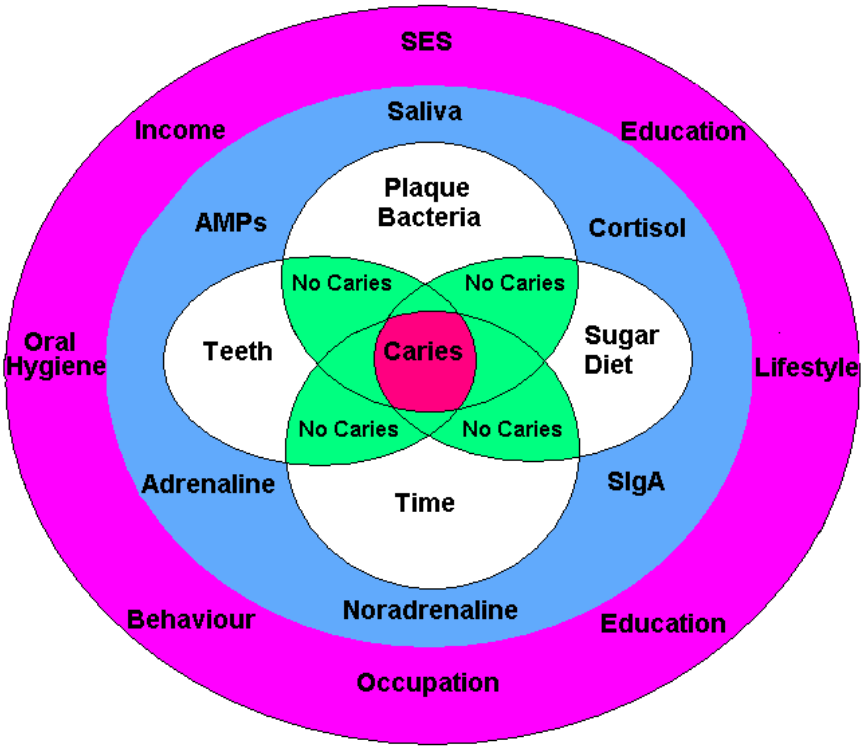


Figure 1-1: Complex, multi-factorial aetiology of dental caries

Diagrammatic representation of the complex and multi-factorial aetiology of dental caries. The presence of specific microorganisms in conjunction with a cariogenic diet, the nature of the host immune response together with lifestyle and behavioural factors can all culminate in the development of dental caries.

Table 1-1: Summary table of studies exploring the biological and socioeconomic risk factors for dental caries in young adults and children

Study Design	*n* (age)	Clinical Examinations	Findings	Reference
Longitudinal study to assess caries risk in very young children of low SES	*n* = 128, (six- to 24-months-old and their primary caregivers)	Caries examinations and MS levels were recorded at baseline, 9- and 18-month intervals from children and their caregivers. Dietary and sociodemographic data were collected by questionnaire.	Caries prevalence and MS counts increased over the study period ($p < 0.05$). The presence of visible plaque ($p = 0.015$), MS ($p < 0.001$), consumption of sweetened beverages ($p = 0.001$) and age of children ($p = 0.006$) at baseline were associated with caries at follow-up. Sociodemographic variable were not found to be associated with caries.	(Warren et al. 2009)
Longitudinal study to determine the potential associations between the level of social deprivation/affluence and the frequency isolation of caries-associated microflora in a large cohort of children examined annually.	*n* = 1974 (one- to four-years-old).	DEPCAT was used to measure SES. MS, LB and yeasts were enumerated annually from saliva of children aged one-, two-, three- and four-years. Caries was diagnosed at d_1 and d_3 threshold (enamel and dentine) annually.	Caries prevalence increased with age and social deprivation. Social deprivation and LB were correlated, independently of caries status, in children aged three- and four-years ($p < 0.017$ and $p < 0.0044$, respectively). Correlations between MS and DEPCAT were found in children aged two with enamel lesions ($p = 0.011$) and at age-three with dentine lesions ($p = 0.0034$).	(Radford et al. 2000; Radford et al. 2001)
Longitudinal study to assess the value of employing a multidimensional model to evaluate caries development.	*n* = 184 (four- to five-years-old)	MS levels and dmfs recorded at both ages. Psychosocial, cognitive and behavioural variables were evaluated by interviewing primary caretakers.	Caries development at one-year follow up was strongly dependant on earlier caries experience ($p = <0.05$). Life stresses were found to be predictive of dmfs at baseline ($p = <0.05$).	(Litt et al. 1995)
Retrospective analysis to investigate the significance of social, environmental and biological variables in relation to caries status in young children.	*n* = 89 (10- to 71-months-old)	Data regarding fluoride status and sociodemographic variables were collected from dental records of children previously examined for salivary MS levels.	Multivariate analysis revealed that detectable caries was associated with higher MS levels, a single parent household, lack of fluoride in drinking water and not being covered by dental insurance.	Grindefjord 1995, 1996, 1991

Study Design	*n* (age)	Clinical Examinations	Findings	Reference
Longitudinal study to investigate the immunological and microbiological changes during caries development in young children	*n* = 40 (three-to five-years-old) Children were selected from a larger study (n = 188) based on disease status. Children who developed 3 or more lesions in the one-year follow-up (caries-active group [CA, n = 17]) and caries-free group (CF, n = 23).	Total sIgA and IgA specific for S. mutansvirulence epitopes were measured by Luminex assay. MS, LB and total plaque bacteria were enumerated.	No differences in baseline MS or LB between CA and CF groups ($p > 0.05$). Both MS and LB were higher in CA group at follow-up compared with baseline CA group ($p < 0.05$). LB higher in CA group compared with CF group at follow-up ($p < 0.05$). Total and specific sIgA increased over time in both CA and CF groups ($p < 0.05$). Lower baseline levels of anti-GbpB associated with higher caries at follow-up (OR 7.5).	(Parisotto et al. 2011)
Cross-sectional study to investigate the relationship between dental caries experience, SES, eating behaviours, oral hygiene, dental plaque accumulation and MS levels in children from two areas of Ulaanbaatar city, Mongolia.	*n* = 670 (one- to five-years-old)	MS levels and mean dmft were recorded for each child and their mothers. Psychosocial, SES and behavioural variables were measured by questionnaire	Caries prevalence and mean dmft were high in both areas. Higher family income and education level of mothers was significantly associated with higher caries prevalence.	(Jigjid et al. 2009)
Cross-sectional study to investigate the relationship between the secretory immune response and dental caries	*n* = 49 (three- to five-years-old) Children were grouped according to disease status: caries-free (I, n = 20), one – two decayed surfaces (II, n = 15), rampant caries (III, n = 14)	Caries prevalence was recorded using dmfs. MS was enumerated from stimulated saliva. Total sIgA, anti-*S. mutans* IgA, IgM and IgG were measured in unstimulated saliva by ELISA	MS was lower in group I, compared with groups II and III ($p < 0.005$). No differences in total IgA or anti-*S. mutans* IgA, IgM or IgG between groups.	(Naspitz et al. 1999)
Cross-sectional study to investigate how specific maternal health beliefs, behaviours, and psychosocial factors relate to young children's ECC status in a low-income African-American population.	*n* = 1021 (children aged-six-years and their primary caretakers)	ECC status of child based on the International Caries Detection and Assessment Criteria (ICDAS). Caretakers undertook interviews and completed questionnaires to provide info regarding their oral health beliefs, behaviour and psychosocial risk factors.	One third of children had ECC of which 20% had S-ECC. Higher levels of parenting stress were significantly and inversely related with ECC incidence ($p = <0.05$) Higher parental education and income were found to be protective.	(Finlayson TL 2007)

Study Design	*n* (age)	Clinical Examinations	Findings	Reference
Cross-sectional study to assess the relationship between socioeconomic factors, behaviours and the severity of ECC in Thai children.	*n* = 520 (six- to 19-months-old)	Demographic, SES and behavioural data was collected by questionnaire. Caries prevalence of mother-child pairs was assessed using the dmft/DMFT index and MS levels in saliva were assessed.	Children from low-income families, those with low education and mother's and care givers with decayed teeth had higher ECC scores ($p = <0.05$). Breast fed children and those with high MS counts also had higher ECC scores ($p = <0.05$).	(Vachirarojpisan et al. 2004)
Cross-sectional study to evaluate the prevalence of caries and associated risk factors in outpatients of the Pediatric Ambulatory Pedro Ernesto University Hospital	*n* = 80 (zero- to 36-months-old)	SES, behavioural and dietary data were collected from parents by questionnaire. Dental examination was assessed by a single investigator and included assessment of caries, dental biofilm and gingival bleeding.	Prevalence of caries was 41.6% and the mean dmfs was 1.7 (± 2.5). No significant associations were found between the prevalence of caries and socioeconomic status.	(Santos and Soviero 2002)
Cross-sectional analysis to investigate the relationship between stress-related psychobiological factors and high prevalence f dental caries among children of low SES in the Francisco Bay are of California.	*n* = 94 (five- to six-years)	SES was estimated using mothers education levels. Financial stressors were assessed by questionnaire. Basal salivary cortisol was measured and salivary MS and LB were enumerated.	SES was inversely associated with basal salivary cortisol ($p < 0.05$). High MS and LB were associated with dental caries ($p < 0.001$). Low SES, high concentrations of basal salivary cortisol and high level of MS and LB were predictive of caries lesions in a multiple logistic model ($p < 0.001$). Children with the highest number of lesions had the highest combinations of basal cortisol concentrations and high MS and LB counts.	(Boyce et al. 2010)
Cross-sectional analysis to estimate the prevalence and prediction factors for dental caries in children in Rome.	*n* = 1494 (three- to five-years-old)	DMFT index was used to assess caries experience. Behavioural and socioeconomic variables were collected from parents by questionnaire. MS counts, diet and nutritional status were investigated for their association with rampant ECC.	Caries prevalence was 27.3%. Low social class ($p = 0.03$) and high salivary MS counts ($p = <0.1$) were significantly associated with rampant ECC.	Petti 2000

Study Design	*n* (age)	Clinical Examinations	Findings	Reference
Cross-sectional analysis to investigate the caries-related microflora in saliva of Turkish children with the prevalence of ECC and associations with mother's SES and feeding habits.	*n* = 101 (15- to 35-months-old)	Saliva samples were assessed for the presence of LB, MS and Candida albicans. DMFS criteria were used to assess dental status of mother and child pairs. Questionnaires were used to collect SES and dietary data from mothers of participants.	Data indicated that mother's DMFS scores, low level of education and poor feeding habits were strong risk factors for colonisation with caries-related microflora and ECC.	(Ersin et al. 2006)
Cross-sectional analysis to examine the relationship among microbiological composition of dental plaque, sugar exposure and social factors in young children with different stages of caries.	*n* = 169 (three- to four-years-old) Children were divided into three groups based on disease status: caries-free (n = 53), early caries lesions (ECL, n = 56), cavitated caries lesions (CCL, n = 60).	Caries measurements included dmft and ECL. Visible plaque was recorded and collected for enumeration of MS, LB and total bacteria. Sugar consumption was collected by questionnaire.	High MS, total sugar consumption and presence of visible plaque were significantly associated with ECL ($p < 0.05$). High total plaque bacteria, frequency of sugar intake and high LB were significantly associated with CCL ($p < 0.05$).	(Parisotto et al. 2010a)

MS: Mutans streptococci, LB: Lactobacillus spp., DEPCAT: area-based measure of deprivation category , dmft/s or DMFT/S: decayed, missing, filled teeth/surfaces in primary or permanent teeth, repectively , (S)ECC: (severe) early childhood caries, sIgA: salivary IgA antibodies

1.2 Development of the oral biofilm from birth to three-years

Dental plaque is an oral biofilm comprised of a diverse and complex microbial community. Traditional culture techniques have estimated that around 700 bacterial types exist in the human oral microbiome. Recent advances in molecular sequencing methods put this figure at closer to 25,000 phylotypes in the global oral human microbiome (Belda-Ferre et al. 2011). At the moment of birth the oral cavity of the newborn is a sterile environment, however, it immediately begins to become colonised by pioneer microbial species, which are quick to take advantage of this new environment (Pearce et al. 1995; Rotimi and Duerden 1981). These pioneer organisms attach to the mucosa of the oral cavity of the newborn and modify the habitat, creating an environment that supports the growth of new microbial species (Liljemark and Bloomquist 1996). *Streptococcus salivarius*, *S. mitis* biovar 1 and *S. oralis* are dominant pioneer organisms of the oral mucosa (Kononen et al. 2002). The microorganisms which colonise the oral cavity of newborns are thought to be largely derived from the primary caregiver, usually the mother (Berkowitz 2006; Liljemark and Bloomquist 1996; Smith et al. 1993; Smith and Taubman 1992). *S. anginosus*, *S. gordonii* and *S. mitis* biovar 2 are also present in the oral cavity at this time, but at lower levels than the aforementioned species (Lucas et al. 2000; Pearce et al. 1995).

The microbial species that colonise the oral cavity at this time persist and become members of the indigenous microbiota and influence the colonisation of subsequent populations through their metabolic activities (Kononen 2000). This process is influenced by environmental factors and the host immune system. The oral biofilm continues to evolve and with the emergence of teeth, new microbial habitats are provided with unique characteristics and the oral biofilm continues to increase in diversity and complexity (Hardie and Bowden 1975; Taubman and Nash 2006).

Teeth provide unique non-shedding attachment sites for *S. sanguinis* and later Mutans streptococci in addition to numerous other species, including anaerobic

species belonging to the genus *Prevotella*, *Veillonella and Neisseria* and aerobic species, such as *Actinomyces spp*. (Caufield et al. 2000; Liljemark and Bloomquist 1996). Anaerobic species are able to survive in the oral cavity through their physical interactions with aerobic and facultative anaerobic species that metabolise the available oxygen (Kolenbrander 2000). The anaerobic organism *Fusobacterium nucleatum* is also an early coloniser (<6 months). This organism is important for the maturation of oral biofilms. Its ability to co-aggregate with numerous species of the oral microbiota enables this organism to bridge the gap between colonisation of early and late colonisers (Kolenbrander 2000; Kononen 2005).

This population succession (Table 1-2) continues until all available niches become colonised and the oral biofilm ecology becomes relatively stable and is maintained in a state of homeostasis (Marcotte and Lavoie 1998). Disruption to this balance can lead to disease states, such as dental caries.

Table 1-2: Timing of oral colonisation by commensal bacteria

Time of colonisation (age)	Aerobic/facultative aerobic spp	Obligatory anaerobic spp
1-2 days	*S. salivarius*	-
0-3 months (predentate)	Neisseria spp *S. mitis biovar I and II* *S. oralis* *Actinomyces odontolyticus* *S. parasanguinis*	Veillonella spp *Prevotella melaninogenica* *Fusobacterium nucleatum*
6-12 months (dentate)	*S. sanguinis* *S. gordonii* *S. anginosus* Staphylococcus spp	Corroding rods
1-3 years	*S. mutans* *S. sobrinus* Lactobacillus spp	Spirochetes Peptostreptococcus spp

1.2.1 Mutans Streptococci and dental caries

There is substantial evidence indicating a causative relationship between dental caries and the Mutans streptococci, with many studies demonstrating that the development of caries is preceded by increased colonisation with the Mutans streptococci (Hamada and Slade 1980; Loesche 1986; Tanzer et al. 2001). *S. mutans* (serotypes c, e and f) and *S. sobrinus* (serotypes d and g) are implicated as the primary aetiological agents associated with the initiation and progression of dental caries (Kristoffersson et al. 1985). These strains are facultatively anaerobic, non-motile, Gram positive cocci (van Houte 1994). Mutans streptococci are routinely isolated from the mouths of children and adults, suggesting that dental caries is the most widespread infectious disease of humans (Taubman and Nash 2006). Other oral bacterial species can also produce acids and thus be cariogenic (Carlsson et al. 1975), although the Mutans streptococci possess unique biochemical features that render them extremely efficient at developing carious surfaces. These include the ability to rapidly produce copious amounts of lactic acid, while at the same time tolerating extremes of sugar concentration, ionic strength and pH (Hamada and Slade 1980).

S. mutans has been associated with the initiation of carious lesions on tooth surfaces, while *S. sobrinus* is thought to enhance the progression of lesions (Law et al. 2007; Marchant et al. 2001). *S. mutans* are commonly recovered in far greater numbers from dental plaque than *S. sobrinus* (Kishi et al. 2009; Yano et al. 2002) and caries prevalence is commonly higher in children who are colonised by both *S. mutans* and *S. sobrinus* compared with children from whom only *S. mutans* or *S. sobrinus* can be recovered (Babaahmady et al. 1998; Hirose et al. 1993). *S. mutans* has been isolated from 95% of children with dental caries and was found to comprise up to 30-50% of the plaque microbiota in carious lesions (Berkowitz et al. 1984). In contrast, only 1% of the oral microbiota was found to comprise *S. mutans* in caries free children (Law et al. 2007). Further evidence implicating the Mutans streptococci in the development of dental caries has come from animal studies, in which the development of caries was attributable to the presence of *S. mutans* (Hamada et al. 1978; Smith and Taubman 1996).

1.2.1.1 Acquisition of Mutans streptococci

It is generally agreed that children acquire *S. mutans* from their mother or primary caregiver and several studies have provided evidence in support of this (Berkowitz 2006; Berkowitz and Jones 1985). Initial colonisation by *S. mutans* in the oral cavity of children has been reported to occur anytime between the ages of seven-months to three-years or older (Law et al. 2007). One study reported that initial colonisation by *S. mutans* occurs during a "window of infectivity" at a mean age of 26 months (Caufield et al. 1993). This finding has been reported by others (Alaluusua and Renkonen 1983; Carlsson et al. 1975) and coincides with the eruption of the primary dentition, again reported to be required for colonisation by *S. mutans* (Caufield et al. 2000). However, there have also been reports of mucosal colonisation by *S. mutans* in predentate infants (Law et al. 2007; Wan et al. 2001, 2003). This finding is clinically significant as there is evidence to suggest that caries risk increases with earlier acquisition of *S. mutans* (Law et al. 2007; Nogueira et al. 2005). Moreover, *S. mutans* colonisation has been shown to increase with increased age of children (Alaluusua 1983; Fujiwara et al. 1991). Thus, colonisation with the Mutans streptococci is a critical event in the pathogenesis of dental caries and represents important targets for therapeutic interventions.

1.2.1.2 Mutans streptococcal virulence factors and dental caries

As the primary agent of caries *S. mutans* has developed multiple mechanisms to colonise tooth surfaces and become numerically significant in the dental biofilm (Figure 1-2). Initially, *S. mutans* must attach to the tooth surfaces. The salivary dental pellicle is a thin layer of salivary proteins, lipids and glycoproteins which coats tooth surfaces and is the first step in plaque formation (Taubman and Nash 2006). Attachment of *S. mutans* to the salivary pellicle is mediated via an adhesin known as antigen I/II, and *S. sobrinus* via Spa A and represents an important first step in colonisation of the tooth surface by the Mutans streptococci (Hajishengallis et al. 1992; Jenkinson and Lamont 1997). Next, *S. mutans* accumulation occurs. This process is dependent on the presence of sucrose together with expression of glucosyltransferases (Gtfs) and glucan binding proteins (Gbps). *S. mutans* produces at least 3 known Gtfs encoded by the genes *gtfB, gtfC* and *gtfD*. These enzymes synthesise extracellular glucan

polymers from directly from sucrose. GtfB synthesises water-insoluble glucans, while GtfC synthesises a mixture of water-soluble and water-insoluble glucans and GtfD synthesises a water-soluble glucan (Wen et al. 2010). Each of these glucans are structurally distinct and thus, contribute distinct roles in the formation of dental plaque (Bowen and Koo 2011). As a result *S. mutans* synthesises large quantities of insoluble-glucans, conversely *S. sobrinus* synthesises primarily water-soluble glucans. Both *S. mutans* and *S. sobrinus* express an array of Gbps. These receptor-like proteins are distinct from Gtfs and specifically bind to glucans. Gtfs also have glucan-binding domains and so can also function as receptors for extracellular glucans. Binding of extracellular glucans by Gtfs and Gbps facilitates adhesion of Mutans streptococci to tooth surfaces and constitutes the sucrose dependent pathway which is of critical importance in plaque formation and development of dental caries (Banas and Vickerman 2003). Thus, through the synthesis of polymeric glucans together with expression of Gtfs and Gbps the Mutans streptococci produce an extracellular matrix that provides a protective environment in which the Mutans streptococci and other oral bacterial species are shielded from the host immune response, mechanical stresses and antimicrobial agents (Shemesh et al. 2010). In the final stage, large quantities of lactic acid are produced by *S. mutans* biofilms in the presence of sucrose as a metabolic end-product of anaerobic respiration (Banas 2004). Accumulation of lactic acid leads to demineralisation of tooth surfaces and eventually leads to dental caries.

A substantial body of literature supports the importance of the contribution of Gtfs and Gbps to caries development. Disruption of the genes encoding Gtfs by mutagenesis reduced the amount of glucans produced and the efficiency of sucrose-dependent adhesion to tooth surfaces and reduced cariogenicity in animal models (Tanzer et al. 1974).

Another important virulence property of the Mutans streptococci is their acidogenicity and acidurance, allowing cariogenic bacteria to tolerate the low pH environment generated from the production of acids in the oral cavity, while non-aciduric species cannot survive (Marcotte and Lavoie 1998). This ability is derived from the presence of ATPase proton pumps which actively transport hydrogen ions from the cytoplasm (Dashper and Reynolds 1992). *S. sobrinus* has been shown to be more acidogenic than *S. mutans* (Kohler et al. 1995).

However, *S. sobrinus* is unable to metabolise N-acetylglucosamine, a component of peptidoglycan which consequently inhibits the ability of *S. sobrinus* to proliferate except in circumstances of extreme acidity and in the presence of high sucrose concentrations (Homer et al. 1993). This is believed to account for the finding that *S. sobrinus* is recovered only from a minority of individuals and is usually found in conjunction with and outnumbered by *S. mutans*. Overall, a high-sucrose diet alters the microbial ecology to support the growth of acid-producing and acid-tolerant species associated with dental caries, such as Mutans streptococci and Lactobacilli spp (Beighton 2005). Thus, the dental biofilm associated with caries is differentially distinct from the microbial ecology of the healthy oral biofilm (Aas et al. 2008).

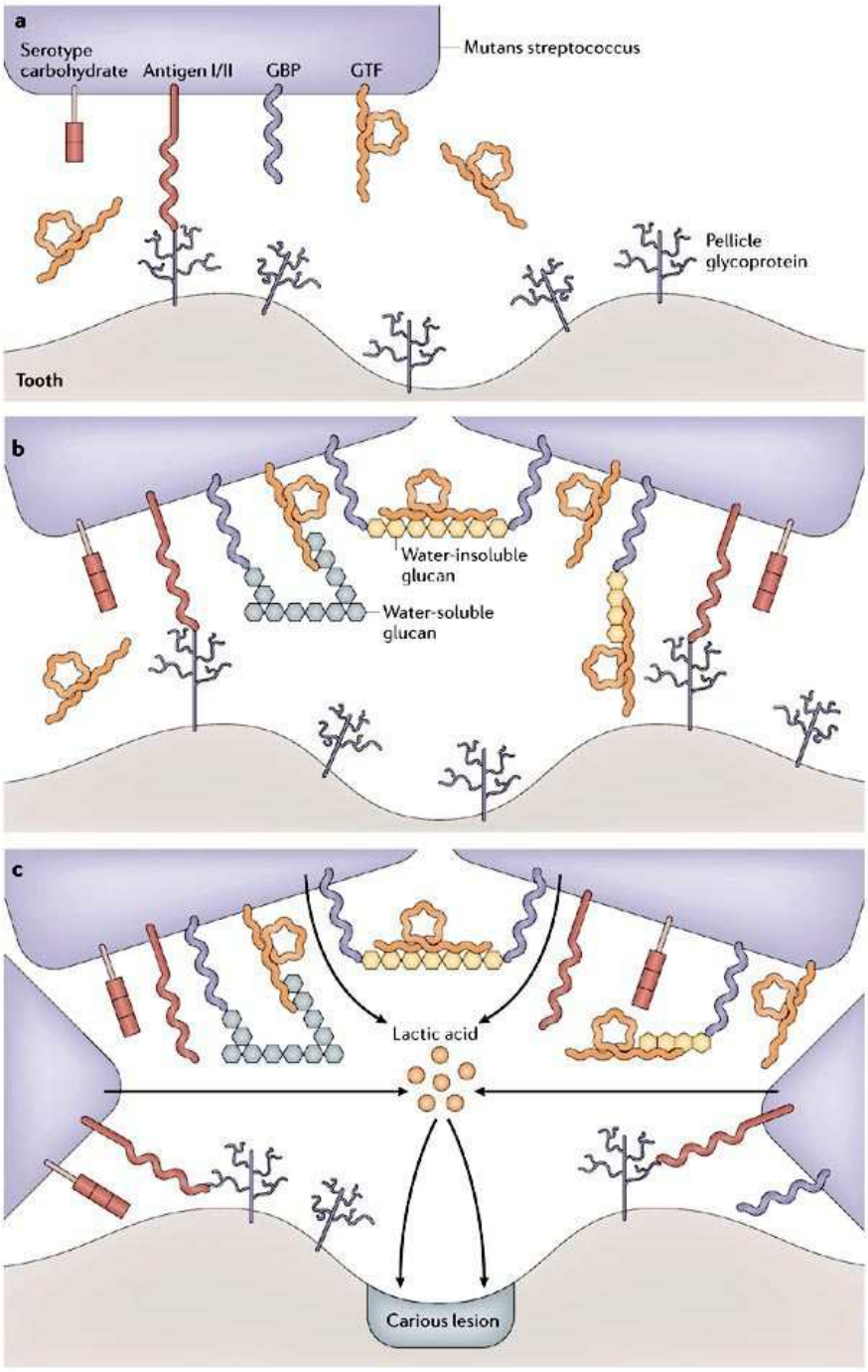


Figure 1-2: Mutans streptococci pathogenesis of dental caries

Schematic representation of the sequential formation of *S. mutans* biofilms on tooth surfaces. (a) Initial adherence and accumulation are mediated by surface antigen I/II and expression of Gtfs and Gbps. (b) subsequent production of water soluble and insoluble glucans. (c) In the presence of sucrose, significant accumulations of *S. mutans* produce large quantities of lactic acid, culminating in the demineralisation of tooth surfaces and development of dental caries. Image courtesy of Dr Martin Taubman.

1.2.2 The role of other bacterial species in dental caries

Despite extensive evidence in support of the role of Mutans streptococci as the primary agents of dental caries, a number of studies have documented caries in the absence of the Mutans streptococci (Loesche and Straffon 1979), and indeed Mutans streptococci can be detected in the absence of caries (Belda-Ferre et al. 2011). A variety of bacterial species can produce acids from carbohydrate fermentation in the oral cavity, including non-mutans streptococci such as *S. gordonii*, *S. mitis, S. oralis* and *S. anginosus*. These bacterial species outnumber Mutans streptococci and thus could contribute to caries initiation (van Houte et al. 1996). A study utilising 16s DNA checkerboard hybridisation to quantify numbers of oral bacteria associated with health and disease in children identified a strong relationship with *S. mutans* and caries but additionally identified numerous other bacterial species thought to be important in caries initiation and progression, including *Actinomyces gerensceriae*, Veillonella spp, *S. salivarius, S. constellus, S. parasanguinis* and *Lactobacillus fermentum*. A novel species of Bifidobacterium was also associated with deep caries lesions (Becker et al. 2002). These studies highlight the complexity of the oral microbial ecology associated with dental caries initiation and progression.

In recent years, the advancement in metagenomics and next generation sequencing techniques has allowed investigators to apply a holistic approach to oral microbial ecology in health and disease. These studies provide evidence in support of a polymicrobial aetiology of dental caries (Alcaraz et al. 2012; Belda-Ferre et al. 2011).

1.3 Development of the salivary immune response from birth to three-years

Development of the oral biofilm is influenced by a variety of factors, including environmental and behavioural factors such as oral hygiene, and the host immune response to infection. It therefore follows that caries susceptibility can be influenced by host immune factors, particularly those found in saliva. The protective role of saliva in the maintenance of oral health is well established (Dowd 1999; Tabak 2006). Saliva contains a complex mixture of proteins, enzymes and innate and adaptive immune mediators all of which have a significant impact on the colonisation of microorganisms in the oral cavity. In excess of 70 salivary components have been described (Mogi et al. 1986a; Mogi et al. 1986b). These components regulate microbial colonisation by a number of mechanisms, including binding and promoting aggregation to enhance bacterial clearance, inhibiting surface mediated binding to host surfaces, inhibiting microbial growth and mediating direct bacterial killing (Table 1-3). Additionally, salivary flow rate and buffering capacity facilitate oral clearance, neutralisation of acid production and maintain tooth integrity (Tenovuo 1997). However, salivary proteins can also serve to provide attachment sites for bacterial adhesion, such as formation of the salivary pellicle and can provide nutritional substrates for microorganisms (Scannapieco 1994).

1.3.1 Non-specific immunity

Saliva contains numerous non-specific, protective factors such as lysozyme, lactoferrin, histatins, mucins and peroxidases (Table 1-3), all of which are present and functional in saliva from birth, although their concentrations are increased in adult saliva compared with saliva from infants (Hyyppa et al. 1989). Additionally, antimicrobial peptides provide non-specific innate immune defence. Antimicrobial peptides have received a great deal of attention in recent decades for their protective functions at mucosal surfaces and their potential for development of future therapeutic strategies. Antimicrobial peptides are natural antibiotics with broad-spectrum antimicrobial properties against Gram-positive and Gram-negative bacteria, yeasts and viruses. Moreover, antimicrobial peptides have chemotactic and immunomodulatory functions,

which function to bridge innate and adaptive immune responses at mucosal surfaces (Dale and Fredericks 2005).

In saliva, several families of cationic antimicrobial peptides can be detected. These include the ß-defensins (HßD 1-3), the α-defensins (also known as human neutrophil peptides [HNPs 1-4]), the cathelicidin LL37 and calprotectin (also known as calgranulin). Sources of these peptides in the oral cavity include constitutive and inducible expression by the oral epithelium and salivary glands, and infiltration of leukocytes via the gingival crevicular crevice, such as neutrophils (Dale and Fredericks 2005). Thus, the oral epithelium plays an active role in orchestrating innate immune defences in the oral cavity. Furthermore, antimicrobial peptides have synergistic and additive functions, enhancing the antimicrobial activities of each other and other salivary proteins, such as lactoferrin, lysozyme and sIgA antibodies. Thus, antimicrobial peptides together with other non-specific and specific immune mediators in saliva provide an effective antimicrobial barrier at mucosal surfaces.

1.3.2 Antimicrobial proteins and their role in dental caries

1.3.2.1 Lactoferrin

Lactoferrin is an iron-binding protein found in exocrine secretions including saliva, tears and breast milk. Its non-specific antibacterial action is mediated primarily through its iron-sequestering properties, making iron unavailable for bacterial growth. In addition, apo-lactoferrin (iron-free lactoferrin) has been demonstrated to have direct antibacterial action (Weinberg 2003). Sources of lactoferrin in the oral cavity include expression and secretion by acinar epithelial cells and lactoferrin is a component of secondary granules of neutrophils (Kalmar and Arnold 1988). In young children lactoferrin can be detected in saliva, although at concentrations significantly lower than are generally detected in adult saliva (Hyyppa et al. 1989). Lactoferrin has been shown to have direct antimicrobial activity against *S. mutans* and it is functional even in low pH environments, such as those created by *S. mutans* in the presence of fermentable carbohydrates (Arnold et al. 1981; Berlutti et al. 2004).

1.3.2.2 LL37

The human cathelicidin, LL37 is the only cathelicidin identified in humans. It is proteolytically cleaved from the holoprotein (hCAP18) to generate the mature peptide, with biological activity. It is expressed by epithelial cells of the oral cavity, skin and other mucosal surfaces and is also present in the granules of neutrophils and other immune cells, including B cells, monocytes and mast cells (Zanetti 2004). The antimicrobial activity of LL37 is mediated through its amphipathic structure, allowing it to insert into and permeabilise bacterial cell membranes (Henzler Wildman et al. 2003). The protective role of LL37 at mucosal surfaces is highlighted by diseases in which LL37 is absent, such as Morbus Kostmann syndrome in which LL37 is deficient in neutrophil granules and individuals develop early onset chronic periodontal disease (Putsep et al. 2002).

Highly variable concentrations of LL37 have been detected in saliva of adolescents; however, less information is available of the levels of LL37 in saliva of young children (Tao et al. 2005). One study indicated that the concentration of LL37 in saliva increased with increased age of children and that low concentrations of LL37 were associated with dental caries (Davidopoulou et al. 2012). In a separate study, *S. mutans* strains isolated from adolescents with caries were more resistant to the antimicrobial effect of LL37 compared with strains isolated from caries-free individuals (Phattarataratip et al. 2011). Thus, low concentrations of LL37 in saliva may affect an individual's susceptibility to caries.

1.3.2.3 Human neutrophil peptides

The HNPs 1-4 are a family of structurally related peptides with antimicrobial activities. HNPs are expressed within the granules of neutrophils and are released into the oral cavity upon degranulation and participate in non-oxidative microbial death of metabolically active bacteria (Ganz et al. 1985). More recently, expression of the HNPs 1-3 was identified in salivary glands (Tao et al. 2005). Thus, both sources contribute to the concentration of HNPs in saliva. Levels of HNPs in saliva have been shown to be highly variable in adults (Goebel et al. 2000) and adolescents (Tao et al. 2005), although no information exists in relation to their concentrations in the saliva of young children. Notably,

expression of HNPs is also defective in Morbus Kostmann syndrome (Putsep et al. 2002). High concentrations of the HNPs 1-3 were associated with caries-free status in children aged 11- to 15-years (Tao et al. 2005). Additionally, concentrations of the HNPs 1-3 in saliva of adolescents was found to be significantly positively correlated with Mutans streptococci counts from plaque (Phattarataratip et al. 2011). Thus, low concentrations of the HNPs may influence the susceptibility of young children to dental caries.

1.3.2.4 Calprotectin

Calprotectin is a heterodimeric calcium and zinc-binding protein, also referred to as S100A8 and S100A9 (Nacken et al. 2003). Calprotectin is a major component of neutrophil granules, monocytes, macrophages and is constitutively expressed by the oral epithelium (Dale and Fredericks 2005). Calprotectin's antimicrobial action is mediated by its ability to sequester zinc, making it unavailable for microbial growth (Brandtzaeg et al. 1995). Additionally, expression of calprotectin by the oral mucosa inhibits bacterial adherence. Calprotectin is upregulated in the gingival crevicular crevice during periodontal disease (Becerik et al. 2011). There are no reports of the concentration of calprotectin in the saliva of children with or without dental caries. The significant level of expression of calprotectin in the oral cavity may influence the development of the oral biofilm and thus may alter caries susceptibility.

1.3.3 Initiation of an adaptive immune response to S. mutans

The initiation of an adaptive immune response begins with antigen recognition. Cells of the innate immune system, such as neutrophils, macrophages and dendritic cells express a limited range of receptors that recognise conserved microbial motifs, known as pathogen-associated molecular patterns (PAMPs). The receptors which recognise PAMPs are called pattern recognition receptors (PRRs) and include the family of TLRs. These receptors allow the innate system to recognise self from non-self. Recognition of foreign antigens activates cells of the innate system which in turn function to initiate adaptive immune responses.

Dendritic cells are the major antigen presenting cells. They recognise, capture, process and present foreign antigen to naïve T cells. These in turn perform

effector functions in peripheral tissues and provide help to and activate B cells within lymphoid tissues, promoting affinity maturation and antibody class switching (Banchereau and Steinman 1998). Thus, antigens present at mucosal surfaces must first be transported across the epithelium before they can stimulate an immune response.

Both oral and intestinal mucosa dendritic cells (DCs) are strategically located to sample antigens from oral and intestinal fluids (Ito et al. 1998; Neutra et al. 2001). Thus, it is likely that in oral mucosal tissues DCs fulfil functions of antigen capture, processing and presentation, such as occurs in intestinal lymphoid tissues. However, little is known about antigen capture by DCs in the oral cavity. Murine studies have provided evidence that buccal DCs can capture antigen and migrate to the lymph nodes draining the oral cavity (Aramaki et al. 2011). Upon arrival at DLN, DCs present antigen to T helper cells (Itano et al. 2003; Lane and McConnell 2001). However, T cell responses to DCs migrating from the oral mucosa have not been shown directly.

Activated T cells produce a variety of cytokines, TGFβ is particularly important for activating B cell switching from IgM to IgA producing plasma cells (Brandtzaeg 2010). T cells isolated from peripheral blood of individuals with caries have been shown to respond to Mutans streptococcal Gtf and SA I/II, although it is unclear whether they have a role in protection of caries other than to provide help to B cells for the synthesis of IgA (Taubman and Nash 2006).

It has long been held that initial stimulation of mucosal B cells takes place mainly in the mucosa-associated lymphoid tissue (MALT), particularly in the Peyer's patches and other gut-associated lymphoid tissues (GALT). Thus, mucosal stimulation with oral antigens or Mutans streptococci that reach the gut via ingestion, results in the activation and migration of antigen-specific IgA-producing B cells to effector sites, such as salivary glands. This is followed by their subsequent differentiation and maturation into plasma cells and secretion of sIgA across the epithelium into saliva. There is compelling evidence demonstrating the migration of activated B cells from the GALT to salivary glands in both animals and humans (Jackson et al. 1981; Mestecky et al. 1978). However, evidence also suggests that the intestinal immune response is not ideally configured for the induction of sIgA antibodies (Jertborn et al. 1986).

Thus, it remains to be established which part of the common mucosal immune system is most important for the induction of sIgA antibodies directed against oral bacteria. More recently, several studies have suggested that B cell homing to effector sites is more compartmentalised than previously thought and indicates that the nasopharyngeal-associated lymphoid tissues (NALT) may be more important than the GALT for the induction of B cell homing to salivary glands (Brandtzaeg 2007; Wu et al. 1997). Thus, the sIgA response to oral bacteria may be induced by two mechanisms; oral bacteria present in saliva may stimulate the proliferation and differentiation of B cells locally in salivary glands, and ingestion of oral bacteria and subsequent antigen uptake in the GALT is followed by the migration of activated B cell precursors to the salivary glands where they exert their effector functions.

Alternative routes for immune induction in the oral cavity have been proposed (Cutler and Jotwani 2006). Lymphoid tissues are located within salivary glands and Waldeyer's ring which consists of the tonsils and adenoids (Wu et al. 1997). In the intestine, specialised epithelial cells called M cells are involved in antigen sampling of the gut lumen (Mowat 2003). M cells have also been identified in the lymphoid tissues of Waldeyer's ring and have been shown to be functionally and structurally similar to M cells in Peyer's patches (Fujimura 2000). Very little is known about immune induction sites to orally exposed antigens. Studies investigating the functions of M cells in the NALT have primarily focussed on the induction of immune responses to inhaled antigens (Fujimura et al. 2004). NALT structures represent prototypical immune inductive sites and could theoretically transport antigen to underlying DCs. However, this has not been shown directly.

Oral lymphoid foci, located within interdental papilla have been proposed as oral effector sites. These structures contain large numbers of DCs in close proximity to T cells (Jotwani et al. 2001). These structures are reminiscent of isolated lymphoid follicles identified in the murine small intestine (Hamada et al. 2002), but oral lymphoid foci lack germinal centres. These structures are believed to develop in response to chronic inflammation initiated by the subgingival biofilm in diseases such as gingivitis and periodontitis (Cutler and Jotwani 2004) and thus are likely absent in the oral cavity of very young children who do not typically suffer from periodontal diseases, except in rare circumstances (Defraia and Marinelli 2001).

Thus, relatively little is known about the induction of adaptive immunity in the oral cavity and even less is known about the induction of adaptive immunity directed against oral bacteria, such as the Mutans streptococci (Figure 1-3). However, the potential exists to raise an antibody response to S. *mutans* under certain circumstances. It will be crucial to better understand the early stages of the initiation of adaptive immunity, which are a prerequisite to antibody generation. Further characterisation of these early responses to S. *mutans* will aid development of targeted and therapeutic interventions.

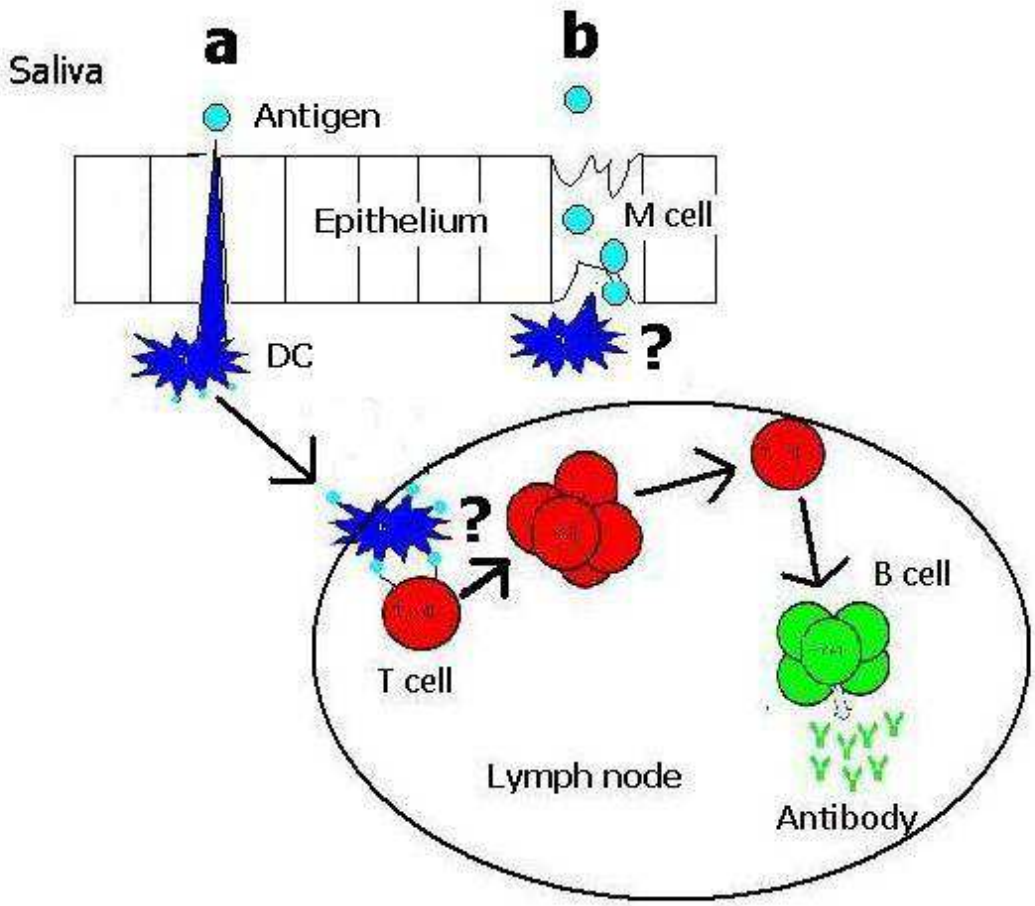


Figure 1-3: Antigen uptake in the oral cavity
Schematic representation of potential routes of antigen uptake in the oral cavity. (a) DCs are strategically placed to sample antigens from the oral mucosa (Ito et al. 1998) and evidence has shown DCs migrate to the DLNs upon antigen uptake (Aramaki et al. 2011). However, the influence DC migration on T effector responses to orally derived antigens has not been shown. (b) M cells located in NALT structures are functionally and structurally similar to M cells of the Peyer's patches (Fujimura 2000). It has not been shown if they can sample bacterial antigens directly in the oral cavity to induce mucosal immune responses.

1.3.4 Secretory immunity

Secretory or salivary IgA (sIgA) is the principle component of the adaptive immune system found in saliva. Salivary IgA exists as a polymeric molecule of two or more IgA monomers attached by a J chain and a secretory component. Secretory component, expressed on the basolateral surface of mucosal epithelial cells, is a receptor which allows for the transepithelial transport of polymeric IgA antibodies and subsequently protects the molecule from proteolytic degradation (Brandtzaeg 1995). In humans there are two subclasses of polymeric IgA: IgA1 and IgA2 and both can be found in saliva. IgA1 is the predominant subclass usually representing about 60% of IgA in saliva (Mestecky and Russell 1986). These two subclasses differ in only 16 amino acids, located in the hinge region, this difference renders IgA2 resistant to cleavage by the IgA proteases expressed by a number of oral bacteria, including early streptococcal colonisers of the oral cavity (Kilian et al. 1983). Salivary IgA is secreted by plasma cells located in the vicinity of salivary glands. Serum IgG can also be detected in saliva and enters the oral cavity via the gingival crevicular crevice. Together these antibodies make up 5 – 15% of proteins in saliva and represent a broad spectrum immune defence system (Van Nieuw Amerongen et al. 2004).

The biological functions of sIgA in the oral cavity include the inhibition of bacterial adherence, neutralisation of toxins and enzymes, agglutination of bacteria which facilitates their clearance from the oral cavity and immune exclusion (Table 1-3). The ability of sIgA to bind to and exclude microbial antigens and limit their penetration across mucosal surfaces is likely the most important role of sIgA and thereby limits hyper-stimulation of mucosal immune responses (Marcotte and Lavoie 1998). IgA is a poor activator of complement and complement components are absent in saliva, thus activation of complement is not a major biological function of sIgA. Furthermore, cell-mediated killing by leukocytes in response to opsonised bacteria is also not likely to be a major role fulfilled by sIgA. Leukocytes entering the oral cavity via the gingival crevicular crevice may be active short distances from the crevice but these cells cannot withstand the osmotic pressure of saliva. Serum-derived IgA and IgG may stimulate cell-mediated responses within and close to the gingival crevicular crevice. Nonetheless, oral bacteria have been found to be coated with sIgA

antibodies and this likely represents agglutination and inactivation of adhesins and bacterial enzymes.

At birth the oral cavity is sterile and devoid of sIgA (Gahnberg et al. 1985). At this time maternally derived IgG is the major component of secretory immunity in the oral cavity and low concentrations of IgM and IgD have been detected (Cripps et al. 1987; Gleeson et al. 1987). In the first few months of life the numbers of IgA producing plasma B cells increase and thus, levels of sIgA increase to become the dominant component of secretory immunity in the oral cavity (Smith et al. 1993). Initially, IgA in the saliva of newborns is predominantly of the IgA1 subclass, but as levels increase the proportion of IgA2 antibodies increases and may reflect the increased colonisation by IgA1 protease expressing early colonisers (Smith et al. 1989).

As microorganisms begin to colonise the oral cavity sIgA antibodies with new specificities are directed towards them and can be detected in saliva. Salivary IgA antibodies specific for *S. mitis* and *S. salivarius* antigens can be detected in the saliva of infants as young as five-weeks-old and reflects the early colonisation by these species (Smith et al. 1990). Similarly, sIgA antibodies specific for Gtf from *S. sanguinis* and *S. mutans* can be found in the saliva of children aged one-year and three-four years, respectively and coincides with the acquisition of these bacterial species (Gahnberg et al. 1985). At this time these antibodies are thought to be cross-reactive with low antigenic specificity. The sIgA antibody response increases with age and is of a level similar to adults by the age of four- to five-years (Smith et al. 1998). Coincidentally, the specificity for bacterial antigens increases with the increased length of exposure to oral bacteria (Parisotto et al. 2011; Smith and Taubman 1995). Thus, bacterial colonisation of the oral cavity occurs in an environment that is immunologically responsive to microbial challenge.

1.3.5 Can secretory immunity protect against dental caries?

Salivary IgA antibodies can control the colonisation of Mutans streptococci in the oral cavity by a number of mechanisms (Table 1-3). As previously discussed, bacteria must first adhere to host tissues or to each other in order to colonise

the oral cavity. Salivary IgA antibodies can interfere with this process by binding to and blocking the action of adhesins. *In vitro* studies investigating the role of sIgA in limiting bacterial adherence have demonstrated inconsistent and contradictory results. In one study, *S. mutans* adherence was inhibited by sIgA antibodies specific for SA I/II (Hajishengallis et al. 1992). Conversely, a separate study reported no effect of sIgA antibodies on *S. mutans* adherence to hydroxyapatite (a constituent of bones and teeth) (Kilian et al. 1981). Salivary IgA antibodies form a significant part of the salivary pellicle and are found to compose up to 2% of the dry weight of dental plaque (Orstavik and Kraus 1974). This has lead some to hypothesise that sIgA antibodies can even promote adherence of some bacterial species investigated (Kilian et al. 1981). Salivary IgA antibodies can also function to bind to and inhibit bacterial enzymes; sIgA specific for Mutans streptococcal Gtfs can inhibit the production of glucans and reduce dental plaque formation (Klein et al. 1977). Additionally, antibodies directed against Mutans streptococcal Gbps are predicted to influence bacterial adherence to polymeric glucans synthesised in the presence of sucrose. Rats immunised with a Gbp-derived peptide developed sIgA and serum IgG antibodies. Moreover, the development of dental caries was reduced in immunised animals, although colonisation by Mutans streptococci was unaffected (Taubman et al. 1995). Clinical studies have also demonstrated that topical application of monoclonal antibodies specific for *S. mutans* SA I/II prevents re-colonisation of the oral flora with *S. mutans* and the subsequent development of dental caries (Ma et al. 1987).

Additional studies have attempted to correlate the levels of naturally occurring IgA antibodies specific for Mutans streptococci with dental caries experience. These studies, often cross-sectional in design, have reported conflicting data with some studies reporting a positive (Challacombe 1980), negative (Camling and Kohler 1987) or no correlation (Hocini et al. 1993). Similarly, conflicting correlations of levels of specific sIgA antibodies and recovery of *S. mutans* were reported in these studies.

Table 1-3: Overview of salivary component and their functions in saliva

Salivary component	Activities in saliva	Reference
Salivary proteins		
mucins	formation of salivary pellicle, bacterial aggregation, inhibition of bacterial adherence	(Amerongen and Veerman 2002; Bennick et al. 1983; Gibbons et al. 1988; Scannapieco 1994; Van Nieuw Amerongen et al. 2004)
lysozyme	antibacterial, bacterial aggregation, inhibition of bacterial adherence	
lactoferrin	iron chelator, antibacterial	
peroxidases	antibacterial, acid and H_2O_2 neutralisation	
proline-rich proteins	formation of salivary pellicle, mineral homeostasis	
statherins	mineral homeostasis, formation of salivary pellicle	
cystatins	protease inhibitors	
Antimicrobial peptides		(Amerongen and Veerman 2002; Dale and Fredericks 2005; Nisapakultorn et al. 2001b; Ramos et al. 2011)
histatins	antibacterial/antifungal, formation of salivary pellicle	
LL37	antibacterial, chemotactic properties, wound repair, angiogenesis	
ß-defensins	antibacterial, antifungal, antiviral, chemotactic	
α-defensins	antibacterial, antifungal, antiviral, chemotactic	
calprotectin	antibacterial, chemotactic, inhibition of bacterial adherence	
Antibodies		(Amerongen and Veerman 2002; Smith and Taubman 1992)
IgA, IgG, IgM	bacterial aggregation, neutralisation of antigens, inhibition of bacterial adherence, salivary pellicle formation	

Additionally, a number of studies have attempted to correlate dental caries experience in children with IgA deficiency. These studies have also produced conflicting results with studies reporting higher caries rates in children with sIgA deficiency (Tar et al. 2008) or lower rates compared with healthy aged-matched controls (Fernandes et al. 1995). It is extremely difficult to delineate the contribution of sIgA antibodies to caries susceptibility due to compensatory mechanisms, such as increased expression of sIgM in IgA-deficient individuals (Fernandes et al. 1995; Nikfarjam et al. 2004).

Maternally derived serum IgG specific for oral bacteria can be detected in the saliva of newborns (Smith and Taubman 1993). Levels of IgG antibodies rapidly decrease in the first months of life and become undetectable in the majority of infants by three- to four-months of age (Smith et al. 1989). With the eruption of the primary dentition serum IgG, IgM and IgA can enter the oral cavity via the gingival crevicular crevice. These antibodies are synthesised in response to microbial challenge (Smith and Taubman 1992). Serum IgG specific for *S. mutans* and *S. sobrinus* can be detected only in very low levels in children aged one- to three-years, coinciding with their acquisition in dental plaque (Luo et al. 1988). The protective role of serum IgG in saliva is also met with controversy, although an inverse relationship between the levels of serum IgG directed against *S. mutans* and their numbers in dental plaque and subsequent caries development have been reported (Aaltonen et al. 1987). However, the contribution of serum derived antibodies to secretory immunity in the oral cavity is minimal when compared with sIgA (Russell et al. 1999).

These studies indicate that the potential does exist to mount a secretory IgA response that can inhibit processes required for Mutans streptococcal plaque formation, including sucrose-independent adhesion to the salivary pellicle, mediated by SA I/II, accumulation of Mutans streptococci mediated by glucan production by Gtfs in the presence of sucrose and adhesion to glucans mediated by Gbps. However, the cross-sectional nature of these studies complicates interpretation of the results, not least because caries takes months to years to manifest clinically. Therefore, collection of saliva at a single point in time may not reflect the past or present disease status. Thus, the role of sIgA in protection against caries remains controversial. Longitudinal studies are required to identify whether naturally elicited protective antibody responses can be

generated. Thus far the limited knowledge of the immune response to infection has hindered the advancement of therapeutic strategies.

1.4 Childsmile

The human samples obtained in the following study were from participants in 'Childsmile.' Childsmile is a national dental health improvement programme for Scotland. The programme was initiated by the Scottish Government's (then Executive) 2005 action plan for improving oral health and modernising dental services in Scotland (Macpherson et al. 2010a). The programme was developed in response to the consistently high levels of dental caries among young children in Scotland, with over half of Scottish five-year-olds experiencing significant levels of decay, with an average dmft (decayed, missing [due to caries] or filled teeth) of five in these caries experienced children. A significant proportion of these figures were attributable to those children of low SES, who suffer from the greatest burden of disease. Moreover, with extremely low rates (30%) of very young children registered with dental practitioners there is a need to access these young children and provide anticipatory and preventative care, particularly to those from deprived backgrounds who are less likely to access dental services (Shaw et al. 2009). Thus, the overarching aim of Childsmile is to improve the oral health of children across Scotland and to reduce inequalities in dental health and access to dental services.

Initially two targeted demonstration programmes were established beginning in January 2006, one in the East and one in the West of Scotland. In the East, Childsmile was designed to provide additional preventative clinical care through NHS dental practices, specifically aimed at children aged three-years and over and attending nurseries or schools in the most deprived areas. In the West, Childsmile was set up to target children from birth and to promote oral health and caries prevention, in dental practice and local community settings. Newly born infants identified as at risk of developing dental caries by their health visitors are referred to dental health support workers, who visit families in their homes to promote attendance at Childsmile dental services, encourage good oral hygiene and provide additional advice as required. Extended duty dental nurses,

trained in oral health promotion and fluoride varnish application, provide additional care to families as required.

In 2009, these demonstration phases of the Childsmile programme were rolled out Nationwide (Turner et al. 2010). Early evidence indicates that Childsmile intervention is associated with a reduction in the prevalence of dental caries among young children, decreasing from 26% in 2006/7 to 17% in 2009/10. Moreover, the reduction in decay experience was observed across the socioeconomic spectrum (McMahon et al. 2011).

The Childsmile programme provides children with specific health interventions depending on need, from birth and up to 16-years of age. The combination of Childsmile's unusually young target age group and the multiple visit interventions provides a unique opportunity to investigate the evolution of the oral biofilm coincident with the development of the salivary immune response.

1.5 Objectives

There are fundamental gaps in current knowledge of the interrelationships between caries-associated risk factors. In particular how the immune system interacts with the evolving cariogenic oral biofilm in young children, and how this may contribute to inequalities in dental caries. This study aimed to unravel the relationship between development of the cariogenic oral biofilm, coincident with the evolution of the salivary immune response, specifically:

1. Investigate the biological caries-associated risk factors in a cross-sectional clinical study of one-year-old Childsmile participants.

2. Investigate the biological caries-associated risk factors in a longitudinal clinical study of one- to three-year-old Childsmile participants.

3. Investigate the initiation of the adaptive immune response to *S. mutans,* using as series of *In vitro* and *In vivo* experimental models.

4. To develop and optimise the use of an *In vitro S. mutans* biofilm model.

Chapter 2 Materials and Methods

2.1 Study Participants and Ethical Considerations

Study participants were recruited from the Childsmile Initiative. Parental consent was obtained at baseline from parents of children aged between 12 and 24 months and attending a Childsmile appointment within the Glasgow and Clyde area (Parental information sheet and consent are shown in Appendix I). Plaque and saliva samples were collected from children as detailed in sections 2.3.1 and 2.3.2, respectively. Sociodemographic data was collected by questionnaire. Patients were recalled approximately 18 months later and invited to bring their child to a follow-up appointment. Ethical approval was amended to include the use of a validated food frequency questionnaire, for which additional parental consent was obtained. Plaque and saliva samples were collected and clinical disease data (dmft) were also recorded by a national dental inspection program (NDIP) calibrated dentist. In the first collection the study group comprised 63 individuals with a median age of 16 months and at follow-up the study group comprised 23 individuals with a median age of 35 months. This study (Dr Shauna Culshaw, 'Unraveling the relationship between the oral biofilm and the host immune response' was reviewed and received ethical approval from the West of Scotland Research Ethics Committee (08/S0703/139, Appendix II) and NHS Greater Glasgow and Clyde R&D Management (YN08DN369, Appendix III).

2.2 Reagents

The source of reagents is given in the text. All chemicals were obtained from Sigma, Poole, UK and all cell culture media and supplements from Life Technologies, Paisley, UK unless otherwise indicated.

2.3 Clinical sample collection

2.3.1 Plaque

Plaque was collected from the buccal surface of the upper molars using a sterile nylon elution swab and placed into 1 ml Liquid Amies transport medium (Eswab [Copan, Brescia, Italy]). If the deciduous molars had not erupted at time of examination, plaque was collected from the incisors. Samples were stored at

4˚C and transported to the microbiology laboratory at Glasgow Dental School and Hospital within hours for immediate processing.

2.3.2 Saliva Collection

Saliva was collected using cotton sorbettes (Salimetrics, Europe LTD), specially designed for the collection of saliva from infants. This product was discontinued prior to the follow-up sample collection and replaced with the Children's Oral Swab (Salimetrics, Europe LTD). This long swab is made from a synthetic durable polymer and is designed to collect saliva from children under the age of six-years. Sorbettes and oral swabs were placed in the mouth for up to 60 s, pending child cooperation, and allowed to absorb saliva. Saturated sorbettes and swabs were then placed into appropriately labelled collection tubes (Salimetrics, Europe LTD), stored at 4˚C and transported to the microbiology laboratory at Glasgow Dental School and Hospital within hours for immediate processing.

2.4 Questionnaires

2.4.1 Sociodemographic

A socioeconomic demographic questionnaire (University of Glasgow Dental School: version 2 December 2008 [Appendix IV] was designed to collect information regarding the parental profile of children recruited to the study. Postcodes were recorded for each study participant and used to determine the Scottish index of multiple deprivation (SIMD) 2009. SIMD identifies small area concentrations of multiple deprivation across Scotland in a consistent manner. SIMD ranks are area based measures that provide a relative measure of deprivation for every postcode sector using 38 indicators across seven domains, including income, employment, health and education (The Scottish Government). SIMD ranks are often divided by deciles or quintiles. In this study quintiles were used to assess the relative deprivation of each postcode. The most deprived quintile (quintile 1) includes the most relatively deprived 20% of Scottish postcode areas.

Socioeconomic data collected by questionnaire contained multiple categories for some of the variables. In a change to this format, data regarding annual household income, the proportion of income received as benefits, the level of parental education and SIMD quintiles were dichotomised as illustrated in Table 2-1. The cut-off points for the dichotomisation were arbitrarily chosen in an attempt to compare the most deprived families with those who were relatively less deprived.

Table 2-1: Dichotomisation of measures of socioeconomic status

Demographic measure	Dichotomised categories	
	Most deprived	Relatively less deprived
Annual household income	< £10,000	≥ £10, 000
Proportion of income received as benefits	Half - all	None - about a quarter
Parental education	Secondary school	6th form or above
Healthboard SIMD 2009	SIMD 1	SIMD 2-5

2.4.2 Food frequency questionnaire

A validated Scottish Collaborative Group Food frequency questionnaire (FFQ: version C2 for use in three- to 11-year olds [Appendix V] (Sheehy et al. 2008), was used to estimate non-milk extrinsic sugar (NMES) intake from three-year old study participants at the follow-up collection. This version lists 140 different foods and drinks each with a measure to represent a small portion of each item. Examples of portion sizes were shown in colour images on the front cover of the FFQ. Parents or guardians of participants were asked to estimate the frequency and amount of each item consumed over a typical week. The options for selection ranged from 'rarely or never' to '7 or more portions a day'. Data from completed questionnaires were entered into an ACCESS database file. Nutrient intakes were calculated from the FFQ using a calculation program developed by the University of Aberdeen.

2.5 Caries scores

Caries experience for each tooth was calculated by a national dental inspection program calibrated (NDIP) dentist. Summary measures of $d_3$mft (decayed into dentine, missing due to caries and filled teeth) were then calculated for each child. Dental examinations were performed in line with BASCD criteria (Hinds and Gregory. J 1995).

2.6 Microbiology

2.6.1 Plaque

The plaque samples in 1 ml Liquid Amies were vortex mixed for 30 sec to release bacteria from nylon swab. A 100 µl volume of plaque suspension was removed and serially diluted 10-fold in sterile phosphate buffered saline (PBS). The CFU/ml was determined using the technique described by Miles and Misra (Miles et al. 1938). Aliquots of 20 µl volumes of 10^{-1} to 10^{-4} dilutions were spotted in triplicate, onto Columbia base agar (Oxoid, Basingstoke, UK) supplemented with 5% defibrinated horse blood (hence referred to as blood agar, BA), for the enumeration of total aerobic flora; mitis salivarius agar (MSA, [Sigma]), supplemented with 1 ml of 1% potassium tellurite per litre (Fluka™ Analytical, Sigma, UK) for total oral streptococci; MSA supplemented with 0.2 units/ml bacitracin and 150 g sucrose per litre (MSB) for Mutans streptococci (Gold et al. 1973); and Rogosa agar supplemented with 0.1% Tween 80 (Invitrogen, Paisley, UK) and 1.32 ml/L acetic acid for enumeration of total lactobacillus spp.

BA plates were incubated at 37˚C for 24 h in an atmosphere of 95% air and 5% CO_2 [Binder GmbH, Tuttlingen, Germany]. MSA, MSB and Rogosa plates were incubated at 37˚C for 48 h under anaerobic conditions, in an atmosphere of 85% N_2, 10% CO_2 and 5% H_2.

The remaining suspension of plaque bacteria in 900 µl of Liquid Amies was pelleted by centrifugation at 10, 000 x g for 10 min. The supernatant was discarded and the cell pellet was stored at -80˚C until required for quantitation of cariogenic bacteria by TaqMan® QPCR (section 2.6.8).

2.6.2 Saliva

Saliva was harvested from sorbettes and oral swabs by centrifugation at 1, 500 x g for 10 min at 4˚C. Collected saliva was stored at -80˚C and retained for immunological investigations. The cell pellet was also retained and stored at -80˚C until required for quantitation of cariogenic bacteria by TaqMan® QPCR (section 2.6.8). At this time the total volume of saliva collected from each patient was recorded.

2.6.3 Colony counting

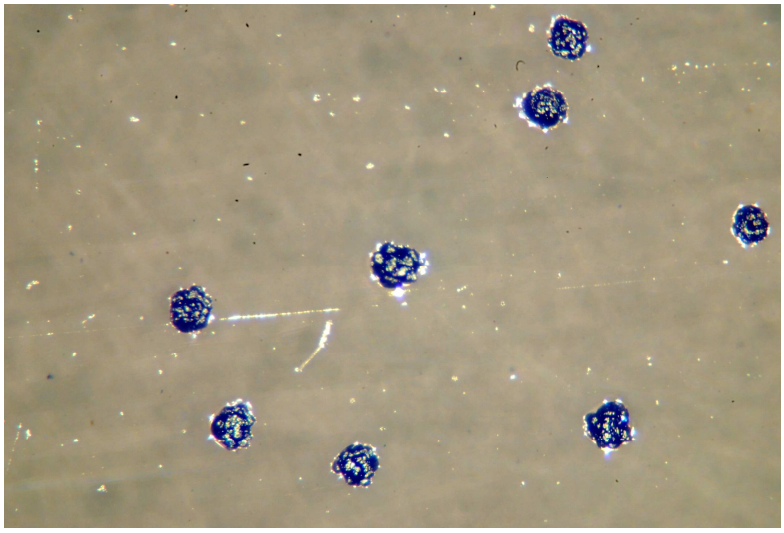
All colonies on BA and MSA were counted and recorded for enumeration of total aerobic flora and oral streptococci respectively, using the formula:

No. of colonies x dilution factor x 50 (volume to 1 ml) = CFU/ml

All colonies with granular "frosted-glass" morphology (Figure 2-1a), characteristic for *S. mutans* on MSB agar (Emilson 1983; Gold et al. 1973) were counted, recorded and the CFU/ml was calculated. The minimum detection level was 1 x 10^3 CFU/ml suspended plaque. Putative *S. mutans* isolates were Gram-stained and a positive staining reaction and appearance of streptococci was considered indicative of *S. mutans*. Further confirmation was carried out using Rapid ID 32 Strep API (BioMérieux, UK LTD), according to the manufacturer's instructions (Figure 2-1b).

All colonies growing on Rogosa were considered to be lactobacilli spp. Identification was confirmed with a Gram positive staining reaction and appearance of bacilli.

a



b

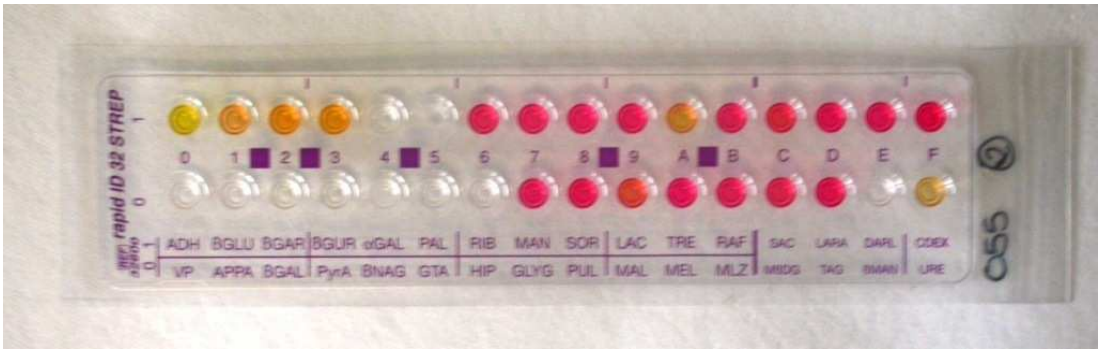


Figure 2-1: Identification of *S. mutans*

(a) Characteristic granular "frosted glass" morphology of *S. mutans* colonies grown on MSB agar. (b) Rapid ID 32 Strep API

2.6.4 Bacterial strains and culture conditions

S. mutans NCTC 10449 and UA159, *S. sobrinus* NCTC 33478, *S. mitis* NCTC 12261 and *S. sanguinis* NCTC 7863 were stored on Protect® (Technical Service Consultants Ltd, UK) at -80˚C at Glasgow Dental School and Hospital, Scotland, UK, throughout the duration of the study. Strains were maintained at 37˚C in 5% CO_2 on BA for 24 – 48 h. For subculture, individual colonies were inoculated into 10 ml brain heart infusion broth (BHI [Oxoid]) and cultures were grown at 37˚C in 5% CO_2 for 24 h.

S. sobrinus 6715 and *S. mutans* 28BE3 stock cultures were maintained in Todd Hewitt Broth (Difco, USA) in 10% glycerol at – 70 ˚C at the Forsyth Institute,

Boston, USA. Three ml of BHI broth (Difco, USA) was inoculated with a 'scraping' of stock culture and incubated anaerobically at 37˚C for 8 h. Following incubation, a 0.1 ml volume was transferred to 3 ml fresh BHI and incubated at 37˚C for 8 h.

For experiments requiring heat-killed bacteria, *S. mutans* UA159 were incubated at 60˚C for 30 min.

All microbiological procedures were performed using aseptic technique.

2.6.5 Sucrose-dependent biofilm formation

Mutans streptococcal biofilm formation was assessed using 96-well peg lids in conjunction with 96-well polypropylene plates (Figure 2-2, [Nunc™, Fisher Scientific, Loughborough, UK]) with low protein-binding characteristics to ensure bacterial cells bound only to pegs and not to wells during the incubation stage. Peg lids were submerged in 248 µl BHI containing 1.5 x 10^7 CFU/ml of bacteria grown to exponential phase, 0.4% sodium azide, 0.25% sucrose in a pH adjusted to pH 6.8, unless otherwise indicated in figure legends. *S. mutans* and *S. sobrinus* biofilms (the strains used are indicated in figure legends) were formed over 4 h, under anaerobic conditions (80% N_2; 10% H_2; 10% CO_2) with orbital shaking at 575 rpm.

Biofilm formation was assessed in the presence of various mediators or inhibitory molecules as detailed in figure legends. Positive control antibodies used for Mutans streptococcal biofilm inhibition have been previously described (Taubman et al. 1995). Briefly, rat IgG anti-*S. sobrinus* Gtf and rat IgG anti-*S. mutans* Gtf were prepared by active immunization with *S. sobrinus* Gtf or *S. mutans* Gtf, respectively. Animals were injected subcutaneously in the vicinity of the salivary gland with 50 µg of Gtf from *S. sobrinus* or 25 µg of Gtf from *S. mutans* in complete Freund adjuvant. One week later animals were reinjected, in the same vicinity with the same antigen and dose in incomplete Freund adjuvant. One week after the second injection animals were bled from the retroorbital plexus and serum was collected. Serum IgG collected from sham-immunized rats served as control group. SS2 was prepared by phage display, described in section 6.1 (Sui et al. 2008; Sui et al. 2009).

2.6.6 Biofilm quantification

Following biofilm formation, pegs were washed twice in 250 µl PBS to remove non-adherent cells. Biofilm formation on pegs was then quantified using the following methods.

2.6.6.1 Crystal violet assay

Biofilms formed on pegs were stained in 250 µl/well of 0.2% crystal violet (w/v in dH_20) for 10 min. Following staining, pegs were air dried for 30 min then washed three times in PBS before bound crystal violet was eluted from pegs in 250 µl/well of elution solution (70% ethanol; 5% acetic acid). The eluate was measured with plate reader KC junior (Forsyth Institute, Boston, USA) or Omega Fluostar (BMG Labtech, Glasgow Dental, School and Hospital, UK) absorbance was measured at OD 570 nm with water blank.

Biofilm inhibition, expressed as '% inhibition of biofilm formation' was calculated according to the following equation:

100 – ((Absorbance of test antibody/absorbance of no antibody control) x 100)

2.6.6.2 CyQuant assay®

Pegs were removed with pliers and transferred to sterile eppendorfs containing one ml of sterile PBS. Biofilms were disrupted from pegs by sonication for five min at 35 kHz. Sonication did not to affect *S. mutans* cell viability. Pegs were discarded and the resulting cell suspension was pelleted by centrifugation at 10, 000 x g for 10 min. The cell pellet was frozen to -70˚C for 1 h and thawed to room temp. Next, cells were resuspended in 1 ml of CyQuant® GR dye/lysis buffer (Invitrogen). Simultaneously, a standard curve was prepared from planktonic *S. mutans* cells in the range of $1x10^6$ – $1x10^1$ CFU/ml. All samples were incubated with the dye for 5 min in the dark at room temperature. Following incubation, 200 µl volumes of cell suspensions, including standards were added to wells of a black fluorescent microtitre plate (Thermo Fisher Scientific, UK) and fluorescence of samples was measured using an Omega

Fluostar®. A standard curve was created using non-linear regression analysis and used to extrapolate the number of CFU/ml of *S. mutans* forming biofilms.

2.6.6.3 TaqMan® QPCR

Individual pegs were removed using pliers and transferred to sterile eppendorfs. Enzymes for cell lysis (outlined in section 2.6.7) were added and eppendorfs containing pegs were subjected to sonication for 5 min at 35 kHz. Therein DNA digestion was performed as outlined below (section 2.6.7) and TaqMan® QPCR using *S. mutans* specific primers and probe was performed as outlined in section 2.6.8.

2.6.6.4 AlamarBlue® Assay

The AlamarBlue® assay (Invitrogen) incorporates a fluorometric growth indicator based on the detection of metabolic activity. The system incorporates an oxidation-reduction (REDOX) indicator that fluoresces in response to chemical reduction of the growth media resulting from cell growth. Following *S. mutans* biofilm formation on peg lids, pegs were washed as previously described and transferred to a fresh microtitre plate (Costar) containing 250 µl/well of PBS and 1/10 dilution of AlamarBlue®. Biofilms were incubated with AlamarBlue® solution at 37˚C, in 5% CO_2 for 4 h. Following incubation, pegs lids were removed and the change in fluorescence was measured using an Omega FluoStar® plate reader. The raw fluorescence intensity data was averaged for each condition with increased fluorescence intensity being indicative of an increased reduction of the growth media and thus represents increased metabolic activity.

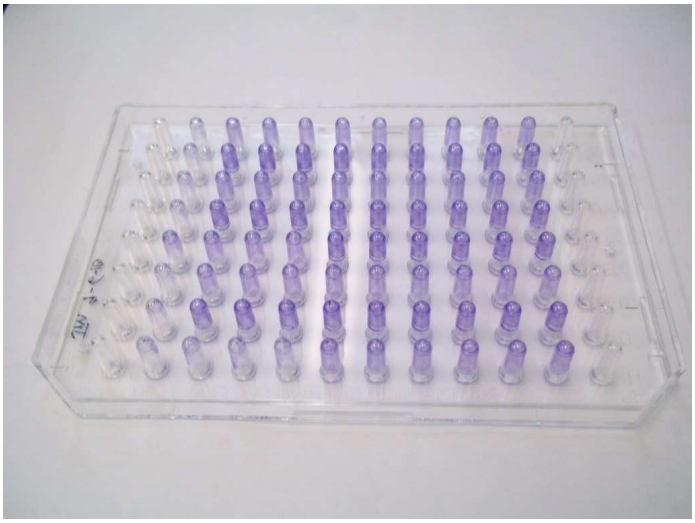


Figure 2-2: *S. mutans* biofilm formation on peg lids

S. mutans sucrose-dependent biofilm formation on peg lids, visualized with crystal violet incorporation.

2.6.7 DNA extraction

S. mutans NCTC 10449 and *S. sobrinus* NCTC 33478 cultures were grown to exponential phase in BHI for 8 h. Cultures were adjusted to absorbance 0.05 (OD 570 nm), equivalent to approximately 5 x 10^7 CFU/ml and serially 10-fold diluted down to 5 x 10^0 CFU/ml. One ml volumes of each culture were pelleted by centrifugation at 10, 000 x g for 10 min. DNA extraction was carried out using Masterpure™ Gram positive DNA purification kit (Epicentre Biotechnologies, USA) in accordance with manufacturer's instructions. Briefly, 1 ml of culture was pelleted by centrifugation, and resuspended in 150 µl TE buffer. For bacterial lysis, 1 µl Ready-Lyse Lysozyme® and an additional 20 U mutanolysin were added to the resuspended cell pellet and incubated for 1 hr at 37˚C. Next, 150 µl of Gram positive lysis solution, containing 3 µg proteinase K was added to each sample and incubated at 65˚C for 15 min. Samples were cooled to 37˚C and then placed on ice before addition of 175 µl MPC Protein Precipitation Reagent. Samples were vortex mixed vigorously before centrifugation at 4˚C for 10 min at 10,000 x g (Microfuge® 22R Centrifuge; Beckman Coulter). Supernatants were transferred to clean Ambion® DNase-free eppendorfs, 5 µg of RNase A was added to each sample before 30 min incubation at 37˚C. One volume of isopropanol was added and tubes were inverted 30-40 times. DNA precipitate was harvested

by centrifugation for 10 min at 10,000 x g at 4˚C. Isopropanol was removed, taking care not to dislodge the DNA pellet. The pellet was then washed in 70% ethanol, which was removed by pipetting; remaining ethanol was allowed to evaporate then DNA was resuspended in 35 µl of DNase free water. The quantity and quality of DNA was measured using a Nanodrop spectrophotometer (Nanodrop 1000, Thermo Scientific). The DNA from serially diluted cultures was used as standards to quantify the absolute CFU/ml of *S. mutans* and *S. sobrinus* in saliva of clinical samples by TaqMan® QPCR. Cell pellets from clinical plaque and saliva samples were removed from -80˚C storage and allowed to thaw prior to DNA extraction performed as above.

2.6.8 Quantitative PCR

TaqMan® real-time QPCR was performed using an Mx3000P (Agilent Technologies Inc) according to the manufacturer's instructions. The principle of the method is outlined below (Figure 2-3).

Primers and fluorescent probes, previously described (Table 2-2) were purchased from Eurogentec, Belgium. Fluorescent probes contained a reporter dye (FAM) covalently attached to the 5' end and a quencher dye (TAMRA) attached to the 3' end. Primers and probes were HPLC purified. Extension from the 3' end of the probe was blocked by the attachment of a 3' phosphate group. The specificity of primers and probes were confirmed (Table 2-3).

Each reaction contained:

12.5 µl Taqman® Platinum mix UDG (Invitrogen, Paisley, UK)

200 nM each primer

250 nm fluorescent probe

1 µl DNA template

total volume = 25 µl.

Amplifications of DNA were performed in triplicate using an initial cycle of 50˚C for 2 min and 95˚C for 10 min followed by 60 cycles of 95˚C for 15 s and 58˚C

for 1 min. Data analysis was performed using MxPro QPCR Software (Agilent Technologies Inc) to calculate the threshold cycle (Figure 2-4a). The Ct value represents the PCR cycle at which fluorescence can be first detected above a defined threshold. DNA extracted from *S. mutans* and *S. sobrinus* cultures of known CFU/ml were used as standards and were amplified in duplicate alongside clinical samples. Standard curves were created and the linear phase of amplification (Figure 2-4b) was used to calculate the absolute numbers of *S. mutans* and *S. sobrinus* from saliva samples. For each saliva sample, the recorded volume of saliva originally collected was used as a multiplication factor to calculate the absolute number of *S. mutans* and *S. sobrinus* CFU/ml saliva.

DNA extracted from plaque samples was used to estimate the relative amount of *S. mutans* and *S. sobrinus* in relation to total Gram positive plaque flora, estimated by 16S universal primers specific for Gram positive bacteria, using the ΔΔCT method. Using this method the fold difference was calculated. A multiplication step was performed to determine *S. mutans* and *S. sobrinus* as a percentage of Gram positive plaque flora for each individual.

Table 2-2: Primers and probes used to detect and quantify absolute and relative numbers of *S. mutans* and *S. sobrinus* in samples

Primers and Probes	Sequence (5'-3')	Target	Reference
S. mutans-Forward primer	GCCTACAGCTCAGAGATGCTATTCT	*gtfB*	(Yoshida et al. 2003a)
S. mutans-Reverse primer	GCCATACACCACTCATGAATTGA		
S. sobrinus-Forward primer	TTCAAAGCCAAGACCAAGCTAGT	*gtfT*	
S. sobrinus-Reverse primer	CCAGCCTGAGATTCAGCTTGT		
S. mutans probe	FAM-TGGAAATGACGGTCGCCGTTATGAA-TAMRA	*gtfB*	
S. sobrinus probe	FAM-CCTGCTCCAGCGACAAAGGCAGC-TAMRA	*gtfT*	
Universal-Forward primer	CAACGCGAAGAACCTTACC	16s DNA	(Wu et al. 2008)
Universal-Reverse primer	ACGTCATCCCCACCTTCC		
Gram +ve probe	FAM-ACGACAACCATGCACCACCTG-TAMRA		

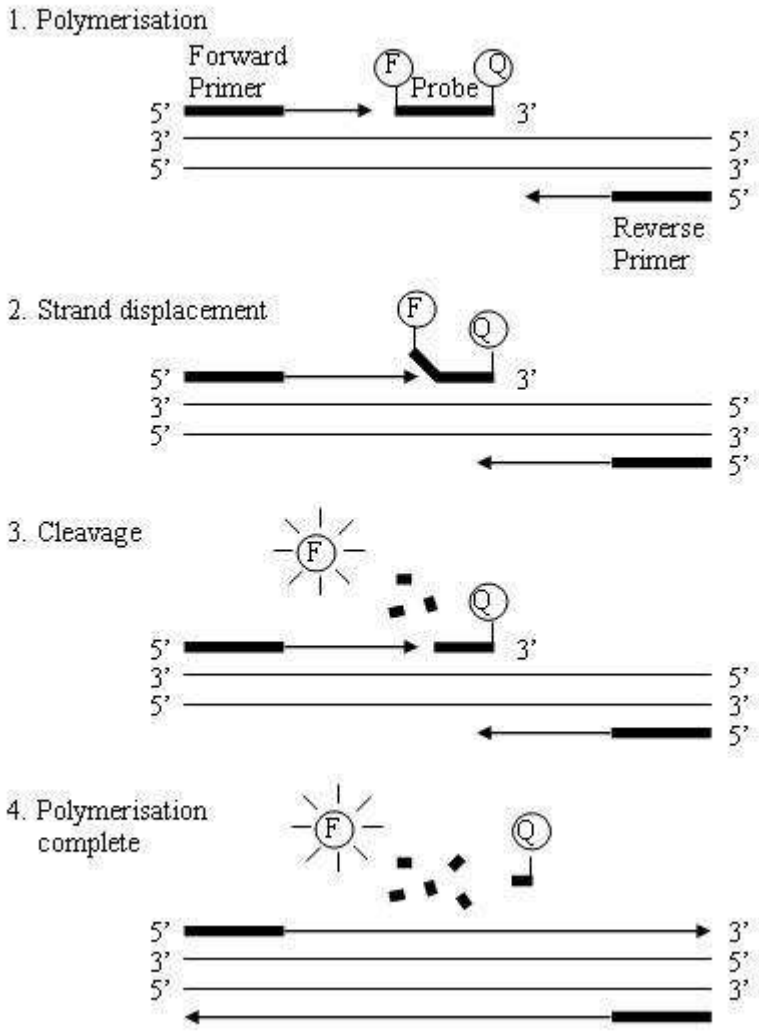


Figure 2-3: Schematic representation of TaqMan® QPCR

A reporter dye, FAM was covalently attached to the 5' end of the probe (F). A quencher (Q), TAMRA was attached to the 3' end. PCR product amplification resulted in cleavage of the probe, thus removing the inhibitory action of the quencher from the reporter.

Table 2-3: Amplification of DNA from a panel of bacterial species using TaqMan primers and probes

Bacterial species (strain)	*S. mutans*	*S. sobrinus*	16S DNA (+ Gram +ve probe)
S. mutans (UA159)	+	-	+ (+)
S. mutans (10449)	+	-	+ (+)
S. sobrinus (33478)	-	+	+ (+)
S. mitis (12261)	-	-	+ (+)
S. sanguinis (7863)	-	-	+ (+)
S. salivarius (7366)	-	-	+ (+)
Porphyromonas gingivalis (33227)	-	-	+ (-)
F. nucleatum (10953)	-	-	+ (-)
Aggregibacter Actinomycetemscomitans (11123)	-	-	+ (-)
Pseudomonas aeruginosa (Pg)	-	-	+ (-)
P. aeruginosa (PA01)	-	-	+ (-)
P. aeruginosa (PA14)	-	-	+ (-)

Primers and probes were assessed for their specificity against a panel of Gram-positive (shaded grey) and Gram-negative (white) bacterial species. +/- indicates an amplification product or no amplification product, respectively following PCR.

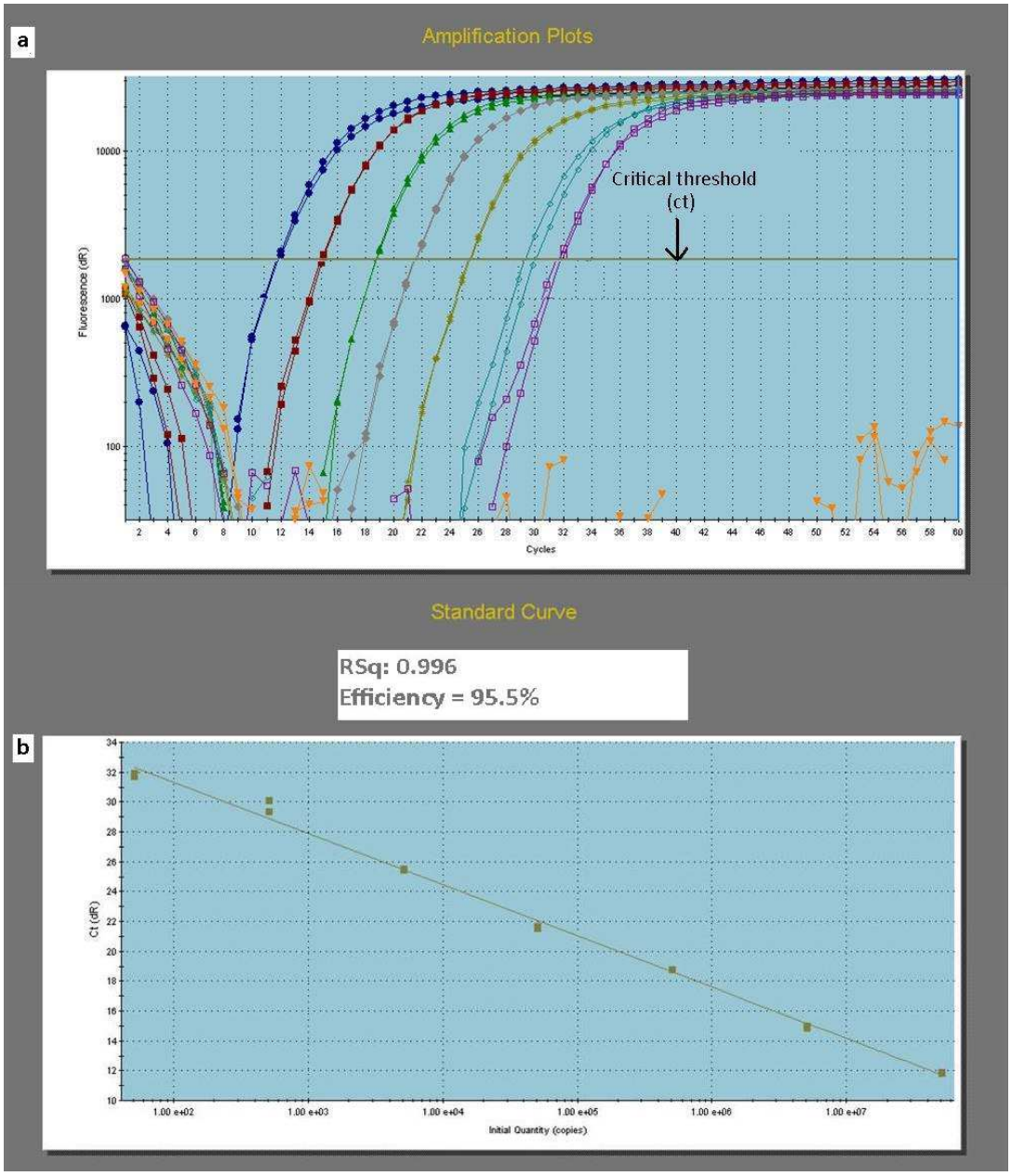


Figure 2-4: Determination of the critical threshold used to quantify *S. mutans* or *S. sobrinus* from DNA standards

Representative images of (a) amplification plots showing the critical threshold (ct) at which the fluorescent signal can be detected above background fluorescence for duplicates of reactions with serially diluted template and (b) standard curve showing the linear reaction efficiency of DNA extracted from cultures of *S. mutans* containing 5 x 10^8 CFU/ml and subsequent ten-fold dilutions of bacteria down to 5 x 10^1 CFU/ml. Thus, data confirm the linear reaction efficiency of the assay and the efficiency of DNA extraction.

2.7 ELISA

2.7.1 Salivary antimicrobial protein assays

The concentrations of antimicrobial proteins (Lactoferrin, LL37, calprotectin and the human neutrophil peptides [HNPs] 1-3) in clinical saliva samples were estimated by sandwich ELISAs. The antibody used to detect the HNPs does not discriminate between peptides 1, 2 and 3. The commercial ELISA kits were used in accordance with the manufacturer's instructions (Hycult Biotech). Clinical saliva samples and assay standards were assayed in duplicate at an appropriate dilution factor as indicated in (Table 2-4). The absorbance of samples were measured at OD 450 nm using an Omega FluoStar® plate reader and the concentrations of antimicrobial proteins were determined by interpolation from a standard 4-parameter fit curve using Omega Data Analysis Software (BMG Labtech).

2.7.2 Cortisol assay

The concentration of salivary cortisol was estimated by competitive ELISA. The ELISA kit was used in accordance with the manufacturer's instructions (Salimetrics, USA) (Table 2-4). Clinical saliva samples and assay standards were assayed in duplicate. The absorbance of samples and standards were measured at OD 450 nm with correction at 630 nm using an Omega Fluostar® plate reader. The percentage of bound cortisol for each sample, standard and control was calculated by dividing the average of the duplicate OD by the average OD of the no cortisol wells. The concentrations of samples and controls were determined by interpolation from a standard 4-parameter fit curve using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, California, USA).

Table 2-4: Dilution factors and sensitivity range for commercial ELISA kits

ELISA	Supplier	Dilution Factor	Sensitivity Range
LL-37 (cathelicidin)	Hycult Biotech	Neat	0.1 – 100 µg/ml
HNPs 1-3 (α-defensins)	Hycult Biotech	1/100	41 – 10,000 pg/ml
Calprotectin (S100A8/A9 complex)	Hycult Biotech	1/100	1.56 – 100 µg/ml
Lactoferrin	Hycult Biotech	1/100	0.4 – 100 µg/ml
Cortisol	Salimetrics	Neat	0.012 – 3 µg/dL

2.7.3 Salivary IgA

The concentration of salivary IgA (sIgA) antibodies specific for a panel of oral streptococci was estimated by whole cell bacterial ELISA.

2.7.3.1 Preparation of bacteria

Bacterial strains: *S. mutans* NCTC 10449, *S. sobrinus* NCTC 33478, *S. mitis* NCTC 12261 and *S. sanguinis* NCTC 7863 grown on BA as previously described were harvested using sterile swabs. Each strain was singularly resuspended into PBS containing 0.1mM disodium EDTA (PBSE). The bacterial cells were centrifuged for 20 min at 4,000 x g. The supernatant was discarded and the bacterial cells resuspended in PBSE. This process was repeated a further three times until the supernatant was clear. Bacteria were fixed with 10% formal saline by incubation at room temperature for 16 – 18 h. The fixed bacteria were washed a further two times in PBSE and once in coating buffer (CB:1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ dissolved in 1L distilled water and adjusted to pH 9.6). Sodium Azide [2% w/v in CB] was prepared and 1/10 volume was added to the fixed cells. Cells were stored in CB at 4˚C for up to one month prior to use in ELISA.

2.7.3.2 Estimation of sIgA antibodies

Ninety-six well Immunolon I plates (Dynex Technologies, VA, USA) were used for their low protein binding properties. The optimal concentration of bacterial suspensions and detection reagents were optimised and validated prior to use. For coating, 200 µl CB was added to each well and incubated for 30 min, then discarded and the plate blotted dry by inverting and pounding on paper towels. Next, 100 µl of 0.25 OD 550 nm of whole bacterial suspensions in CB was added and incubated at 4˚C overnight on a rocking platform. All subsequent steps were carried out at 37˚C with reagents diluted in incubation buffer (PBS 0.05% Tween 20 + 5% BSA). Plates were blocked with 200 µl incubation buffer for 1 h. To detect salivary antibody, clinical saliva samples were added to wells in triplicate, at two-fold dilutions ranging from 1/200 -1/6400 for 2 h. The reaction was developed by 1 h incubation with 1/2000 dilution of goat anti-IgA biotinylated detection antibody (Sigma), followed by addition of ExtrAvidin peroxidase conjugate (Sigma) for 1 h. Following each step plates were washed

four times in washing buffer (PBS 0.05% Tween 20 [PBST]). For each wash, wells were filled with wash buffer and allowed to stand for one minute before being inverted. Following the final wash, residual wash buffer was removed by pounding plates on paper towels. Plates were developed with 100 µl TMB (3,3′,5,5'-tetra-methylbenzidine substrate [R & D Systems, Minneapolis, USA]). The extent of colour development was determined at OD 630 nm with an Omega FluoStar® plate reader.

The raw OD data was used to determine the antibody titres to each strain of oral streptococci in the patient's saliva. In brief, the duplicate results for each dilution were averaged and the final titre was expressed as ELISA units (EU) (Gmur et al. 1986). The results were calculated with a regression line and derived equation from the serial dilutions of patient saliva. Control saliva, pooled using a small volume of saliva from every study participants at baseline, acted as a quality control and allowed for correction in day to day assay variance and enabled the reproducibility of the assay to be monitored.

2.8 Animals

C57BL/6 mice (Harlan, Bicester, UK), C57BL/6 'TEa' mice with transgenic T cells which recognise $E\alpha_{52-68}$-MHCII complex (obtained from S. McSorley, University of Minnesota, Minneapolis, USA) and C57BL/6 'OT-II' mice with TCR transgenic T cells which recognise $OVA_{323-339}$-MHCII were maintained at the Central Research Facility (University of Glasgow, UK) under specific pathogen free conditions. Female mice aged 6 – 8 weeks were used in all experiments. All procedures were performed according to local and UK Home Office regulations.

2.9 Isolation and culture of bone marrow derived dendritic cells from C57BL/6 mice

Mice were sacrificed by cervical dislocation prior to aseptic removal of femurs and tibias, which were transferred to ice cold RPMI. Muscle and epiphyses were removed to expose the cavim. Bone marrow was flushed through with 2 ml of RPMI 1640 medium using a 2.5 ml syringe and 23 G needle. Bone marrow was disrupted by passing through a cell strainer and the resulting cell suspension was

resuspended at 2 x 10^6 cells/ml in RPMI, supplemented with 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin, 10% foetal calf serum (complete RPMI) and 10% GM-CSF (obtained from X63 myeloma cells transfected with mouse granulocyte–macrophage colony-stimulating factor cDNA). Bone marrow derived cells were cultured in wells of 6-well plates (Costar, Corning) at a concentration of 2 - 4 x 10^6 cells/well under microaerophilic conditions (5% CO_2) at 37˚C. Fresh medium was added to the cell cultures every three days and DC's were harvested on day seven. Differentiation into DC's was confirmed by staining DC's with anti-CD11c (Table 2-5), on average 70% of the cells in culture were CD11c positive by FACS analysis.

2.10 Isolation of murine lymph nodes and spleens

Mice were sacrificed by exposure to a rising concentration of CO_2, followed by cervical dislocation. Lymph nodes and spleens were removed and placed in RPMI on ice. Single cell suspensions were prepared by passing tissues through a 40 µm cell strainer using a 5 ml syringe plunger in the presence of RPMI, supplemented with 2 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin (incomplete RPMI). For isolation of antigen specific T lymphocytes or DCs, lymph nodes were digested with 80 µg of Liberase™ (Roche Diagnostics, Indianapolis, USA) for 30 minutes and then passed through a cell strainer using a pipette.

Splenocytes were pelleted by centrifugation at 400 x g at 4˚C and resuspended in 5 ml of room temperature red lysis buffer (Invitrogen, UK) for 15 min. Cells were pelleted by centrifugation as before and resuspended in incomplete RPMI.

2.11 *In vitro* DC activation

Bone marrow derived DCs (section 2.9, BMDCs) were washed by aspirating media and replaced with RPMI supplemented with 10% heat-inactivated fetal calf serum (HI-FCS). This was repeated once then cells were left to settle for at least 4 h. BMDCs were stimulated for 4, 18 or 24 h with heat-killed or live *S. mutans* UA159 or their conditioned supernatants or with *E. coli* (DH5A), with or without 'model' antigens; either 100 µg/ml exogenous EaGFP, (EαGFP generated from the EαRFP fusion protein was a kind gift from Dr John Butcher of the University of Glasgow,

and has been previously described (Rush et al. 2009) or OVA peptide, as detailed in figure legends. Heat-killed or live *S. mutans* or *E. coli* or live *E.coli* were added to DCs at ratios of 10:1 or 100:1. Following stimulation DCs were scraped from wells using a cell scraper and transferred to 15 ml falcon tubes. DCs were then stained for cell surface receptors using appropriate antibodies (Table 2-5) and analysed by flow cytometry according to the protocol outlined below (section 2.13). Alternatively, DCs were co-cultured with T cells (section 2.12).

2.11.1 Viability assay

BMDCs were stained with anti-CD11c, followed by annexin-V AF-488 conjugate (Life technologies) to detect apoptotic cells, and 7-AAD (eBioscience, Hatfield, UK) to detect non-viable cells. Cells were washed twice in 200 µl FACS buffer (PBS + 2% FCS + 0.05% NaN_3), pelleted by centrifugation at 350 x g and resuspended in 300 µl FACS buffer and analysed by flow cytometry.

2.12 *In vitro* T cell proliferation

Stimulated BMDCs were adjusted to 1 x 10^6 cells/ml in complete RPMI and 100 µl (1 x 10^5 cells) were added in triplicate to wells of a 96-well microtitre plate (Costar, Corning). One hundred µl, of approximately 1 x 10^5 cells/ml of transgenic OT-II T cells were added to wells with DCs at a ratio of 1:1. As a postive control 2 µg/ml of anti-CD3e was added to T cells co-cultured with DCs not previously stimulated with antigen. Plates were incubated for 48 h at 37˚C in 5% CO_2. Supernatants were removed and stored at -80˚C. Spent media was replaced after each 24 h period. T cell proliferation was detected using Click-iT™ EdU Flow Cytometry Assay Kit (Molecular Probes, Invitrogen). EdU incorporation and detection was performed as per manufacturer's instructions. Cells were incubated with 10 µM EdU (AF 647 or 488) for the final 24 h of culture at 37˚C in 5% CO_2. Cells were stained for cell surface receptors as detailed in section (2.13.1 and Table 2-5) and analysed by flow cytometry.

Table 2-5: Murine antibodies used for flow cytometry

Antigen	Isotype	Supplier (Clone)	Label
CD11c	Ar hamster IgG1	eBioscience (N418)	APC, PE
CD40	Rat IgG2a	BD (3/23)	FITC
CD80	Ar hamster IgG2	BD (16-10A1)	FITC
CD86	Rat IgG2a	BD (GL1)	FITC
CD4	Rat IgG2a	BD (RM4-5)	FITC, APC
Ea_{52-68} peptide bound to MHCII	Mouse IgG2b	eBioscience (Y-Ae)	BIO

Antibodies were used according to the manufacturer's recommendations at dilutions of 1/200 per 5 x 10^5 cells. Biotin-conjugated Y-Ae was detected with 0.2 µg/test streptavidin-FITC or –APC (BD Pharmingen, Oxford, UK)

2.13 Flow cytometry

2.13.1 Cell surface antigen staining

To reduce non-specific antibody binding to Fc receptors, murine cells were resuspended in FACS buffer containing Fc block (2.4G2 hybridoma supernatant) at 5 x 10^5 cells/100 µl for 15 min at 4˚C. Cells were transferred to polystyrene tubes ('FACS tubes' Falcon 2052, BD Pharmingen) and stained with various antibodies or appropriate antibody controls at 1/200 dilution (Table 2-5) for 30 min in the dark at 4˚C. Cells were washed twice in 200 µl FACS buffer, centrifuging at 350 x g for 5 min. Where secondary detection reagents were required, cells were resuspended in 100 µl FACS buffer containing the appropriate secondary reagent (Table 2-5) and incubated in the dark for 30 min at 4˚C. Cells were washed twice in 200 ul FACS buffer, centrifuging at 350 x g for 5 min. Finally, cells were resuspended in 300 µl FACS buffer and passed through a 30 µm nitex nylon mesh (Fisher scientific, Leicestershire, UK). Cells were then analysed.

2.13.2 Flow cytometry acquisition and analysis

Cells were acquired and analysed on a Becton Dickenson FACSCalibur using CellQuest Acquisition software. Prior to running labeled cells, FSC (forward scatter) and SSC (side scatter) were adjusted so that cells of interest could be gated on screen. Unstained samples were used as auto-fluorescence controls, allowing settings to be adjusted so that auto-fluorescence background was roughly within the first decade of the log scale of the fluorescence intensity plot. Positively stained controls were used to adjust compensation settings such that detection of each fluorochrome was brightest using the appropriate detector. Data were analysed using FlowJo software (Treestar, Ashland, OR, USA). Results are expressed as % positive cells or mean fluorescence intensity.

2.14 Statistical analysis

Data were obtained from observations and measurements made on individuals (mice, humans, cells etc). Statistical tests were applied to evaluate how far the

observed evidence differed from what would be expected if the null hypothesis were true. From these statistics, p values were calculated and if $p < 0.05$ the null hypothesis was rejected and the difference considered statistically significant.

Clinical data were investigated to determine frequency distributions prior to statistical analysis. Skewed data were $\log_{10}$ transformed to obtain a normal distribution. Parametric statistical tests were applied to normally distributed data. Independent samples student's t-tests were applied to test whether two groups had the same mean. In every instance, Levene's test for equality of variance was considered and the appropriate p value was reported. Analysis of variance (ANOVA) with Bonferroni corrections was used for clinical data or Tukey corrections were used for experimental data were applied to test whether three or more groups had the same mean and ANOVA linear was applied to investigate the linear trend from three or more groups. Pearson bivariate correlations or Linear regression analysis were applied to test correlations and associations, respectively between two normally distributed scale variables.

When data exhibited skewed distribution non-parametric statistical tests were applied. Mann-Whitney U tests were applied to test the difference between two means. Kruskal-Wallis was applied to test differences between the means of three or more groups and Jonckheere-Terpstra test was applied to test the linear trend between three or more groups.

Fisher's exact test was applied to determine the statistical significance of an association between two categorical variables.

All statistical analyses were performed using IBM SPSS software, PAWS statistics 18 and GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, California, USA). All graphs were produced in GraphPad Prism version 4 for Windows. Box plots display median, 25^{th} and 75^{th} percentiles and whiskers show the minimum and maximum values.

Chapter 3: Cross-sectional analysis of caries-associated biological risk factors in one-year-old children

3.1 Introduction

Oral health is a substantial component of general health, well being and quality of life. Dental caries remains one of the most prevalent chronic diseases of childhood, inflicting a substantial social and economic burden (Marthaler 2004). The WHO estimates that dental caries affects between 60-90% of school age children in developed countries and the vast majority of adults (Petersen 2003). Twenty-five percent of three-year-old children in Glasgow have some caries experience (McMahon et al. 2010) and caries prevalence increases with age, with 36% of five-year-old Scottish children having obvious decay experience, rising to 41.8% in the Greater Glasgow area (Macpherson et al. 2010b). Moreover, adult oral health is predicted by childhood dental health, highlighting the importance of epidemiological studies targeted to include the youngest age groups.

Dental caries is a complex, multi-factorial disease known to be influenced by dietary factors, although the disease results from a bacterial infection and as such caries susceptibility may be altered by the host immune response. There is substantial evidence to indicate a causative relationship between caries and the Mutans streptococci, namely *S. mutans* and *S. sobrinus* and their presence in plaque and saliva has been used as an indicator of caries risk (Kristoffersson et al. 1985; Parisotto et al. 2010b). In children with high caries rates *S. mutans* is the predominant organism isolated from carious lesions (Marchant et al. 2001) and comprises 30-50% of plaque microbiota at these sites (Berkowitz et al. 1984). In contrast, only 1% of the oral microbiota comprises *S. mutans* in caries free children (Law et al. 2007). To date there is little understanding of how infants and children respond to the evolution of the oral biofilm and how the host might influence this evolution. It is crucial to better understand this process if novel therapeutics and prevention strategies are to be developed.

Previously, *S. mutans* was believed to colonise the oral cavity during a discreet 'window of infectivity' reported to occur between 18 to 36 months of age (Caufield et al. 1993). *S. mutans* preferentially colonises tooth surfaces, although can be recovered from the mouths of pre-dentate infants (Wan et al. 2001). However, *S. mutans* are not recovered in significant numbers in children until around two-years of age (Taubman and Nash 2006). Studies have shown

that *S. mutans* colonisation increases with increasing age and that this increase is independent of socioeconomic status (Radford et al. 2001). The time of *S. mutans* colonisation is clinically significant as caries risk increases with earlier acquisition of *S. mutans* (Alaluusua and Renkonen 1983).

Dental caries is strongly associated with socioeconomic status (SES) with those from the lower socioeconomic groups having the highest prevalence of the disease (Levin et al. 2009; Macpherson et al. 2010b; Sisson 2007). Inequalities in oral health begin early in life and the SES/oral health relationship is already apparent in children as young as three-years-old with caries prevalence rising from 16% in the least deprived to 32% in the most deprived areas (McMahon et al. 2010). These disparities in health cannot be fully explained by differences in access to health care, behavioural, dietary or genetic factors. Recent evidence suggests that the physiological stress response may provide a link between low SES and chronic disease, such as dental caries (Adler and Rehkopf 2008; Quinonez et al. 2001).

Thus, there are fundamental gaps in current knowledge of the interrelationships of caries risk factors and in particular how the immune system deals with the evolving oral biofilm in young children, and how this may contribute to inequalities in dental caries.

The combination of Childsmile's unusually young target age group and the multiple visit interventions provides a unique opportunity to investigate the evolution of the oral biofilm coincident with the development of the salivary immune response. These studies therefore sought for the first time to collect, process and analyse biological samples, socioeconomic data, dietary information and dental health status in a longitudinal clinical study of Childsmile participants (Figure 3.1 and 3.2).

Initially, the cross-sectional sample of children aged approximately one-year was investigated. The research objectives for this study were as follows:

1. To describe study participants in terms of their demographics, total plaque bacteria, carriage of *S. mutans* and *S. sobrinus* in plaque and saliva and the

concentrations of salivary lactoferrin, LL37, calprotectin, the HNPs 1-3 and sIgA antibodies specific for a panel of oral streptococci.

2. To investigate if socioeconomic inequalities influence total numbers of plaque bacteria, the detection of *S. mutans* and *S. sobrinus* in plaque and saliva, the concentrations of salivary lactoferrin, LL37, calprotectin, the HNPs 1-3, sIgA antibodies specific for a panel of oral streptococci or cortisol.

3. To investigate if salivary cortisol concentrations influence total plaque bacteria, the detection of S. *mutans* and *S. sobrinus* in plaque and saliva, the concentrations of salivary lactoferrin, LL37, calprotectin, the HNPs 1-3 or sIgA antibodies specific for a panel of oral streptococci

4. To investigate if the concentrations of salivary lactoferrin, LL37, calprotectin, the HNPs 1-3 or sIgA antibodies specific for a panel of oral streptococci influence the total numbers of plaque bacteria and the detection of *S. mutans* and *S. sobrinus* in plaque and saliva

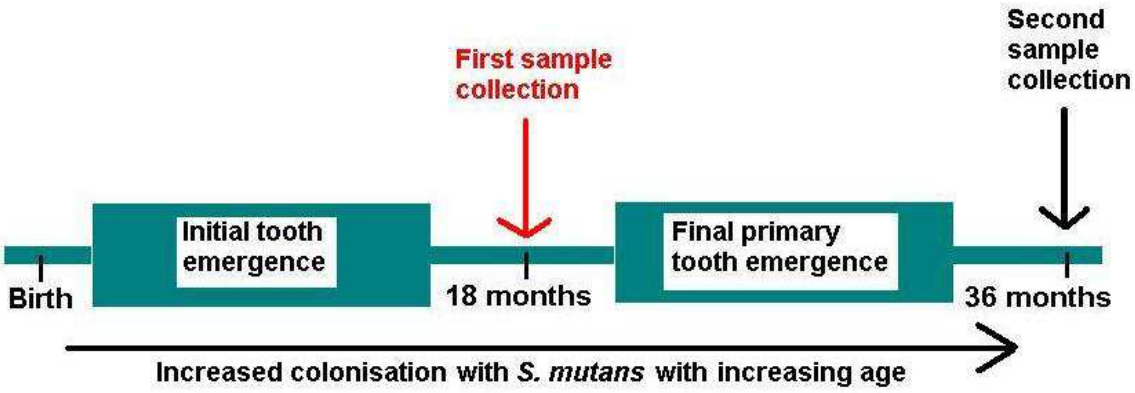


Figure 3-1: Sample collection timeline

Schematic overview of the longitudinal sample collection. Plaque and saliva samples were collected from a cohort of Childsmile participants on two occasions, at approximately 18 and 36 months of age. The present chapter describes the cross-sectional analysis of microbiological and immunological investigations of plaque and saliva, together with demographic data collected from children at the first time point. The timeline for the study was designed to coincide with the eruption of the primary dentition, specifically the molars as these teeth are the preferred attachment sites of *S. mutans* .

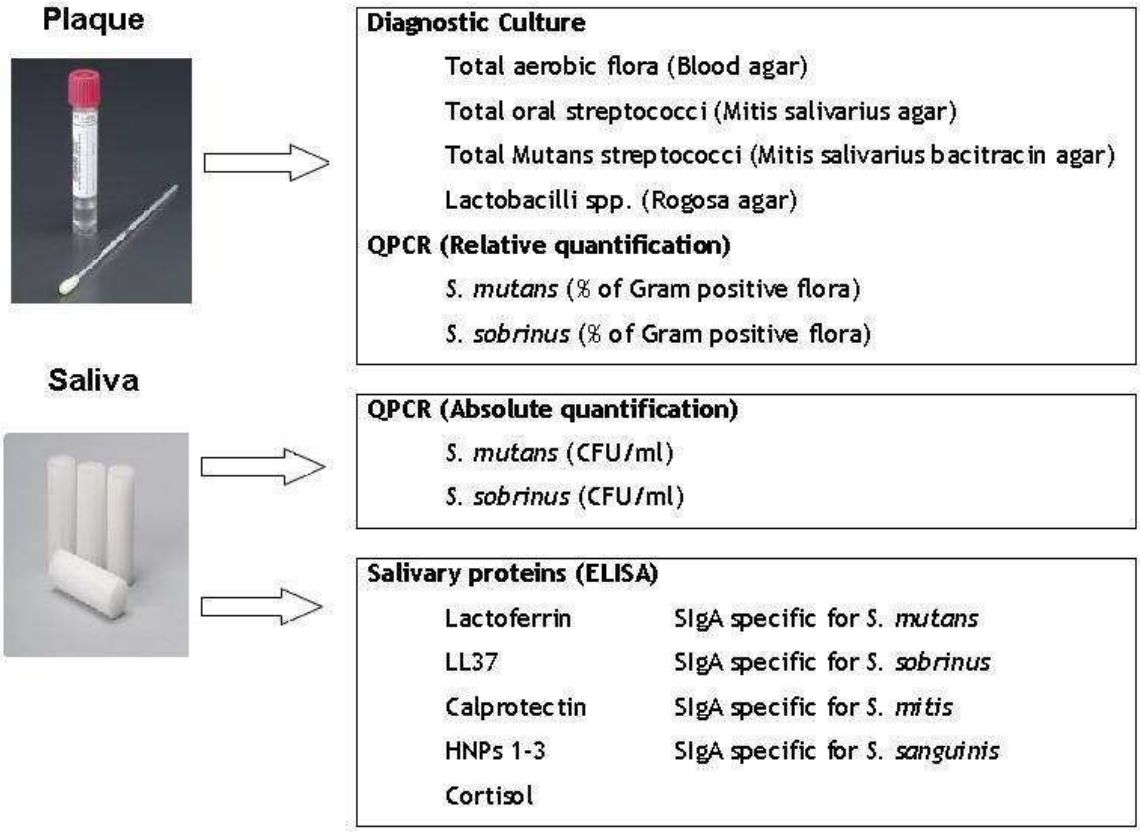


Figure 3-2: Microbiological and immunological investigations of plaque and saliva

Schematic overview of the measures used to investigate the microbiological and immunological characteristics of plaque and saliva samples collected from study participants. Plaque was collected using the Copan Eswab® and was investigated for the presence of oral bacteria by diagnostic culture to determine the CFU/ml of total aerobic flora (as a measure of bacterial load), total oral streptococci and Mutans streptococci. TaqMan® QPCR was used to determine the relative proportions of *S. mutans* and *S. sobrinus* as a percentage of Gram positive plaque flora. Saliva was collected using the Salimetrics children's swab® and was investigated for the presence of cariogenic bacteria. TaqMan® QPCR was used to quantify the absolute numbers of *S. mutans* and *S. sobrinus* from saliva. Saliva was investigated by ELISA for the presence of salivary proteins: antimicrobial peptides (LL37, calprotectin and the HNPs 1-3), the antimicrobial protein lactoferrin and for salivary IgA (sIgA) antibodies specific for a panel of oral streptococci (*S. mutans*, *S. sobrinus*, *S. mitis* and *S. sanguinis*) and salivary cortisol as a surrogate measure of stress.

3.2 Results

Sixty-three children were recruited from the Childsmile programme between April and December 2009 for the present study. The study received approval from the West of Scotland Research Ethics Committee (08/S0703/139) and NHS Greater Glasgow and Clyde R&D Management. Signed parental consent was obtained for each study participant.

3.2.1 Descriptive analysis of demographic data collected by questionnaire

The age of study participants ranged from 12 to 24 months (Table 3-1). This encompassed the target age of 18 months, which was chosen to coincide with the time at which colonisation by *S. mutans* has been shown to increase (Taubman and Nash 2006). The median age of children in the study was 16 months, with peaks at both 12 and 18 months (Figure 3-3). There was no significant difference in gender recruitment for the study with 47% (n = 29) female participants and 53% (n = 33) male participants. The gender of one child was not reported (Table 3-1).

Over 85% (n = 53) of the completed questionnaires were completed by the mothers of study participants, the remainder were completed by fathers. The age of parents who completed the demographic questionnaire was variable ranging from 18 to 44 years with a median age of 29 years. Over 70% (n = 45) of parents reported that they did not smoke (Table 3-1).

Forty-eight percent (n = 30) of parents reported that their child who participated in this study was their only child, while 29% (n = 18) had 2 children, 19% (n = 12) had 3 children and 3.2% (n = 1) reported to have 4 or more children (Table 3-1).

Seventy-one percent (n = 44) of parents reported that their child who participated in the study had not been breastfed. Of those children who were breastfed (n = 18) the length of breastfeeding ranged from 1 to 14 months with a median of 4 months.

Parents reported weaning their child from milk between 4 and 12 months of age with a median of 5 months (Table 3-1). This question asks at what age weaning was started. However, the responses suggest that some parents may have reported the age at which their child was fully weaned, around 8 to 12 months. This is beyond the recommended 6 months to begin weaning.

Parents were asked to provide information regarding the level of education they had received. The level of parental education, particularly mother's education has been used as an indicator of SES and also as an indicator of caries risk (Litt et al. 1995; Warren et al. 2009). In the present study parents reported that they received between 10 and 22 years of full time education, with a median of 12 years (Table 3-1). Education categories were dichotomised as described in (Table 2-1). Thirty-five percent (n = 21) of parents received secondary school education, while the remaining 65% (n = 39) received education to the level of school or college 6th form or above (Table 3-1 and Figure 3-4).

Income categories were dichotomised (Figure 3-5). The original data indicated that children had been recruited to the study from across the socioeconomic scale; however, the low numbers in each category were not useful for any meaningful statistical analyses. Over 43% (n = 23) reported annual household income of below £10,000 and 56.6% (n = 30) reported total income of £10,000 or above. The remaining percentage was divided between those who didn't know, 6.9% (n = 4) and one parent who refused to provide the information (Table 3-1).

Benefit categories were dichotomised (Figure 3-6). Over 37% (n = 22) of households reported that they received half to all of their income from benefits, while the remaining 62.7% (n = 37) received none to about a quarter of their income from benefits (Table 3-1). This data reflects that of the income category in that it suggests that study participants were recruited from across the socioeconomic scale. Furthermore, cross-tabulation of dichotomised income categories against dichotomised benefit categories show a high level of association; as total family income increased the proportion of total income received as benefits decreased ($p = 0.011$ by Fisher's exact).

Postcodes were categorised according to both national and local health board SIMD quintiles. The spread of the data required that these categories were

dichotomised (Figure 3-7). By national SIMD quintiles 64.6% of study participants (for whom postcodes were available, n = 48) lived in postcode sectors ranked within the most deprived quintile (Table 3-1). In contrast, using the local health board SIMD quintiles the proportion of study participants ranked within the most deprived quintile was 42.6% (n = 20). A higher proportion of postcodes were ranked quintiles 2-5 when assessed by local health board SIMD compared to national SIMD (Figure 3-7c & d). The data generated using national SIMD quintiles suggested that recruitment of study participants was highly biased towards those of more deprived areas. On a national level the Greater Glasgow area contains a high proportion of deprived postcode sectors relative to the rest of Scotland. At local level these same areas are relatively less, giving rise to a more even distribution. By local SIMD there was still a bias towards recruitment of children from the relatively more deprived areas of Glasgow. This was not unexpected as these are the families which are specifically targeted by Childsmile for interventional therapy.

Table 3-1: Descriptive analysis of demographic data collected by questionnaire

Demographics	Statistics
Participants	
N	**63**
Age (months) of child	
median, min, max, *Q1, Q3*	**16,** 11, 24, *12, 19*
missing **N**	**1**
Gender of child	
Female **N** (%)	**29** (46.8)
Male **N** (%)	**33** (56.2)
missing **N**	**1**
Age (years) of parent	
median, min, max, *Q1, Q3*	**29**, 18, 44, *24, 37*
missing **N**	3
Gender of parent to complete questionnaire	
Female **N** (%)	**53** (85.5)
Male **N** (%)	**9** (14.5)
Missing **N**	**1**
Parents who smoke	
No **N** (%)	**45** (72.6)
Yes **N** (%)	**17** (27.4)
Missing **N**	**1**
Number of children in families	
1 **N** (%)	**30** (48.4)
2 **N** (%)	**18** (29)
3 **N** (%)	**12** (19.4)
4 **N** (%)	**1** (1.6)
5 **N** (%)	**1** (1.6)
Missing **N**	**1**
Children who were breastfed	
No **N** (%)	**44** (71)
Yes **N** (%)	**18** (29)
Missing **N**	**1**

Demographics	Statistics
Length (months) of breastfeeding	
N	**18**
median, min, max, *Q1, Q3*	**4**, 1, 14, *1, 7*
missing **N**	**1**
Age (months) of weaning	
median, min, max, *Q1, Q3*	**5**, 4, 12, *5, 6*
missing **N**	**11**
Years of parental education	
median, min, max, *Q1, Q3*	**12**, 10, 22, *12, 13*
missing **N**	**3**
Level of parental education	
secondary school **N** (%)	**21**, (35)
college or above **N** (%)	**39**, (65)
missing **N**	**3**
Total household income	
< £10,000 **N** (%)	**23**, (43.4)
> £10,000 **N** (%)	**30**, (56.6)
don't know **N**	**4**
refused **N**	**1**
missing **N**	**5**
Proportion of income received as benefits	
half - all **N** (%)	**22**, (37.3)
none - about a quarter **N** (%)	**37**, (62.7)
missing **N**	**4**
National SIMD quintiles	
most deprived quintile **N** (%)	**31**, (64.6)
quintiles 2-5 **N** (%)	**17**, (35.4)
missing **N**	**15**
Local health board SIMD quintiles	
most deprived quintile **N** (%)	**20**, (42.6)
quintiles 2-5 **N** (%)	**27**, (57.4)
missing **N**	**16**

SIMD: Scottish index of multiple deprivation.

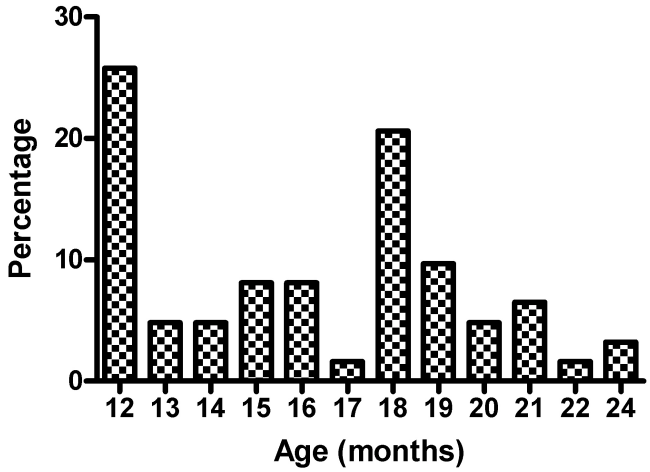


Figure 3-3: Age of study participants

Percentage distribution (n = 63) of the age of study participants (months) at the time of sample collection. The age range encompassed the target age of 18 months, with the greatest proportions of children being recruited to the study at 12 and 18 months of age.

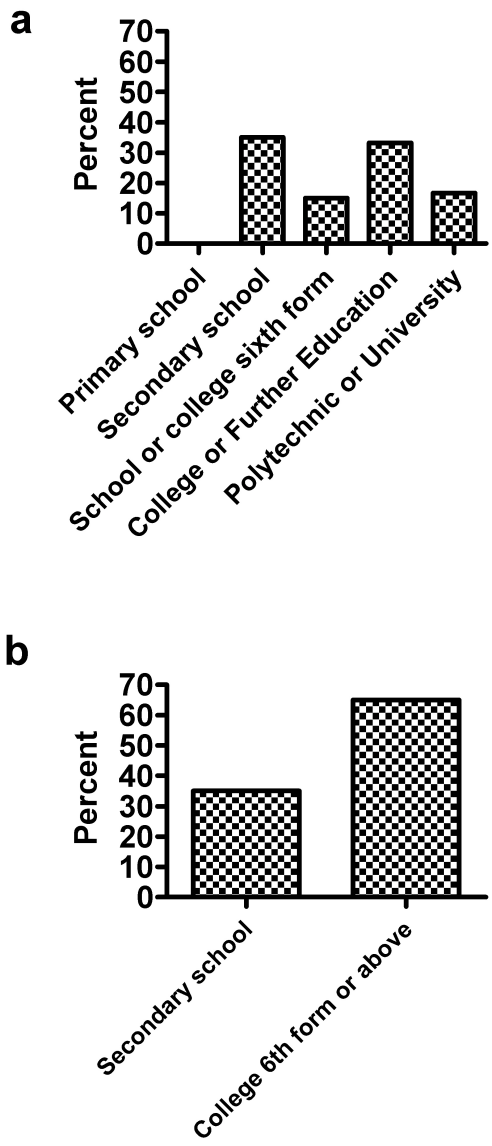


Figure 3-4: Level of parental education obtained

Percentage distribution (n = 60) of the level of parental education obtained. a) Distribution of education categories as collected by questionnaire. b) Dichotomised education categories.

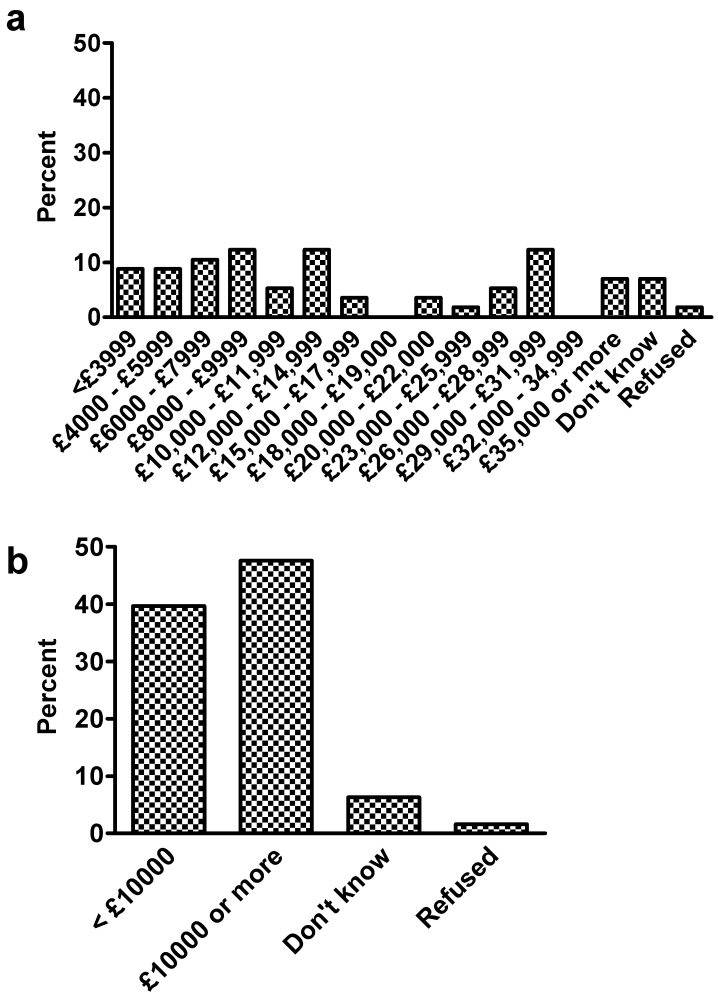


Figure 3-5: Total household income

Percentage distribution (n = 58) of total household income reported by parents of study participants. a) Distribution of income categories as collected by questionnaire. b) Dichotomised distribution of income categories.

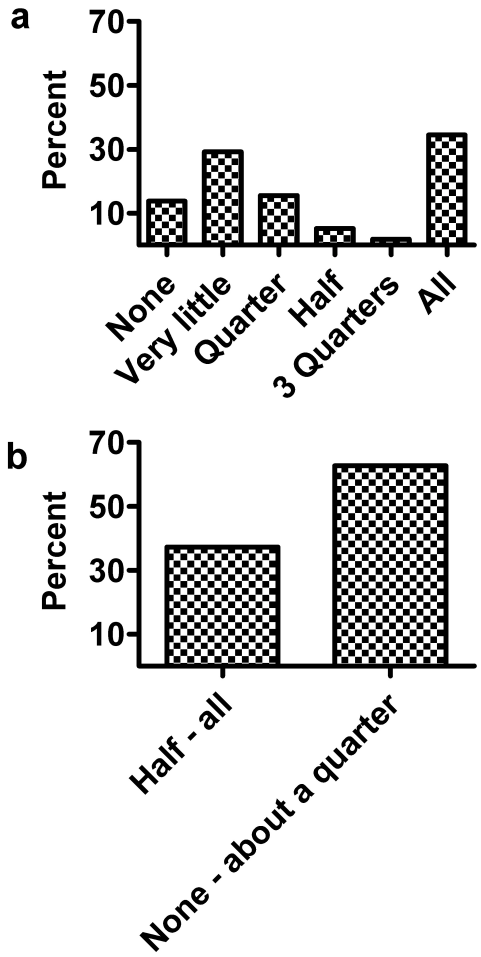


Figure 3-6: Proportion of income received as benefits

Percentage distribution (n = 59) of the proportion of income received as benefits reported by parents of study participants. a) Distribution of the proportion of benefits received by categories as collected by questionnaire. b) Dichotomised benefit categories.

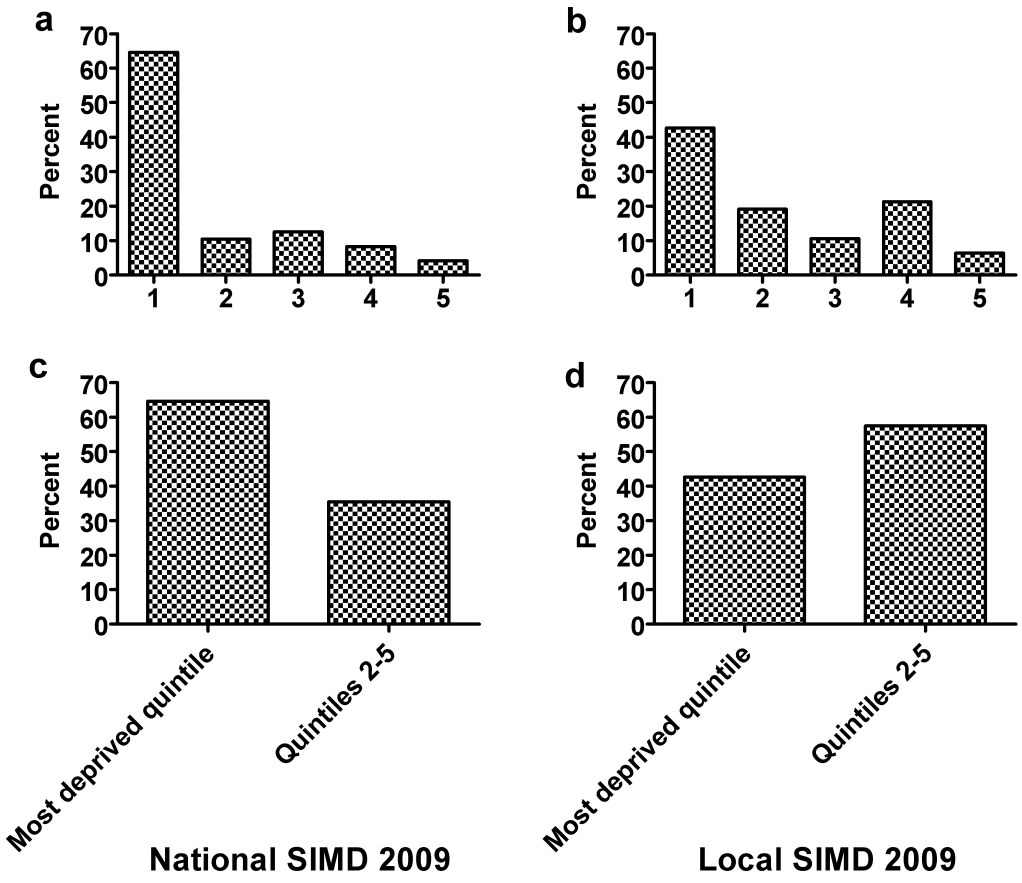


Figure 3-7: National and Local SIMD quintiles 2009

Percentage distribution of postcode sectors ranked by national (n = 48) and local health board SIMD quintiles (n = 48). Distribution of postcodes of study participants by a) national SIMD quintiles, b) local health board SIMD quintiles, c) dichotomised national SIMD quintiles and d) dichotomised local heath board SIMD quintiles.

3.2.2 Descriptive analysis of total plaque bacteria and cariogenic bacteria collected from plaque and saliva of study participants

The numbers of aerobic and cariogenic bacteria were estimated by diagnostic culture of plaque. The study cohort had a geometric mean aerobic bacterial plaque count of 4.8×10^6 CFU/ml, of which approximately 1.9×10^6 CFU/ml were oral streptococci (Table 3-2a), suggesting Streptococci spp. comprised around 40% of the aerobic plaque flora. Mutans streptococci were not isolated from the plaque of over 70% (n = 42) of study participants. In those children who harboured Mutans streptococci the numbers ranged from 1.7×10^3 to 3.2×10^6 CFU/ml with a mean count of 5.2×10^4 CFU/ml, comprising on average 3.9% of oral streptococci (Table 3-2a). Lactobacillus spp. were not isolated from the plaque of study participants.

The relative percentages of *S. mutans* and *S. sobrinus* from plaque were determined by TaqMan® QPCR as a proportion of the Gram positive plaque flora. Both species were detected in the plaque of all children with available samples. The relative percentages ranged from 0.0001 to 1.12% with a mean of 0.012% for *S. mutans* and from 0.0001 to 3.7% with a mean of 0.008% for *S. sobrinus* (Table 3-2a).

Absolute numbers of *S. mutans* and *S. sobrinus* were quantified from saliva by TaqMan® QPCR. Both species were detected in the saliva of all children with measurable samples. The numbers of these organisms in saliva ranged from 204 to 1.2×10^7 CFU/ml with a mean of 8×10^3 CFU/ml for *S. mutans* and 12 to 4.1×10^6 CFU/ml with a mean of 832 for *S. sobrinus* (Table 3-2b).

A Bland-Altman plot was used to determine the level of agreement between the use of diagnostic culture of plaque and TaqMan® QPCR of saliva to quantify Mutans streptococci (Figure 3-8). Overall the mean difference between both methods was 1.8×10^6 CFU/ml as indicated by the level of bias and suggests very little agreement between the two methods. Although, as Mutans streptococci detection in plaque increased so too did detection by TaqMan® QPCR of *S. mutans* in saliva. All culture positive samples were also QPCR positive. However,

the reverse was not true. A number of QPCR positive samples were not detected by culture. Only 14 samples (27.6%) were positive for Mutans streptococci by diagnostic culture of plaque, and so only these samples were available for comparison, thereby limiting the potential results of this test.

The distributions of data within each microbiological data set were investigated using frequency histograms. The percentage distribution of aerobic plaque flora was negatively skewed due to lower than average bacterial counts in some study participants. Oral streptococci were approximately log normal, with a minor negative skew. These distributions are likely an effect of the relatively small sample size (Figure 3-9a & b, respectively). A high proportion of plaque samples were negative for Mutans streptococci by diagnostic culture and so the data were not normally distributed (Figure 3-9c). The distribution of Mutans streptococci counts in children from which it was isolated was log normal (Figure 3-9d).

The distribution of salivary *S. mutans* counts were positively skewed, while salivary *S. sobrinus* counts were approximately log normal (Figure 3-10a & b, respectively). The distributions of *S. mutans* and *S. sobrinus* as relative proportions of Gram positive plaque flora were approximately log normally distributed (Figure 3-10c & d, respectively).

Table 3-2a: Descriptive analysis of microbiological data measured from plaque of study participants

Microbiological measurements	Statistics
Diagnostic culture of plaque (CFU/ml)	
Total aerobic flora **N** (missing)	**62** (1)
not detected **N** (%)	**0** (0)
detected **N** (%)	**62** (100)
mean, min, max	**4.8×10^6**, 1×10^5, 5.5×10^7
95% CIs (lower, upper)	(3.1×10^6, 7.6×10^6)
Total oral streptococci **N** (missing)	**60** (3)
not detected **N** (%)	**0** (0)
detected **N** (%)	**60** (100)
mean, min, max	**1.9×10^6**, 1×10^5, 3.4×10^7
95% CIs (lower, upper)	(1.2×10^6, 2.9×10^6)
Total Mutans streptococci **N** (missing)	**58** (5)
not detected **N** (%)	**42** (72.4)
detected **N** (%)	**15** (27.6)
mean, min, max	**5.2×10^4**, 1.7×10^3, 3.2×10^6
95% CIs (lower, upper)	(1.6×10^4, 1.7×10^5)
Relative quantitation by QPCR (% Gram positive plaque flora)	
S. mutans **N** (missing)	**60** (3)
not detected **N** (%)	**0** (0)
detected **N** (%)	**60** (100)
mean, min, max	**0.0012**, 0.0001, 1.12
95% CIs (lower, upper)	(0.0006, 0.0022)
S. sobrinus **N** (missing)	**60** (3)
not detected **N** (%)	**0** (0)
detected **N** (%)	**60** (100)
mean, min max	**0.0008**, 0.0001, 3.7
95% CIs (lower, upper)	(0.0004, 0.0014)

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

Table 3-2b: Descriptive analysis of microbiological data measured from the saliva of study participants

Microbiological measurements	Statistics
Absolute quantitation by QPCR (CFU/ml)	
S. mutans **N** (missing)	
not detected **N** (%)	**57** (6)
detected **N** (%)	**0** (0)
mean, min, max	**57** (100)
95% CIs (lower, upper)	**8 x 10^3**, 204, 1.2 x 10^7, (3.9×10^3, 1.6 x 10^5)
S. sobrinus **N** (missing)	
not detected **N** (%)	**57** (6)
detected **N** (%)	**0** (0)
mean, min, max	**57** (100)
95% CIs (lower, upper)	**832**, 12, 4.1 x 10^6 (457, 1.5 x 10^3)

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

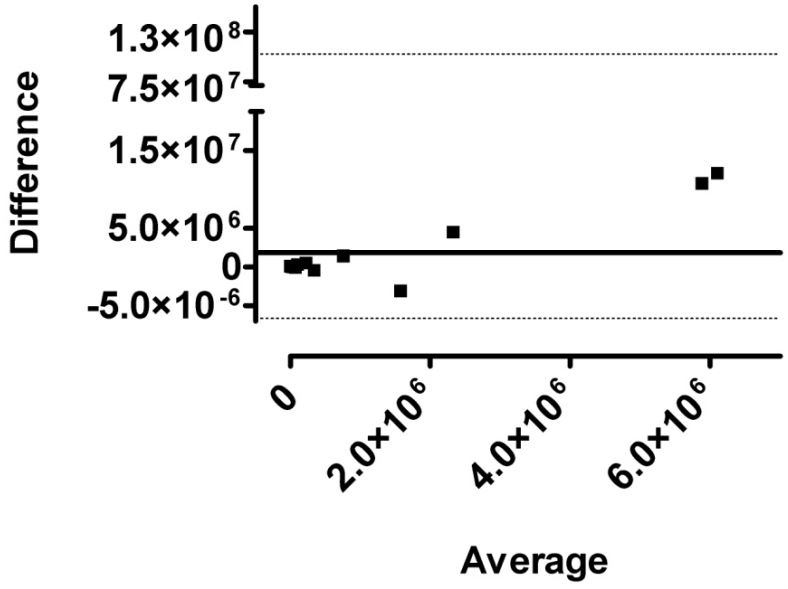


Figure 3-8: Agreement between diagnostic culture of plaque and TaqMan® QPCR to quantify Mutans streptococci

A Bland-Altman plot of the level of agreement between Mutans streptococci (CFU/ml) by diagnostic culture of plaque and *S. mutans* (CFU/ml) by TaqMan® QPCR of saliva. Each data point represents the difference (TaqMan® QPCR of saliva minus culture of plaque) between the methods plotted as a function of the average for each study participant. The solid black line represents the level of bias and the dotted lines represent the 95% limits of agreement.

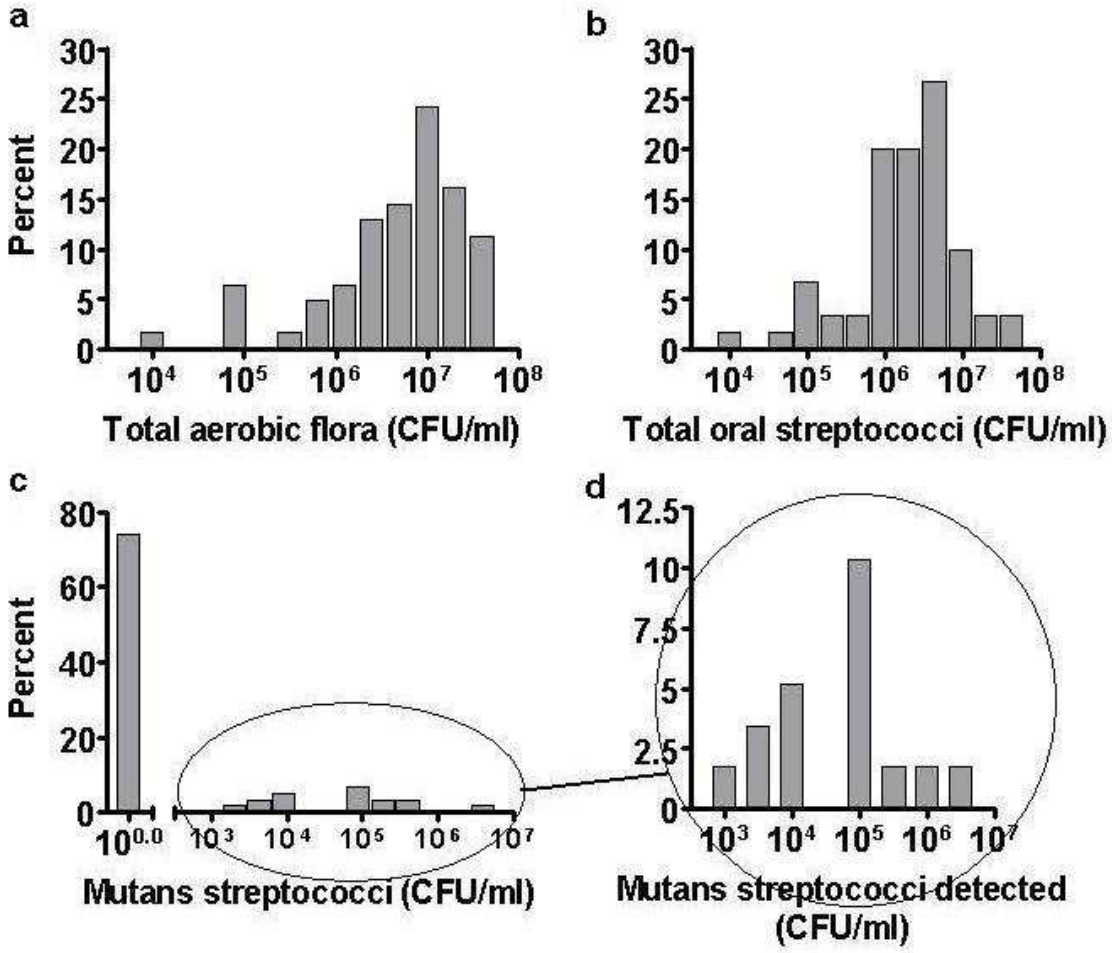


Figure 3-9: Study cohort distribution of plaque bacterial counts estimated by diagnostic culture

Histograms of percentage distribution of $\log_{10}$ transformed bacterial counts from study participants. Plaque was assessed for the presence of a) total aerobic flora (n = 62) b) total oral streptococci (n = 60) and c) Mutans streptococci by diagnostic culture in all children assessed (n = 58) d) Mutans streptococci counts in children in whom it was detected (n = 15).

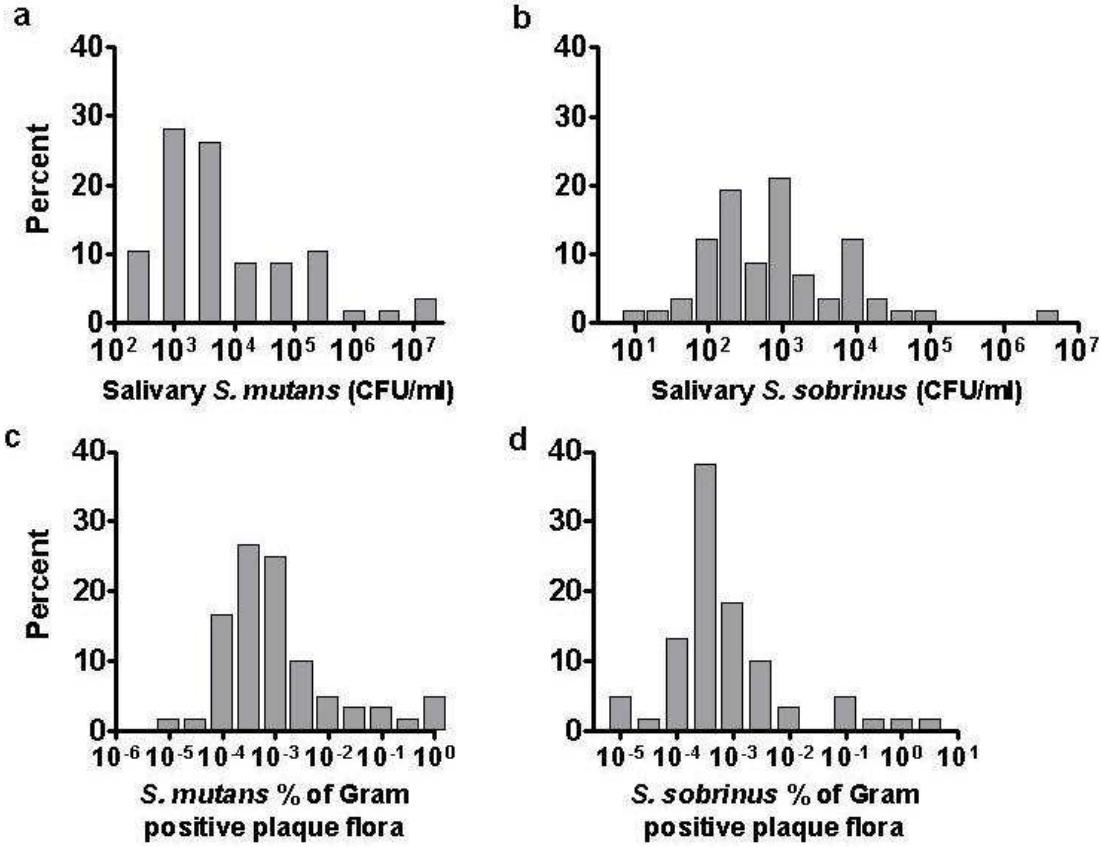


Figure 3-10: Study cohort distribution of bacterial counts estimated by TaqMan® QPCR of saliva and plaque

Histograms of percentage distribution of $\log_{10}$ transformed bacterial counts from study participants. Saliva was assessed for the absolute counts of a) *S. mutans* and b) *S. sobrinus* (n = 57), and plaque was assessed for the relative percentage of c) *S. mutans* and d) *S. sobrinus* (n = 60) by TaqMan® QPCR.

3.2.3 Descriptive analysis of salivary antimicrobial proteins collected from study participants

Saliva samples collected from study participants were investigated by ELISA for the presence of various antimicrobial proteins. Lactoferrin was detected in the saliva of all study participants for whom samples were available (n = 60). The geometric mean concentration of lactoferrin was 1135 ng/ml, ranging from 49.4 to 13,614.5 ng/ml (Table 3-3).

The concentrations of antimicrobial peptides ([AMPs] LL37, calprotectin and the HNPs 1-3) in saliva were assessed. AMPs were detected in the saliva of all study participants assessed, with the exception of LL37 which was undetectable in the saliva of over a quarter of children (n = 12/45). In children for whom peptides were detected, the concentrations were highly variable with around a hundred-fold change in the range for each. LL37 was detected in the range of 0.49 to 40.93 ng/ml, with a geometric mean of 2.53 ng/ml. Calprotectin in the range of 1.3 to 4365 ng/ml, with a geometric mean of 306.9 ng/ml and the HNPs 1-3 in the range of 0.1 to 774.5 ng/ml with a geometric mean of 37.5 ng/ml (Table 3-3).

Titres of sIgA antibodies specific for a panel of oral streptococci were assessed. Salivary IgA antibodies specific for each of *S. mutans*, *S. sobrinus*, *S. mitis* and *S. sanguinis* could be detected in the saliva of all children assessed (n = 51). Salivary IgA antibody titres were highly variable among study participants. However, the ranges and mean EU for each antibody specificity were relatively similar (Table 3-3).

Hypotheses are beginning to emerge that chronic disease, including heart disease, cancers and oral diseases may be influenced by stress responses. Salivary cortisol levels in the saliva of study participants were assessed as a surrogate measure of stress. Cortisol could be detected in the saliva of all children from whom samples were available (n = 57). The study cohort had a geometric mean cortisol concentration of 0.13 µg/dL with a range from 0.02 to 1.15 µg/dL (Table 3-3).

Distributions of the levels of salivary proteins measured from study participants were assessed using frequency histograms. Lactoferrin data were log normally distributed (Figure 3-11). Calprotectin and the HNPs 1-3 had an approximately log normal distribution (Figure 3-12a & b, respectively). In a high proportion of study participants LL37 was not detected and so the data were majorly negatively skewed (Figure 3-12c). In children in whom LL37 was detected the distribution remained negatively skewed (Figure 3-12d). Salivary IgA antibody titres were log normally or approximately log normally distributed (Figure 3-13). Salivary cortisol concentrations were approximately log normally distributed (Figure 3-14).

Table 3-3: Descriptive analysis of salivary proteins collected from study participants

Salivary proteins	Statistics
Lactoferrin (ng/ml) **N** (missing)	**60** (3)
mean, min, max	**1135**, 49.4, 13614.5
95% CIs	844, 1525.8
LL37 (ng/ml) **N** (missing)	**47** (16)
not detected **N** (%)	**12** (25.5)
detected **N** (%)	**35** (74.5)
mean, min, max	**2.53**, 0.49, 40.93
95% CIs	183, 3.50
Calprotectin (ng/ml) **N** (missing)	**60** (3)
mean, min, max	**306.9**, 1.3, 4365.2
95% CIs	211.4, 444.7
HNPs 1-3 (ng/ml) **N** (missing)	**60** (3)
mean, min, max	**37.5**, 0.1, 774.5
95% CIs	24.7, 57.1
S. mutans specific sIgA (EU) **N** (missing)	**51** (12)
mean, min, max	**198.6**, 59, 885.1
95% CIs	169.6, 232.2
S. sobrinus specific sIgA (EU) **N** (missing)	**51** (12)
mean, min, max	**273.5**, 92.9, 981.7
95% CIs	240.2, 310.8
S. mitis specific sIgA **N** (EU) (missing)	**51** (12)
mean, min, max	**189.7**, 47, 970.5
95% CIs	158.6, 226.8
S. sanguinis specific sIgA (EU) **N** (missing)	**51** (12)
mean, min, max	**188.8**, 69, 1285.3
95% CIs	159.4, 224.0
Cortisol (µg/dL) **N** (missing)	**57** (6)
mean, min, max	**0.13**, 0.02, 1.15
95% CIs	0.11, 0.16

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

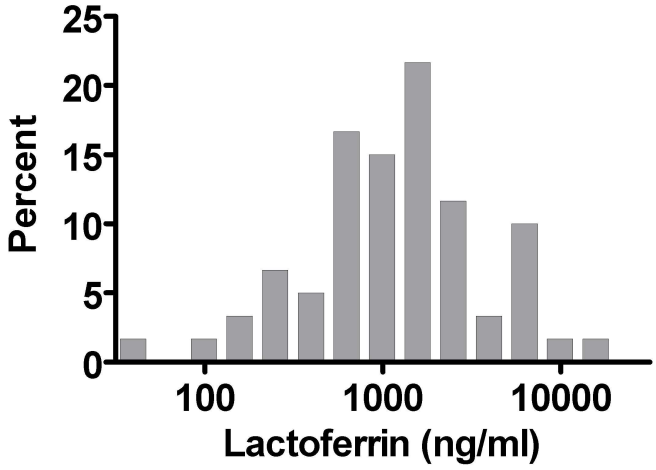


Figure 3-11: Study cohort distribution of lactoferrin

Histogram of percentage distribution (n = 60) of log_{10} transformed lactoferrin concentrations (ng/ml) from saliva of study participants.

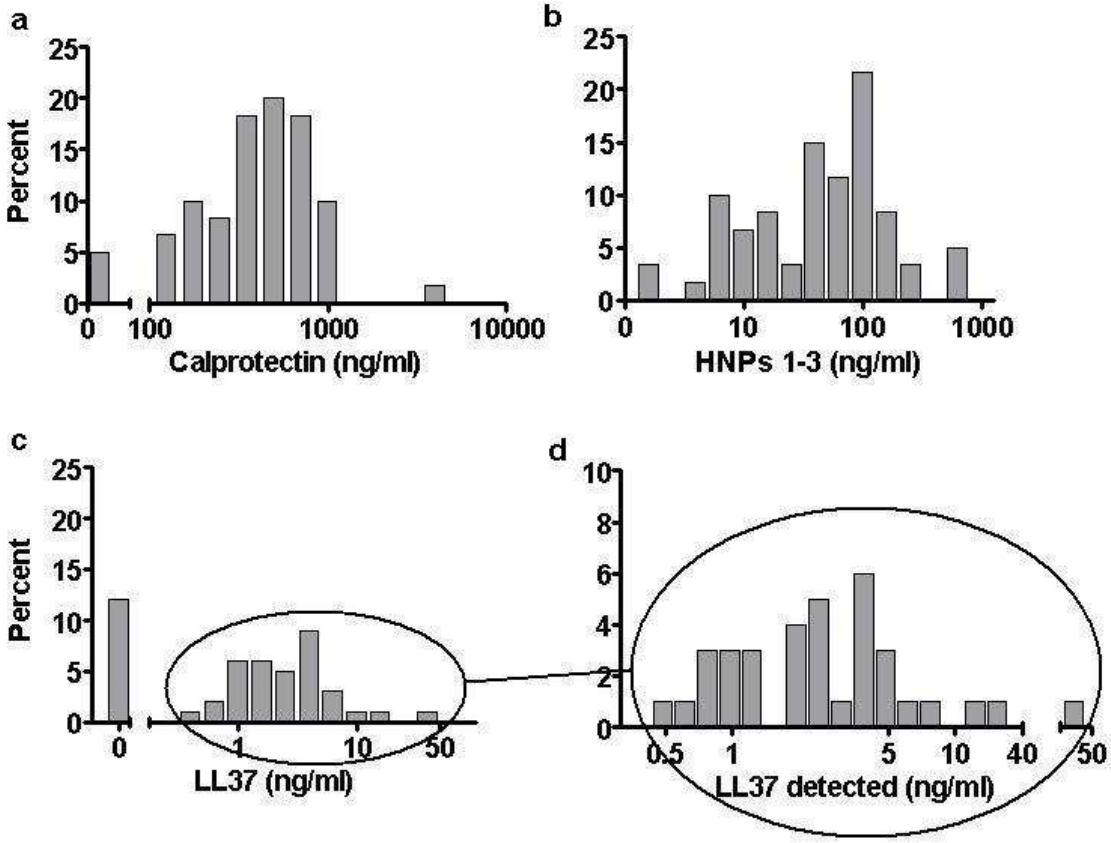


Figure 3-12: Study cohort distribution of antimicrobial peptides

Histograms of percentage distribution of log_{10} transformed concentrations of antimicrobial peptides (ng/ml) from the saliva of study participants. a) calprotectin (n = 60), b) HNPs 1-3 (n = 60), c) LL37 in saliva of all children with measurable samples (n = 47) and d) LL37 only in children from whom LL37 was detected (n = 35).

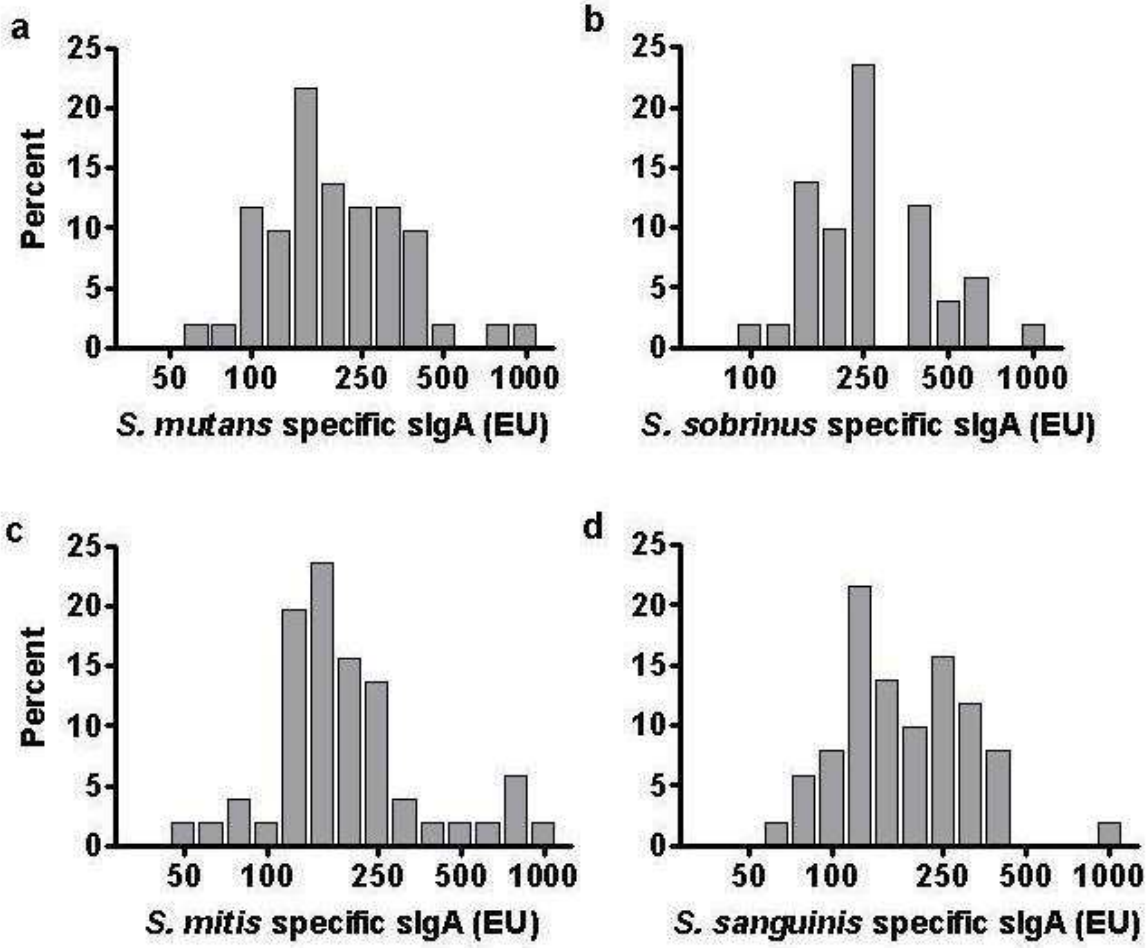


Figure 3-13: Study cohort distribution of sIgA antibodies specific for oral streptococci

Histograms of percentage distribution (n = 51) of $\log_{10}$ transformed titres of sIgA antibodies from study participants specific for a) *S. mutans* b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*.

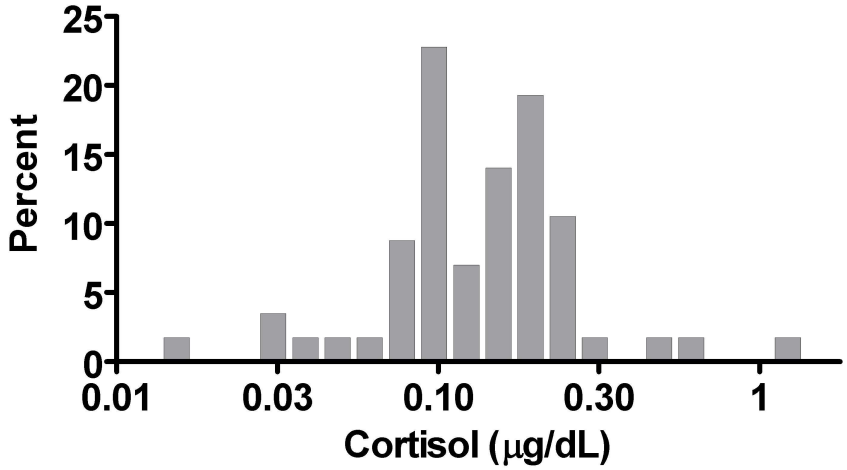


Figure 3-14: Study cohort distribution of salivary cortisol

Histogram of percentage distribution (n = 57) of log_{10} transformed salivary cortisol concentrations (μg/dL) from study participants.

3.2.4 Are there socioeconomic inequalities in bacterial numbers, salivary immune responses and salivary cortisol levels in young children?

It was hypothesised that children of lower SES may have measureable differences in the bacterial numbers of plaque and saliva and in the concentrations of salivary proteins compared with children of higher SES. Therefore, bacterial counts and salivary protein concentrations were assessed in the context of each SES measure (household income, proportion of benefits received, parental education and SIMD).

3.2.4.1 Bacterial counts according to total household income

There were no statistically significant differences in the numbers of bacteria detected by diagnostic culture of plaque from children according to the level of household income received. The geometric mean of *S. mutans* (CFU/ml) detected in saliva by TaqMan® QPCR was marginally higher in children from families earning an annual income of £10,000 or more compared with children from families earning less than £10,000. The difference was not statistically significant (p = 0.788, Table 3-4). Conversely, the geometric mean of *S. mutans* as a proportion of Gram positive plaque bacteria, detected by TaqMan® QPCR, was higher in children from families earning an annual income of below £10,000. The difference was not statistically significant (p = 0.304, Table 3-4).

Mutans streptococci could not be detected in the plaque of a high proportion of study participants by diagnostic culture (n = 42/58, Table 3-2). Cross-tabulation revealed no difference in the proportion of children in whom Mutans streptococci was cultured from plaque compared to children who were culture negative, according to the level of household income, (p = 1, Table 3-5).

The median values for Mutans streptococci (CFU/ml) in plaque by diagnostic culture were equivalent to ‘not detected’ across both income categories. The higher upper percentile and maximum values in children from the lower income category, suggest that in this group the 27.3% of children with detectable counts of Mutans streptococci had higher numbers compared to the 29.6% of children

with detectable Mutans streptococci from the higher income category (Figure 3-15a). This difference was not statistically significant by Mann-Whitney U test ($p = 0.112$), but may have clinical relevance. Indeed the difference in Mutans streptococci (CFU/ml) in only those children in whom it was detected by culture ($n = 15$), compared by the level of household income was found to be statistically significant by Mann-Whitney U test ($p = 0.028$, data not shown).

Graphical summaries of the raw data revealed higher aerobic plaque flora in children from the higher income category had as indicated by the median, upper percentile and maximum values (Figure 3-16a). This finding was in contrast to a higher geometric mean value in children from the lower income category (Table 3-4), although the differences were not statistically significant in either case.

There were no statistically significant differences in the distribution of oral streptococci across the income categories, or in the distribution of *S. mutans* or *S. sobrinus* from the saliva or plaque of children detected by TaqMan® QPCR (Figure 3-16b-f).

Table 3-4: Analysis of bacterial counts from plaque and saliva of study participants with respect to total household income

Bacterial counts by total household income	N	Mean	95% CIs		P
			Lower	Upper	
Diagnostic culture from plaque (CFU/ml)					
Total aerobic flora					
< £10,000	22	4.4×10^6	1.8×10^6	1.0×10^7	0.881
≥ £10,000	30	4.1×10^6	2.1×10^6	7.8×10^6	
Total oral streptococci					
< £10,000	22	2.1×10^6	1.2×10^6	3.7×10^6	0.527
≥ £10,000	29	1.6×10^6	7.8×10^5	3.1×10^6	
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)					
S. mutans					
< £10,000	20	7.4×10^3	1.8×10^3	3.0×10^4	0.788
≥ £10,000	28	9.2×10^3	3.4×10^3	2.5×10^4	
S. sobrinus					
< £10,000	20	840	262	2.7×10^3	0.944
≥ £10,000	28	882	369	1.7×10^3	
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive plaque flora)					
S. mutans					
< £10,000	22	0.0022	0.0007	0.0070	0.304
≥ £10,000	29	0.0010	0.0004	0.0028	
S. sobrinus					
< £10,000	21	0.0015	0.0004	0.0056	0.275
≥ £10,000	30	0.0007	0.0003	0.0017	

Mean bacterial counts in plaque and saliva of children grouped according to the level of total household income were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

Table 3-5: Cross-tabulation of Mutans streptococci detection by dichotomised measures of socioeconomic status

		Mutans streptococci		Total	P
		Not Detected	Detected		
Income					
< £10,000	count	16	6	22	
	%	**72.7**	**27.3**	**100**	
≥ £10,000	count	19	8	27	1.000
	%	**70.4**	**29.6**	**100**	
Total	count	35	14	49	
	%	**71.4**	**28.6**	**100**	
Proportion of income received as benefits					
Half – all	count	18	3	21	
	%	**85.7**	**14.3**	**100**	
None – quarter	count	23	10	33	0.211
	%	**69.7**	**30.3**	**100**	
Total	count	41	13	54	
	%	**75.9**	**24.1**	**100**	
Parental education					
Secondary school	count	18	3	21	
	%	**85.7**	**14.3**	**100**	
6th form or above	count	24	10	34	0.328
	%	**70.6**	**29.4**	**100**	
Total	count	42	13	55	
	%	**76.4**	**23.6**	**100**	
Local SIMD quintiles (2009)					
Most deprived quintile	count	14	5	19	
	%	**73.7**	**26.3**	**100**	
Quintiles 2-5	count	19	7	26	1.000
	%	**73.1**	**26.9**	**100**	
Total	count	33	12	45	
	%	**73.3**	**26.7**	**100**	

Cross-tabulation of Mutans streptococci detection by diagnostic culture of plaque of study participants, grouped according to dichotomised measures of socioeconomic status. P values generated by Fishers exact test

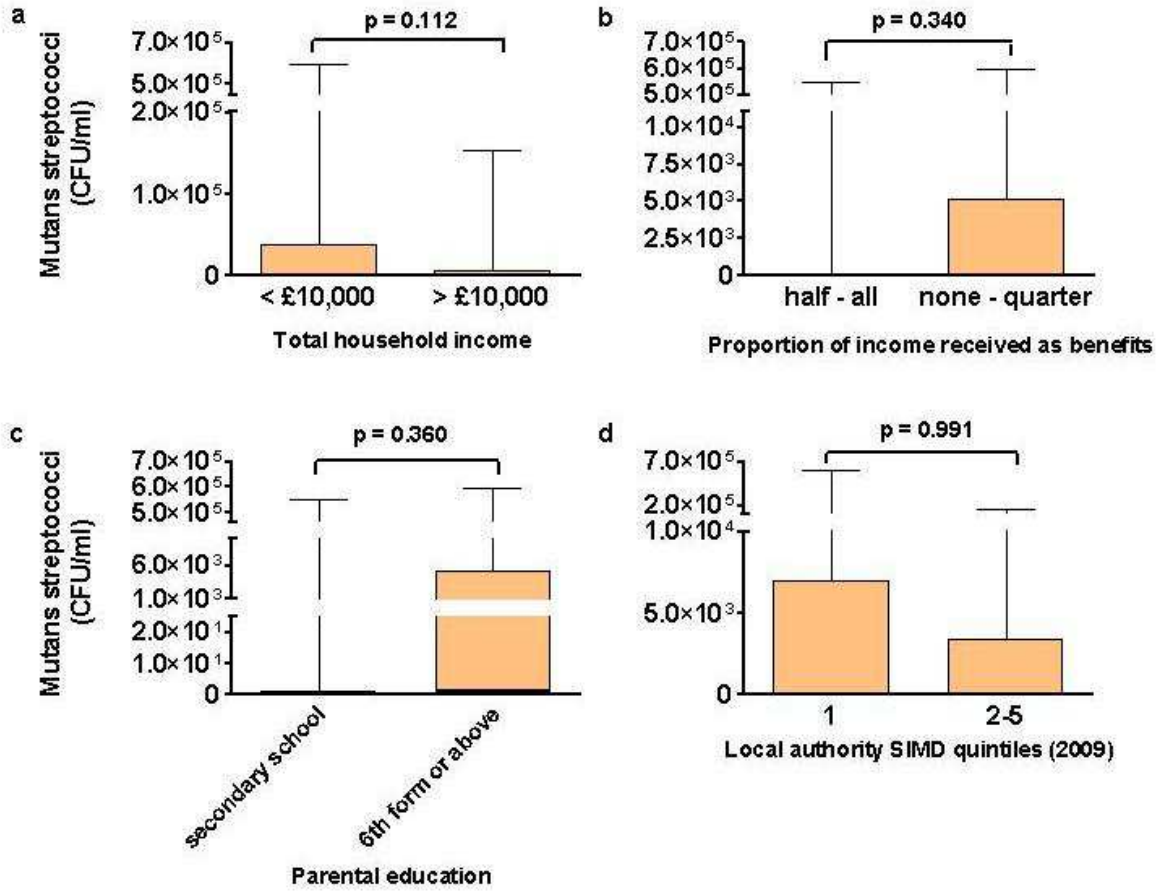


Figure 3-15: Mutans streptococci counts from plaque of children grouped according to measures of socioeconomic inequalities

Box plots of Mutans streptococci (CFU/ml), estimated by diagnostic culture of plaque and grouped according to dichotomised measures of a) total household income (n = 20 vs 27), b) proportion of income received as benefits (n = 16 vs 27), c) level of parental education obtained (n = 16 vs 31) and d) local health board SIMD quintiles (n = 17 vs 26). Raw data were plotted in each instance. Differences were not significant by Mann-Whitney U tests.

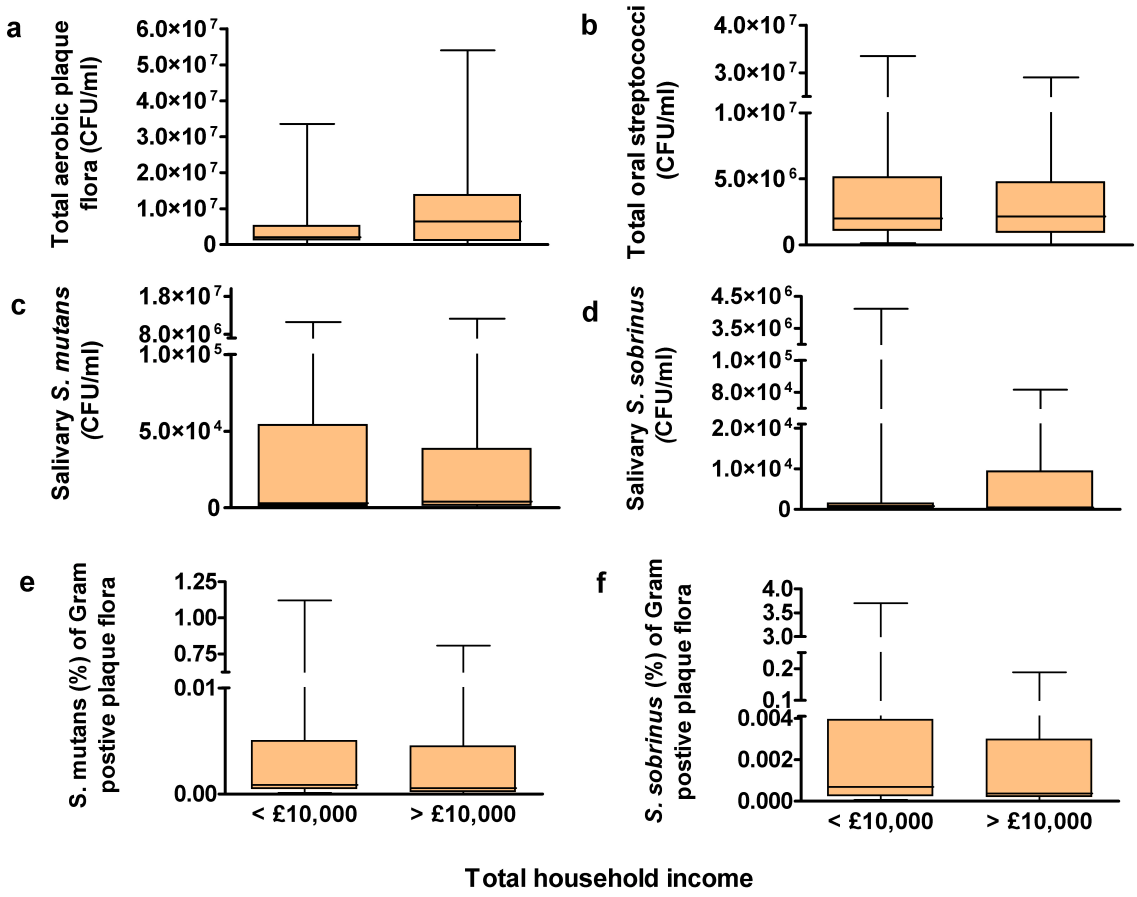


Figure 3-16: Bacterial counts in plaque and saliva of children grouped according to level of total household income

Box plots of bacterial counts from plaque and saliva of study participants grouped according to the level of total household income a) total aerobic plaque flora (CFU/ml [n = 22 vs 30]), b) total oral plaque streptococci (CFU/ml [n = 22 vs 29]), c) salivary *S. mutans* (CFU/ml [n = 20 vs 28]) d) salivary *S. sobrinus* (CFU/ml [n = 20 vs 28]), and e) *S. mutans* (% [n = 22 vs 29]) and f) *S. sobrinus* (% [n = 21 vs 30]) of Gram positive plaque flora. Raw data were plotted in each instance. Differences were not significant by independent samples t-tests (Table 3-4) or Mann-Whitney U tests (data not shown).

3.2.4.2 Bacterial counts according to the proportion of income received as benefits

Independent samples t-tests revealed a significantly higher geometric mean bacterial load, estimated by total aerobic flora, in the plaque of children from families receiving a high proportion of their income as benefits (p = 0.03, Table 3-6). This was also reflected in the geometric mean levels of total oral streptococci which were twice as high in children from families receiving a high proportion of income from benefits compared with those receiving less, although the difference was not statistically significant, (p = 0.129 Table 3-6).

The geometric means were higher for salivary *S. mutans* and *S. sobrinus* in children from families receiving none to about a quarter of their income from benefits, although these differences were not statistically significant (p = 0.263 and 0.715, respectively). Furthermore, the geometric means for the proportion of *S. mutans* and *S. sobrinus* as a percentage of Gram positive plaque flora were also higher in children from families receiving a smaller proportion of benefits (p = 0.287 and 0.793, respectively Table 3-6). While these differences were not statistically significant they reflect the finding that a higher proportion of children, in whom Mutans streptococci were cultured from plaque, were from families who received a smaller proportion of their income from benefits (30.3% vs 14.3% of children from families receiving a high proportion of benefits, p = 0.211 [Table 3-5]). Graphical summary of the raw data revealed no difference in the median values across the benefit categories as a high proportion from each category were negative for detection of Mutans streptococci (half to all [85.7%] and none to about a quarter [69.7%] Table 3-5). However, the higher upper percentile and maximum values for children from families who received a small proportion of their income as benefits indicates that children in whom Mutans streptococci was detected had higher counts than their counterparts in the high benefit category. The difference was not statistically significant by Mann-Whitney U test (Figure 3-15b).

Graphical summaries of the raw data revealed no difference in the distributions of aerobic flora or oral streptococci, detected by diagnostic culture of plaque across the benefit categories (Figure 3-17a & b).

Median salivary *S. mutans* was over three times higher in children from families receiving a smaller proportion of their income from benefits ($p = 0.043$, Figure 3-17c). However, the median values were low (1213 and 4100 CFU/ml for high and low proportion of benefits received, respectively) and are unlikely to be biologically meaningful. Median salivary *S. sobrinus* was also higher in children from families receiving a smaller proportion of benefits, although the difference was not statistically significant (Figure 3-17d and data not shown). Similarly, median values for *S. mutans* and *S. sobrinus* as a proportion of Gram positive plaque flora were higher in children from families receiving a smaller proportion of benefits, although the differences were not statistically significant (Figure 3-17e & f and data not shown).

Table 3-6: Analysis of bacterial counts from plaque and saliva of children with respect to the proportion of income received as benefits

Bacterial counts by the proportion of income received as benefits	N	Mean	95% CIs		P
			Lower	Upper	
Diagnostic culture from plaque (CFU/ml)					
Total aerobic flora					
half - all	22	7.7×10^6	4.6×10^6	1.3×10^7	0.030
none – about a quarter	36	3.1×10^6	1.6×10^6	6.0×10^6	
Total oral streptococci					
half - all	22	2.4×10^6	1.6×10^6	3.8×10^6	0.129
none – about a quarter	35	1.4×10^6	7.2×10^5	2.6×10^6	
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)					
S. mutans					
half - all	20	4.1×10^3	1.1×10^3	1.6×10^4	0.263
none – about a quarter	33	9.4×10^3	4.0×10^3	2.2×10^4	
S. sobrinus					
half - all	20	690	195	2.4×10^3	0.716
none – about a quarter	33	879	419	1.8×10^3	
Relative quantitation of plaque by TaqMan® QPCR (% of Gram positive plaque flora)					
S. mutans					
half - all	21	0.0007	0.0002	0.0021	0.287
none – about a quarter	35	0.0014	0.0006	0.0031	
S. sobrinus					
half - all	21	0.0007	0.0002	0.0022	0.793
none – about a quarter	35	0.0008	0.0003	0.0019	

Mean bacterial counts from plaque and saliva of children grouped according to the proportion of total income received as benefits were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

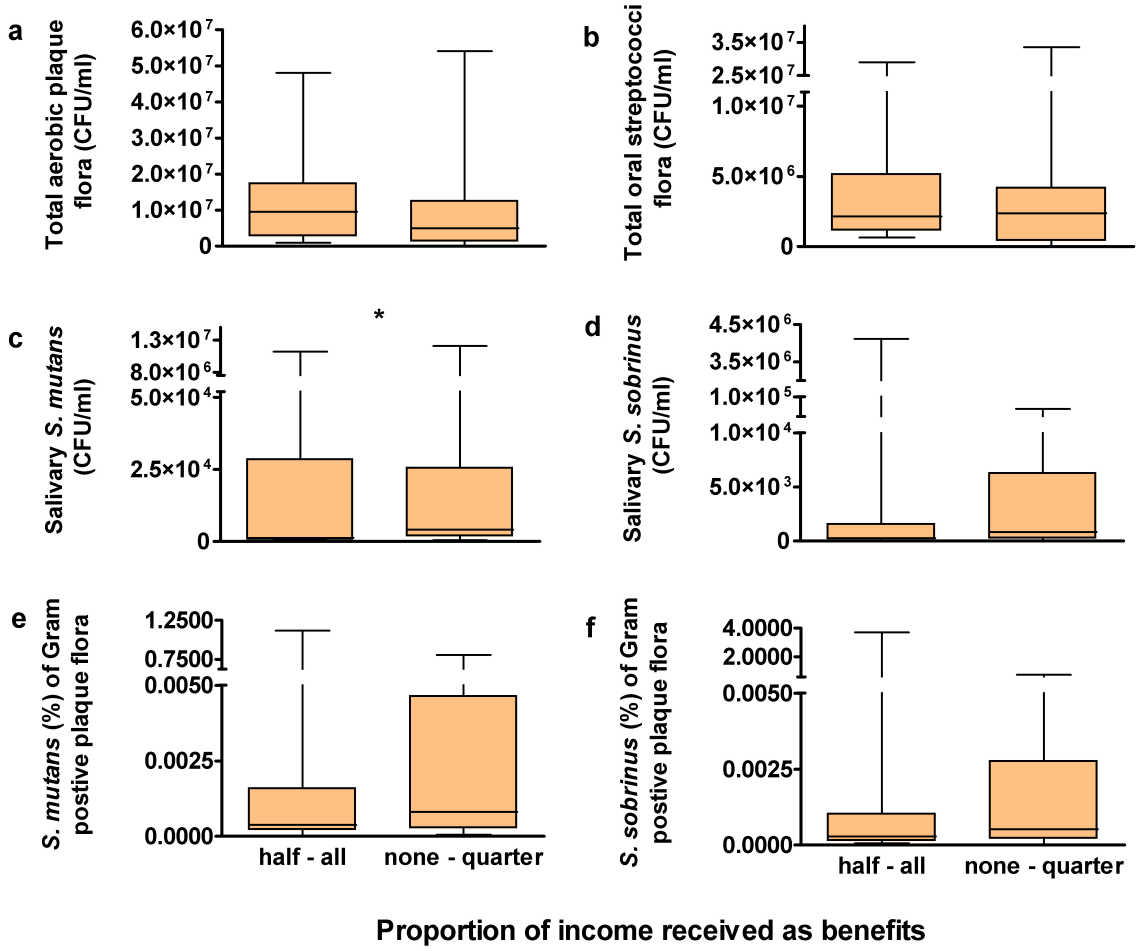


Figure 3-17: Bacterial counts in plaque and saliva from children grouped according to the proportion of household income received as benefits

Box plots of bacterial counts from plaque and saliva of study participants grouped according to the proportion of total household income received as benefits a) total aerobic plaque flora (CFU/ml [n = 22 vs 36]), b) total oral plaque streptococci (CFU/ml [n = 22 vs 35]), c) salivary *S. mutans* (CFU/ml n = 20 vs 33]) d) salivary *S. sobrinus* (CFU/ml [n = 20 vs 33]), and e) *S. mutans* (% [n = 21 vs 35]) and f) *S. sobrinus* (% [n = 21 vs 35]) of Gram positive plaque flora. Raw data were plotted in each instance. Differences were significant only for salivary *S. mutans* (*p = 0.043) by Mann-Whitney U test. Differences were not significant by independent samples t-tests (Table 3-6).

3.2.4.3 Bacterial counts according to the level of parental education

There were no statistically significant differences in the geometric means of aerobic plaque flora or oral streptococci, measured by diagnostic culture of plaque or of *S. mutans* and *S. sobrinus* in plaque and saliva, quantified by TaqMan® QPCR, when compared by the level of parental education (Table 3-7). However, salivary *S. mutans* (CFU/ml) was over twice as high in children with a parent who received a 6th form education or above, the difference was not statistically significant (p = 0.205).

Cross-tabulation revealed a higher proportion of children with detectable levels of plaque Mutans streptococci had a parent who obtained education equivalent to 6th form or above (29.4%) compared to children from parents with only a secondary school education (14.3%). The difference was not statistically significant by Fishers exact test (Table 3-5). The proportion of children who were culture negative for detection of plaque Mutans streptococci was 85.7% and 70.6% from the low and high parental education categories, respectively (Table 3-5). Therefore, there was no statistically significant difference between these groups by Mann-Whitney U test (Figure 3-15c). However, the high upper percentile and maximum value for children of parents with a 6th form education or above indicates that the children with detectable Mutans streptococci had higher counts than their respective counterparts in the low education category.

Graphical analysis of the raw data revealed no differences in the distributions of aerobic plaque flora or oral streptococci across the education categories (Figure 3-18a & b, respectively).

The median value for salivary *S. mutans* in children whose parents obtained education equivalent to 6th form or above was twice as high as children whose parents received only a secondary school education (p = 0.014, by Mann-Whitney U test), although median values were low (4591 and 1891, respectively) and were unlikely to be biologically meaningful (Figure 3-18c).

There were no statistically significant differences for salivary *S. sobrinus* or *S. mutans* or *S. sobrinus* as a proportion of Gram positive plaque flora according to the level of parental education (Figure 3-18d-f).

Table 3-7: Analysis of bacterial counts from plaque and saliva with respect to the level of parental education

Bacterial counts by the level of parental education	N	Mean	95% CIs		P
			Lower	Upper	
Diagnostic culture from plaque (CFU/ml)					
Total aerobic flora					
secondary school	21	5.9×10^6	2.4×10^6	1.5×10^7	0.533
6th form or above	38	4.4×10^6	2.7×10^6	7.3×10^6	
Total oral streptococci					
secondary school	21	1.6×10^6	7.4×10^5	3.3×10^6	0.732
6th form or above	36	1.8×10^6	1.1×10^6	3.0×10^6	
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)					
S. mutans					
secondary school	19	4.5×10^3	1.2×10^3	1.6×10^4	0.205
6th form or above	36	1.2×10^4	4.8×10^3	3.0×10^4	
S. sobrinus					
secondary school	19	830	239	2.9×10^3	0.968
6th form or above	36	853	410	1.8×10^3	
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive plaque flora)					
S. mutans					
secondary school	19	0.0014	0.0003	0.0056	0.711
6th form or above	38	0.0011	0.0005	0.0023	
S. sobrinus					
secondary school	19	0.0011	0.0002	0.0052	0.461
6th form or above	38	0.0006	0.0003	0.0013	

Mean bacterial counts in the plaque and saliva of children grouped according to the level of parental education obtained were compared by independent samples t-tests. Geometric data generated by back transformations of $\log_{10}$ transformed data are shown.

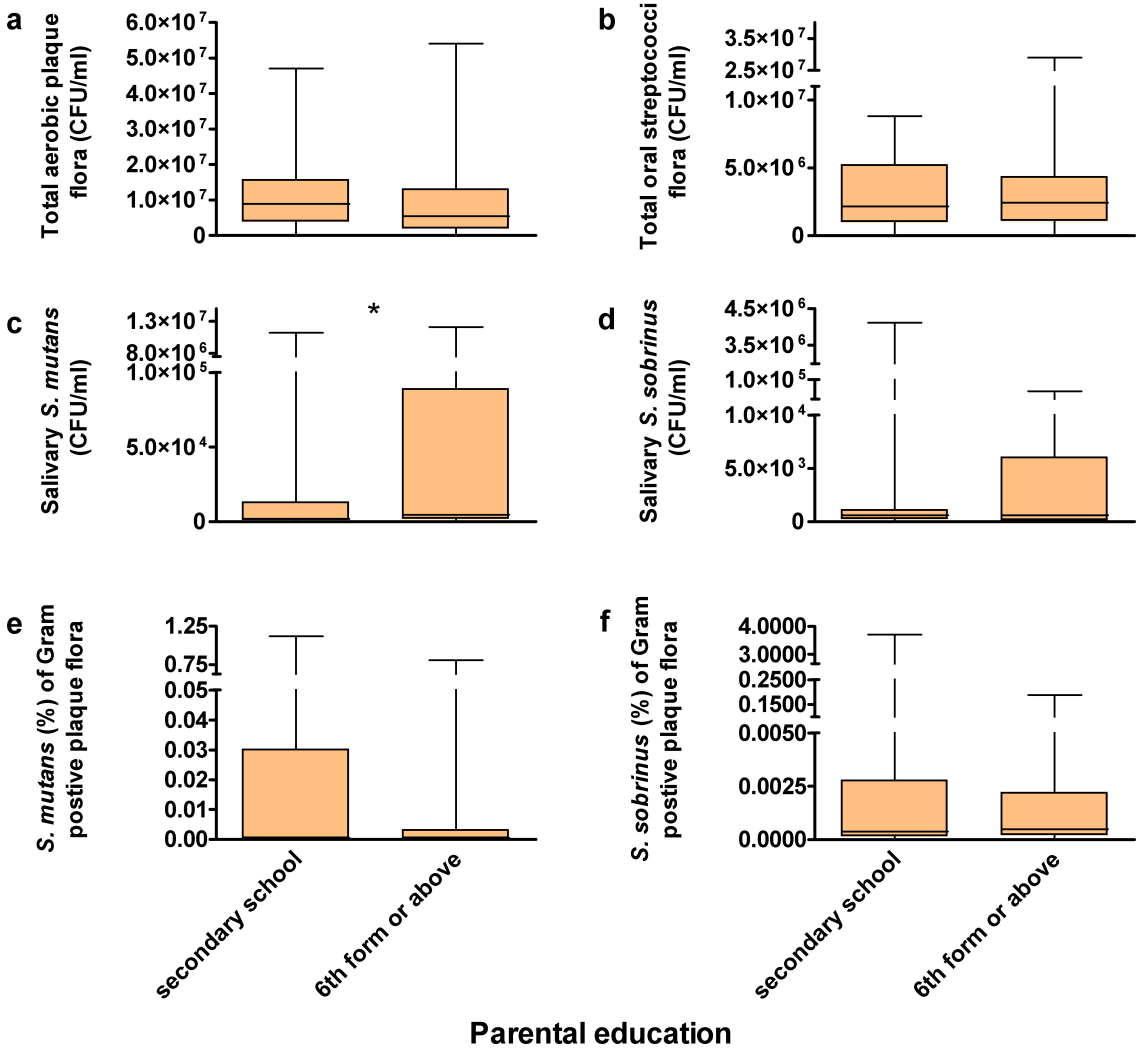


Figure 3-18: Bacterial counts in plaque and saliva of children grouped according to the level of parental education obtained

Box plots of bacterial counts from plaque and saliva of study participants grouped according to the level of parental education obtained a) total aerobic plaque flora (CFU/ml [n = 21 vs 38]), b) total oral plaque streptococci (CFU/ml [n = 21 vs 36]), c) salivary *S. mutans* (CFU/ml [n = 19 vs 36]) d) salivary *S. sobrinus* (CFU/ml [n = 19 vs 36]), and e) *S. mutans* (% [n = 19 vs 38]) and f) *S. sobrinus* (% [19 vs 38]) of Gram positive plaque flora. Raw data were plotted in each instance. Differences were significant only for salivary *S. mutans* (*p = 0.014) by Mann-Whitney U test. Differences were not significant by independent samples t-tests (Table 3-7).

3.2.4.4 Bacterial counts in plaque and saliva according to SIMD

There were no statistically significant differences, with respect to SIMD, in aerobic flora or oral streptococci estimated by diagnostic culture of plaque, or of *S. mutans* or *S. sobrinus* in saliva or plaque estimated by TaqMan® QPCR (Table 3-8). However, the geometric mean for salivary *S. mutans* (CFU/ml) was over 10-fold higher in children living in less deprived areas, compared to those living in the most deprived quintile. Although, this difference was not statistically significant ($p = 0.712$) it reflects a trend from this data for higher Mutans streptococci counts in children from more affluent backgrounds.

Cross-tabulation revealed equal proportions of children with or without detectable levels of Mutans streptococci by diagnostic culture of plaque across the dichotomised SIMD quintile categories ($p = 1$, Table 3-5). Both SIMD categories contained over 50% of children with no detectable numbers of Mutans streptococci. Therefore, there was no statistically significant difference in the median values by Mann-Whitney U test (Figure 3-15d and data not shown).

Graphical summaries of the raw data revealed similar distributions of total aerobic flora and oral streptococci across dichotomised SIMD quintile categories (Figure 3-19a & b, respectively). There were no statistically significant differences for *S. mutans* or *S. sobrinus* in plaque or saliva, estimated by TaqMan® QPCR with respect to dichotomised SIMD quintiles (Figure 3-19c-f).

Table 3-8: Analysis of bacterial counts in plaque and saliva with respect to local health board SIMD quintiles

Bacterial counts by local SIMD quintiles (2009)	N	Mean	95% CIs		P
			Lower	Upper	
Diagnostic culture from plaque (CFU/ml)					
Total aerobic flora					
most deprived quintile	20	4.2×10^6	1.8×10^6	9.5×10^6	0.856
quintiles 2-5	27	4.5×10^6	2.2×10^6	9.4×10^6	
Total oral streptococci					
most deprived quintile	19	2.2×10^6	1.3×10^6	3.9×10^6	0.490
quintiles 2-5	26	1.6×10^6	7.2×10^5	3.4×10^6	
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)					
S. mutans					
most deprived quintile	19	7.2×10^3	2.6×10^3	2.0×10^4	0.712
quintiles 2-5	23	9.8×10^4	2.4×10^3	4.0×10^4	
S. sobrinus					
most deprived quintile	19	791	363	1.7×10^3	0.776
quintiles 2-5	23	672	284	1.6×10^3	
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive plaque flora)					
S. mutans					
most deprived quintile	19	0.0011	0.0004	0.0028	0.742
quintiles 2-5	25	0.0014	0.0005	0.0040	
S. sobrinus					
most deprived quintile	19	0.0009	0.0002	0.0032	0.697
quintiles 2-5	25	0.0007	0.0003	0.0016	

Mean bacterial counts in plaque and saliva of children grouped according to dichotomised local health board SIMD quintile (2009) categories were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

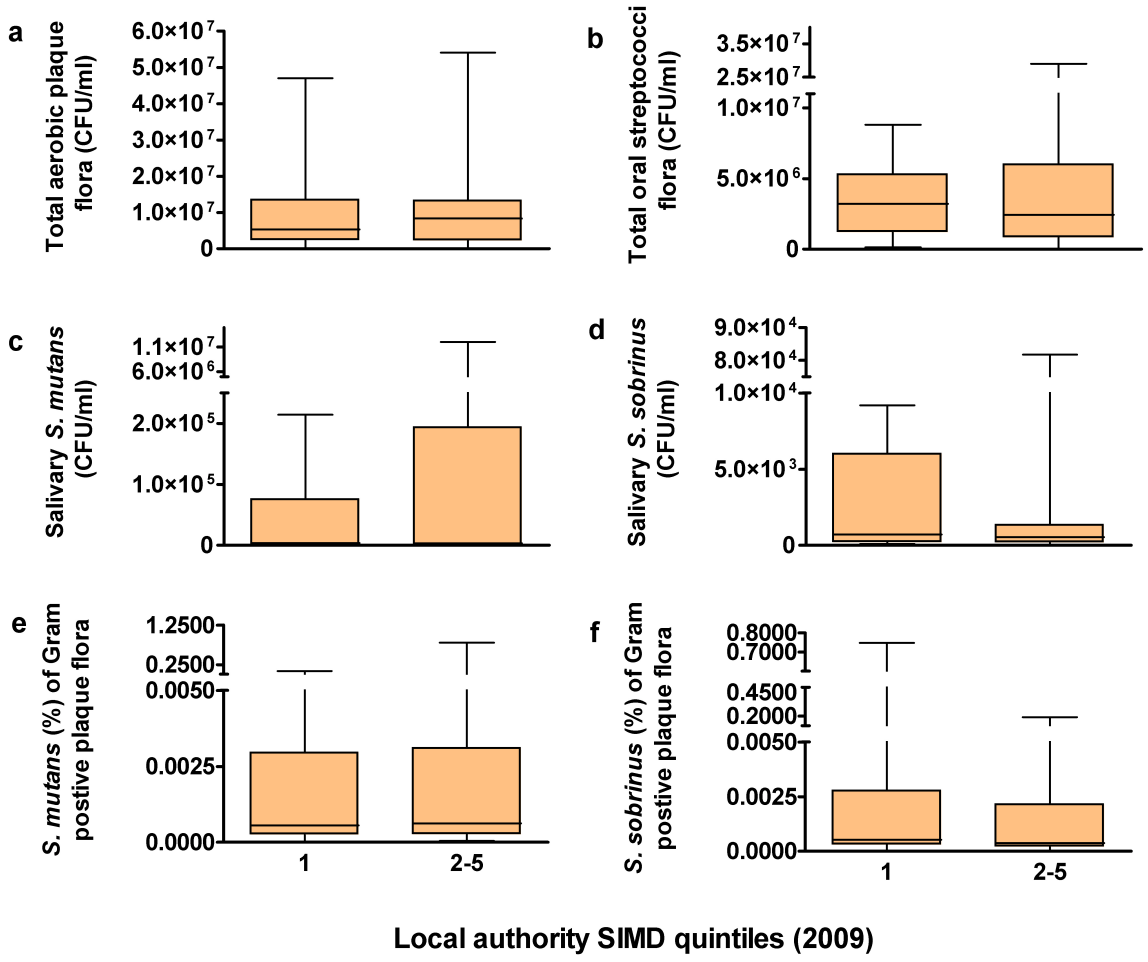


Figure 3-19: Bacterial counts in plaque and saliva of children grouped according to dichotomised SIMD quintile categories

Box plots of bacterial counts from plaque and saliva of study participants grouped according to dichotomised local health board SIMD quintiles (2009). a) total aerobic plaque flora (CFU/ml [n = 20 vs 27]), b) total oral plaque streptococci (CFU/ml [n = 19 vs 26]), c) salivary *S. mutans* (CFU/ml [n = 19 vs 23]) d) salivary *S. sobrinus* (CFU/ml [n = 19 vs 23]), and e) *S. mutans* (% [n = 19 vs 25]) and f) *S. sobrinus* (% [n = 19 vs 25]) of Gram positive plaque flora. Raw data were plotted in each instance. Differences were not significant by independent samples t-tests (Table 3-8) or Mann-Whitney U tests (data not shown).

3.2.4.5 Salivary antimicrobial proteins according to total household income

Geometric mean concentrations of lactoferrin, calprotectin and the HNPs 1-3 were higher in children from families receiving a total household income of £10,000 or more, although the differences were small and not statistically significant (Table 3-9). Graphical summaries of the raw data revealed that median concentrations of these proteins were also higher in children from families within the higher income category, but the differences were not statistically significant (Figure 3-20).

LL37 was below the limit of detection in a high proportion of individuals (25.5%, n =12/45 [Table 3-3]). Cross-tabulation revealed no differences in the proportion of children with or without detectable concentrations of LL37 according to the level of household income received (p = 0.728 [Table 3-10]). Furthermore, graphical summary of the raw data revealed no difference in the concentrations of LL37 in children according to the level of household income (p = 0.879 [Figure 3-21a]).

There was a trend for higher geometric mean concentrations of sIgA antibodies specific for oral streptococci in children of families earning an annual income of less than £10,000 compared to children from families earning £10,000 or more. However, the differences were small and were not statistically significant (Table 3-9). Graphical summaries of the raw data revealed no differences in the distributions of titres of sIgA antibodies specific for oral streptococci in children grouped according to the level of household income received (Figure 3-22).

Table 3-9: Analysis of salivary antimicrobial proteins with respect to the level of total household income

Salivary proteins by total household income	N	Mean	95% CIs		P
			Lower	Upper	
Lactoferrin (ng/ml)					
< £10,000	23	1061	664.5	1693.6	0.585
≥ £10,000	28	1257.2	817.5	1933.3	
Calprotectin (ng/ml)					
< £10,000	23	254.5	141.7	456.9	0.325
≥ £10,000	28	368.2	222.8	608.6	
HNPs 1-3 (ng/ml)					
< £10,000	23	28.4	14.7	54.8	0.465
≥ £10,000	28	40.2	20.0	80.8	
S. mutans specific sIgA (EU)					
< £10,000	19	211.5	155.5	287.1	0.687
≥ £10,000	26	196.3	158.6	244.3	
S. sobrinus specific sIgA (EU)					
< £10,000	19	295.7	231.2	378.2	0.412
≥ £10,000	26	263	220.4	313.7	
S. mitis specific sIgA (EU)					
< £10,000	19	206.4	147.2	289.3	0.330
≥ £10,000	26	171.6	137.0	214.8	
S. sanguinis specific sIgA (EU)					
< £10,000	19	221.6	172.0	285.6	0.113
≥ £10,000	26	164.3	125.5	215.1	

Mean concentrations of salivary antimicrobial proteins in children grouped according to the level of total household income were compared by independent samples t-tests. Geometric data generated by back transformations of $\log_{10}$ transformed data are shown.

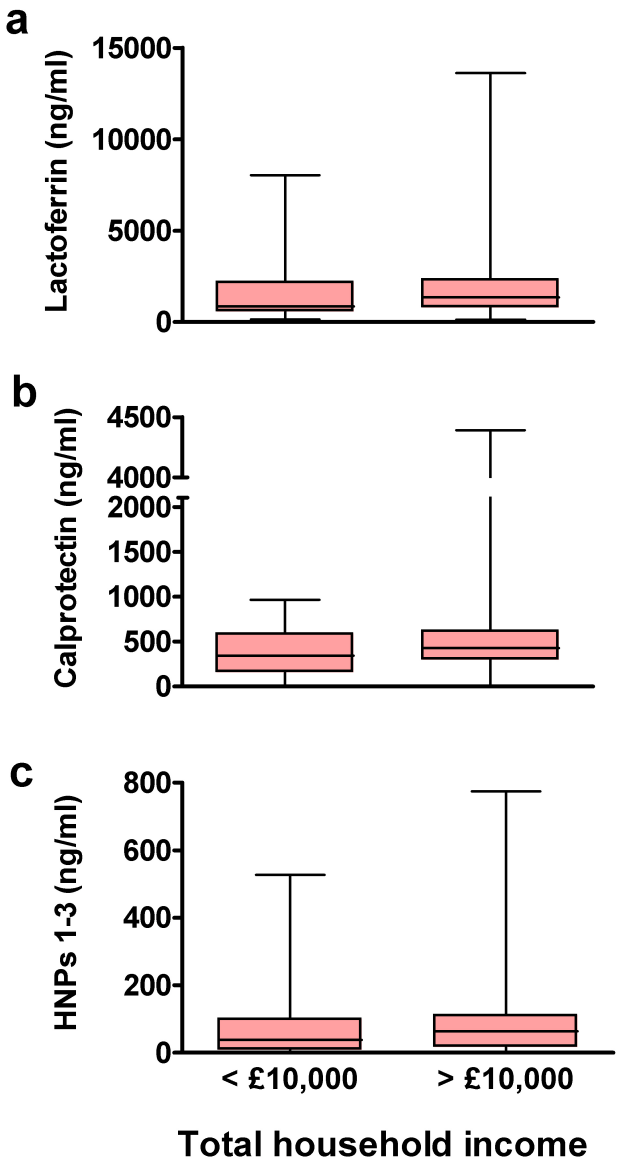


Figure 3-20: Antimicrobial proteins in the saliva of children grouped according to the level of total household income

Box plots of concentrations (ng/ml) of antimicrobial proteins a) lactoferrin, b) calprotectin and c) HNPs 1-3, in saliva of study participants grouped according to the level of total household income (n = 23 vs 28). Raw data was plotted in each instance. Differences were not significant by independent samples t-tests (Table 3-9) or Mann-Whitney U tests.

Table 3-10: Cross-tabulation of LL37 detection by dichotomised measures of socioeconomic status

		LL37		Total	P
		Not Detected	Detected		
Income					
< £10,000	count	5	11	16	
	%	**31.3**	**68.8**	**100**	
> £10,000	count	6	18	24	0.728
	%	**25.0**	**75.0**	**100**	
Total	count	11	29	40	
	%	**27.5**	**72.5**	**100**	
Proportion of income received as benefits					
Half – all	count	10	16	26	
	%	**38.5**	**61.5**	**100**	
None – quarter	count	2	15	17	0.085
	%	**11.8**	**88.2**	**100**	
Total	count	12	31	43	
	%	**27.9**	**72.1**	**100**	
Parental education					
Secondary school	count	4	12	16	
	%	**25.0**	**75.0**	**100**	
6th form or above	count	7	22	29	1.000
	%	**24.1**	**75.9**	**100**	
Total	count	11	34	45	
	%	**24.4**	**75.6**	**100**	
Local SIMD quintiles (2009)					
Most deprived	count	4	11	15	
quintile	%	**26.7**	**73.3**	**100**	
Quintiles 2-5	count	4	13	17	1.000
	%	**23.5**	**76.5**	**100**	
Total	count	8	24	32	
	%	**25**	**75.0**	**100**	

Cross-tabulation was used to assess the proportion of children with and without detectable concentrations of LL37, grouped according to dichotomised measures of socioeconomic status. P values were generated using Fishers exact test.

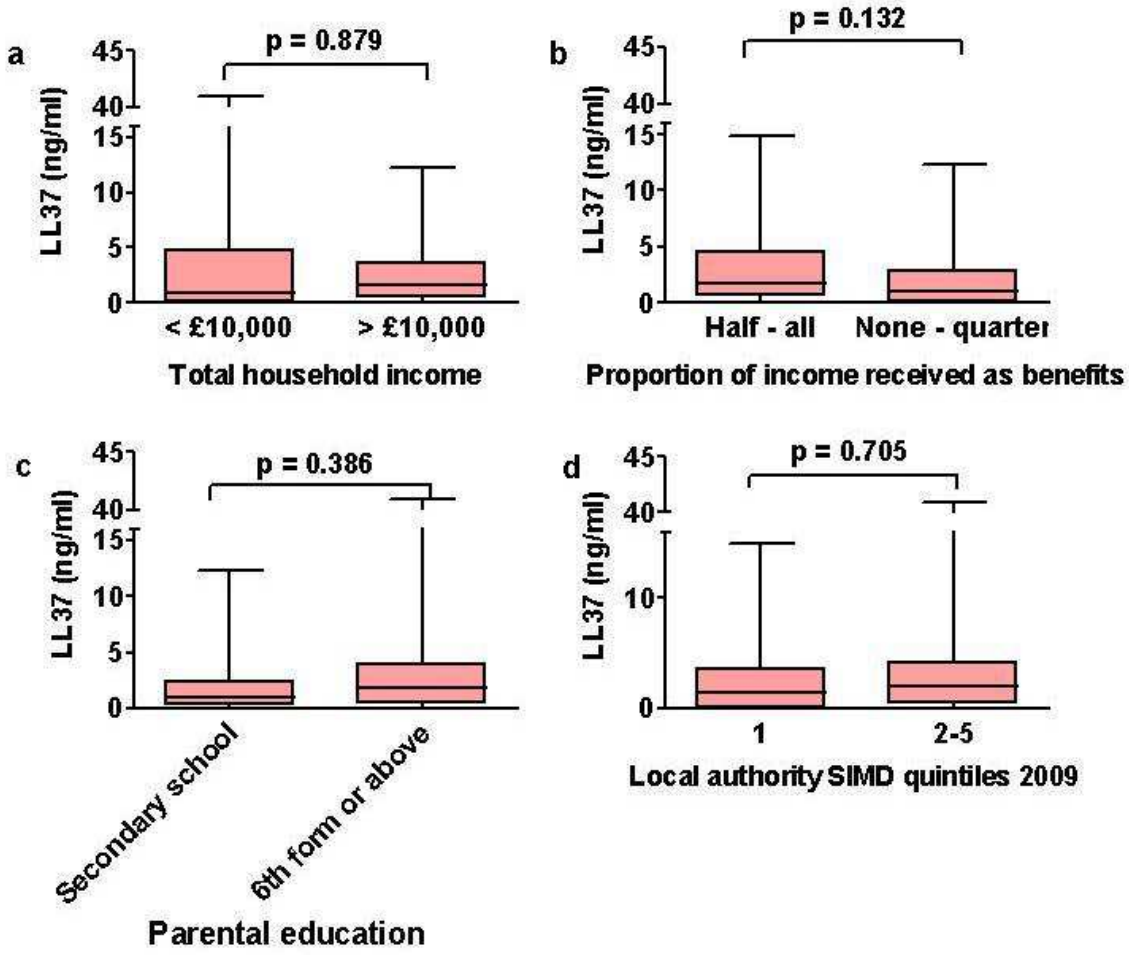


Figure 3-21: LL37 in saliva of children grouped according to dichotomised measures of SES

Box plot of LL37 concentrations (ng/ml) in study participants grouped according to a) total household income (n = 16 vs 24) b) proportion of income received as benefits (n = 26 vs 17), c) level of parental education obtained (n = 16 vs 29) and d) dichotomised SIMD quintile categories (n = 15 vs 17). Raw data were plotted in each instance. Differences were not statistically significant by Mann-Whitney U tests.

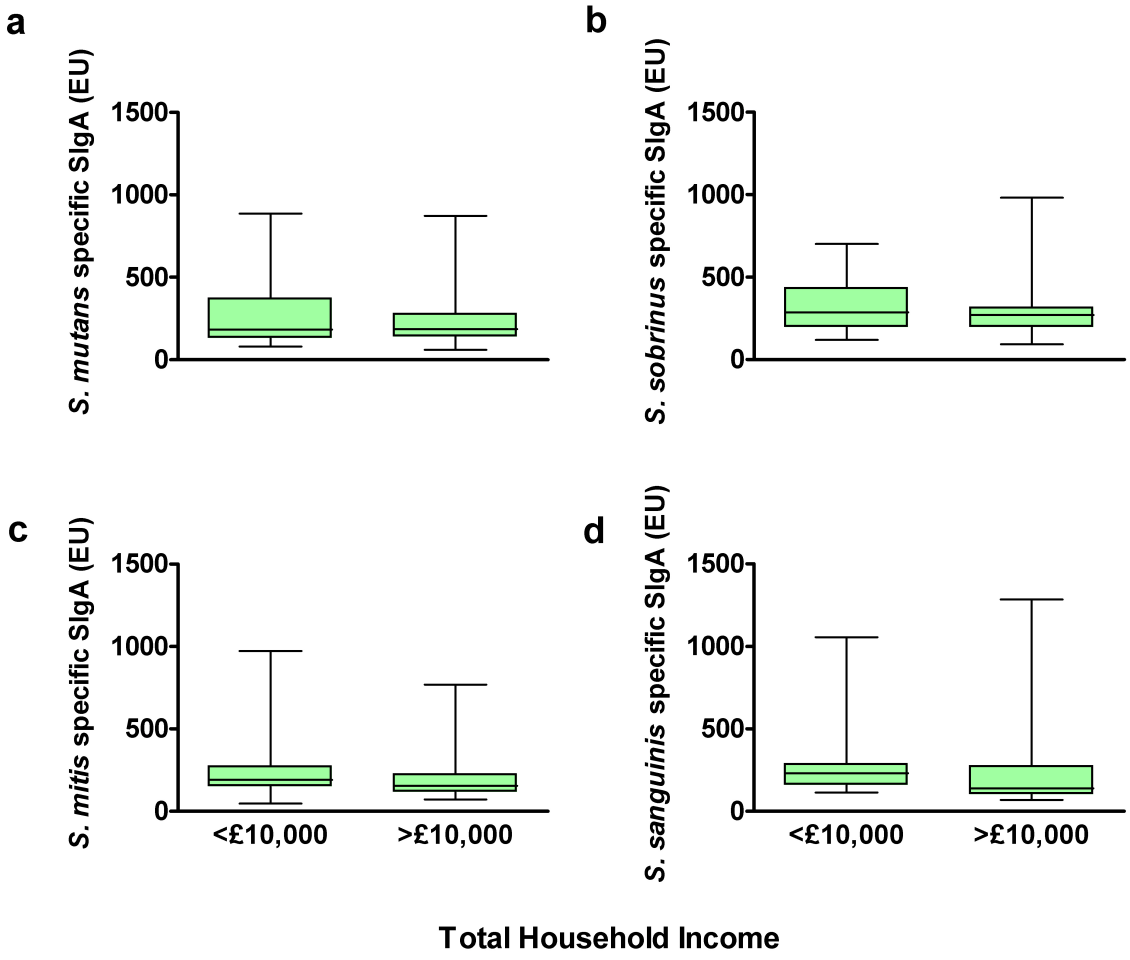


Figure 3-22: Titres of sIgA antibodies specific for oral streptococci grouped according to the level of total household income

Box plots of titres of sIgA antibodies (EU) specific for a) *S. mutans*, b) *S. sobrinus* c) *S. mitis* and d) *S. sanguinis* from study participants grouped according to the level of total household income (n = 19 vs 26). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-9) or Mann-Whitney U tests.

3.2.4.6 Salivary antimicrobial proteins according to the proportion of income received as benefits

There were no statistically significant differences in the geometric mean concentrations of lactoferrin, calprotectin or HNPs 1-3 in the saliva of children according to the level of income received as benefits (Table 3-11). This was confirmed from the graphical summaries of the raw data (Figure 3-23).

Cross-tabulation revealed a higher proportion of children with detectable concentrations of LL37 came from families receiving a small proportion of their income as benefits, compared to those receiving half to all of their income from benefits (88.2% vs 61.5%, p = 0.085 [Table 3-10]).

Graphical summary of the raw data revealed higher median, lower and upper percentiles and maximum values for LL37 concentrations in saliva of children from families receiving a large proportion of their income from benefits, although the difference was not statistically significant (p = 0.132 [Figure 3-21b]).

There were no statistically significant differences in the geometric means of sIgA antibodies in the saliva of children according to the proportion of income received as benefits (Table 3-11). This was supported by analysis of the raw data which revealed no differences in the distributions of sIgA titres in children according to the proportion of benefits received (Figure 3-24).

Table 3-11: Analysis of salivary antimicrobial proteins with respect to the proportion of household income received as benefits

Salivary proteins by the proportion of income received as benefits	N	Mean	95% CIs		P
			Lower	Upper	
Lactoferrin (ng/ml)					
half - all	22	1197.3	739.3	1938.7	0.577
none – about a quarter	34	1002.5	660.1	1522.3	
Calprotectin (ng/ml)					
half - all	22	244.2	133.5	446.8	0.487
none – about a quarter	34	324.1	188.8	556.7	
HNPs 1-3 (ng/ml)					
half - all	22	32.3	16.1	64.9	0.859
none – about a quarter	34	35.0	19.5	62.7	
S. mutans specific sIgA (EU)					
half - all	19	180.8	135.4	241.4	0.380
none – about a quarter	30	209.2	171.3	255.4	
S. sobrinus specific sIgA (EU)					
half - all	19	266.8	211.7	336.2	0.865
none – about a quarter	30	272.0	232.0	321.2	
S. mitis specific sIgA (EU)					
half - all	19	168.3	119.2	237.6	0.321
none – about a quarter	30	203.6	162.6	254.9	
S. sanguinis specific sIgA (EU)					
half - all	19	198.9	151.5	261.1	0.554
none – about a quarter	30	178.6	140.2	227.4	

Mean concentrations of salivary antimicrobial proteins in children grouped according to the proportion of household income received as benefits were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

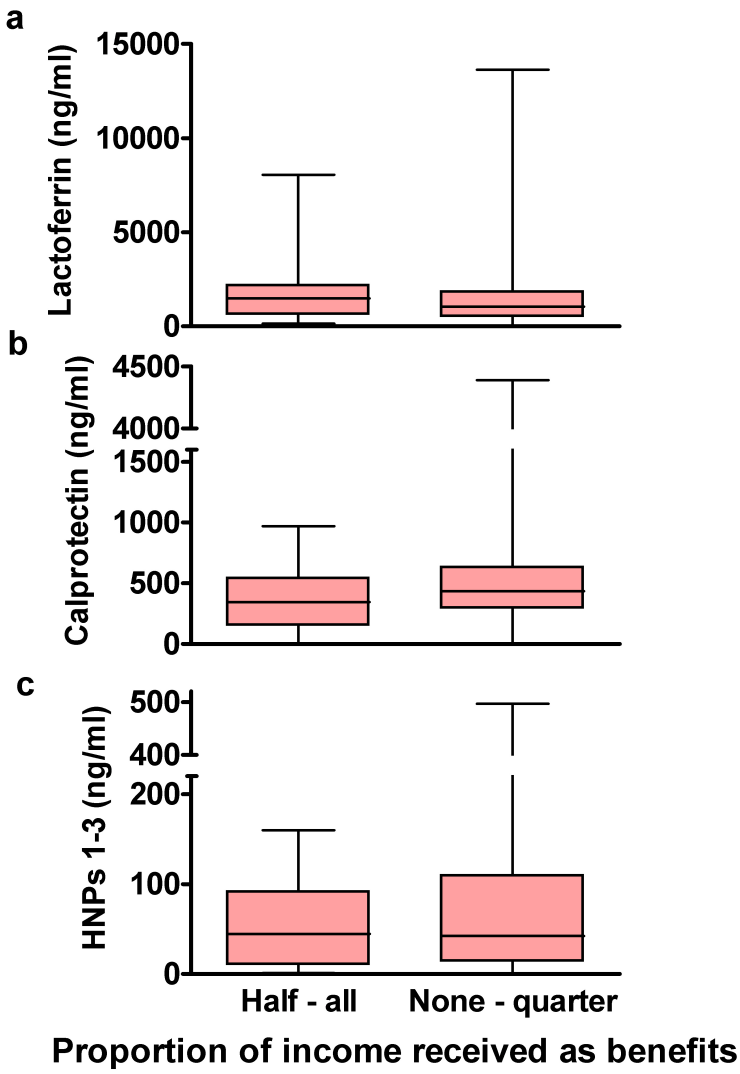


Figure 3-23: Antimicrobial proteins in the saliva of children grouped according to the proportion of income received as benefits

Box plots of concentrations (ng/ml) of antimicrobial proteins a) lactoferrin, b) calprotectin and c) HNPs 1-3 in the saliva of study participants grouped according to the proportion of household income received as benefits (n = 22 vs 34). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-11) or Mann-Whitney U tests.

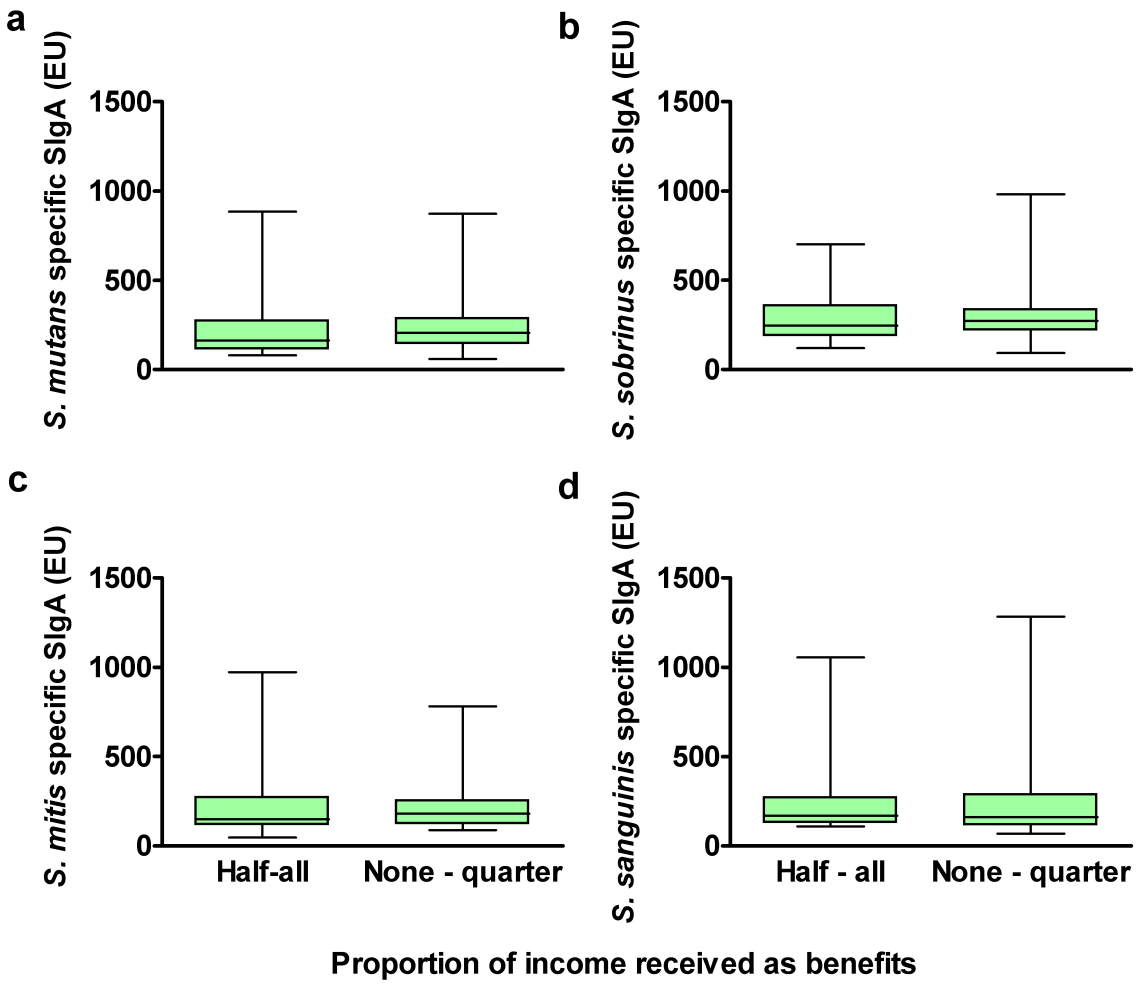


Figure 3-24: Titres of sIgA antibodies specific for oral streptococci grouped according to the proportion of income received as benefits

Box plots of titres (EU) of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus* c) *S. mitis* and d) *S. sanguinis* in study participants grouped according to the proportion of household income received as benefits (n = 19 vs 30). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-11) or Mann-Whitney U tests.

3.2.4.7 Salivary antimicrobial proteins according to parental education

There were no statistically significant differences in the geometric mean concentrations of lactoferrin, calprotectin or the HNPs 1-3 in the saliva of children grouped according to the level of parental education obtained (Table 3-12).

Graphical summaries of the raw data revealed higher median concentrations of lactoferrin, calprotectin and the HNPs 1-3 from the saliva of children whose parents received a relatively higher level of education. The differences were small and not statistically significant by Mann-Whitney U test (Figure 3-25 and data not shown).

There was no difference in the proportion of children with or without detectable concentrations of LL37 across the parental education categories by cross tabulation (p = 1, Table 3-10). There were no differences in the concentrations of LL37 in children whose parents obtained a school or college 6th form education or above (p = 0.386 [Figure 3-21c]).

Geometric mean titres of sIgA antibodies specific for *S. mutans*, *S. sobrinus* and *S. mitis* were higher in children whose parents fell in the higher education category. This was statistically significant only for *S. mutans* specific sIgA (p = 0.048) by independent samples t-test. There was no difference in the mean levels of *S. sanguinis* sIgA antibody titres (p = 0.999 [Table 3-12]).

Graphical summaries of the raw data confirmed a higher median titre of sIgA antibodies specific for *S. mutans* in children whose parents received education equivalent to school or college 6th form or above. The difference remained statistically significant by Mann-Whitney U test (p = 0.032, Figure 3-26a). There were no statistically significant differences in the median titres of sIgA antibodies specific for *S. sobrinus*, *S. mitis* or *S. sanguinis* (Figure 3-26a-c).

Table 3-12: Analysis of salivary antimicrobial proteins with respect to the level of parental education

Salivary proteins by level of parental education	N	Mean	95% CIs		P
			Lower	Upper	
Lactoferrin (ng/ml)					
Secondary school	20	818.8	465.6	1440.1	0.129
6th form or above	37	1326.5	924.5	1903.3	
Calprotectin (ng/ml)					
Secondary school	20	264.7	137.2	510.6	0.631
6th form or above	37	322.9	194.4	536.5	
HNPs 1-3 (ng/ml)					
Secondary school	20	35.2	17.2	71.8	0.865
6th form or above	37	33.1	21.2	68.4	
S. mutans specific sIgA (EU)					
Secondary school	16	159.6	125.0	203.8	0.048
6th form or above	33	224.1	181.9	276.1	
S. sobrinus specific sIgA (EU)					
Secondary school	16	257.3	206.0	321.4	0.459
6th form or above	33	286.0	240.8	339.5	
S. mitis specific sIgA (EU)					
Secondary school	16	167.5	122.8	228.4	0.347
6th form or above	33	202.1	158.9	257.0	
S. sanguinis specific sIgA (EU)					
Secondary school	16	190.0	148.3	243.3	0.999
6th form or above	33	189.9	148.9	242.2	

Mean concentrations of salivary antimicrobial proteins in children grouped according to the level of parental education obtained were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

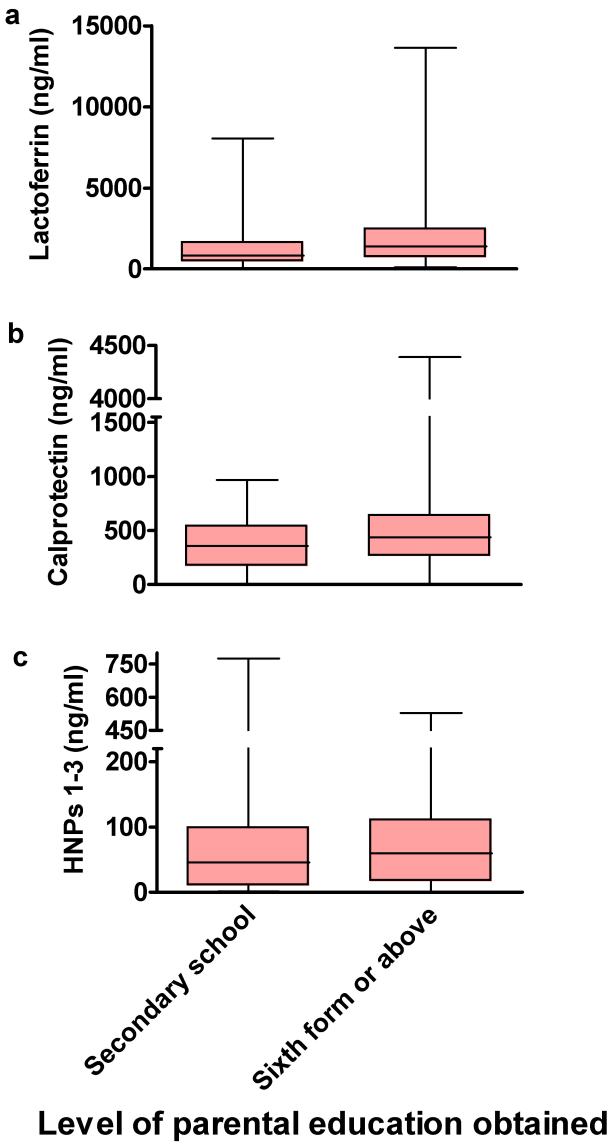


Figure 3-25: Antimicrobial proteins in the saliva of children grouped according to the level of parental education obtained.

Box plots of concentrations (ng/ml) of antimicrobial proteins a) lactoferrin, b) calprotectin and c) HNPs 1-3 in the saliva of study participants grouped according to the level of parental education obtained (n = 20 vs 37). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-12) or Mann-Whitney U tests (data not shown).

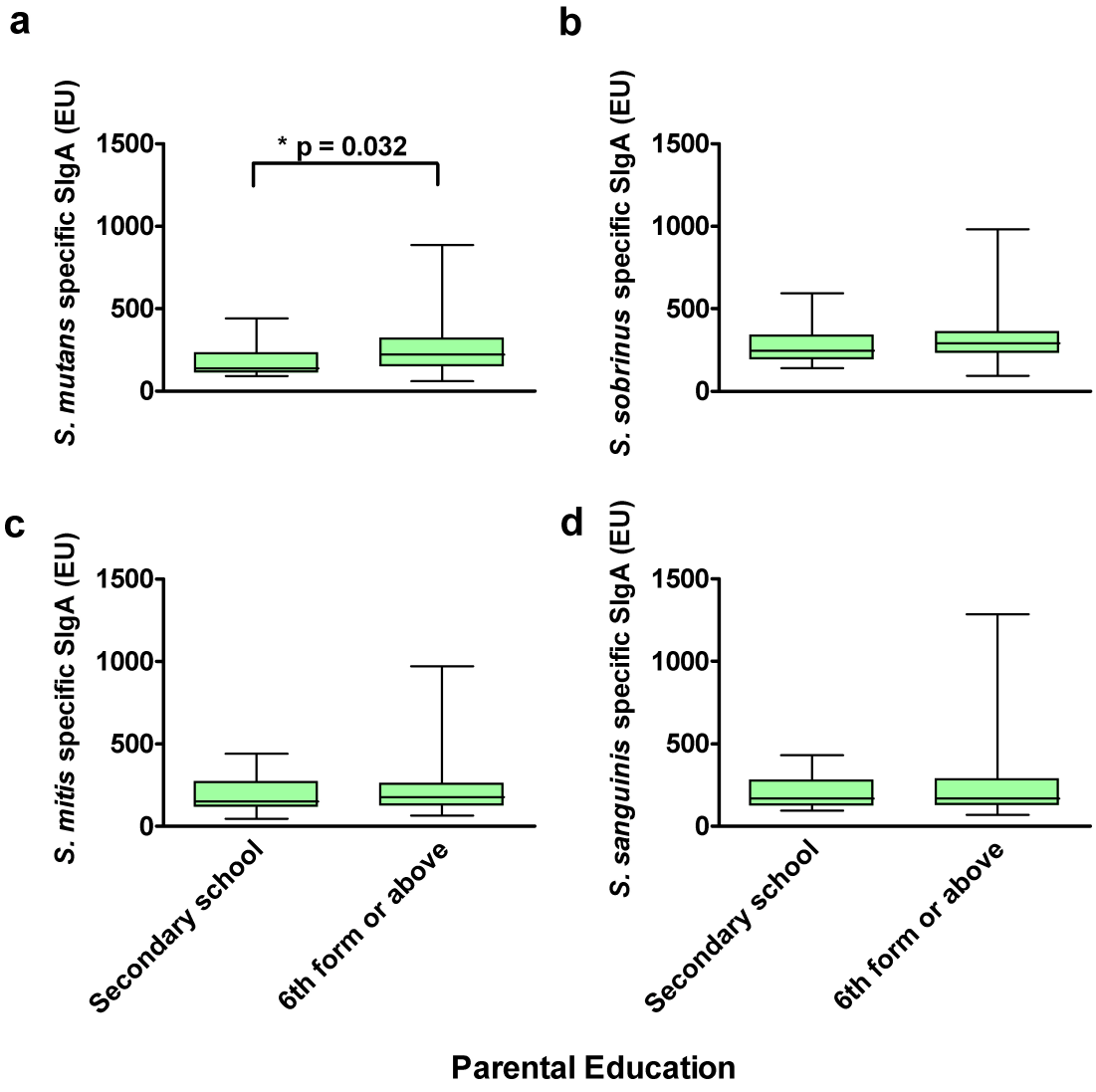


Figure 3-26: Titres of sIgA antibodies specific for oral streptococci grouped according to the level of parental education obtained

Box plots of titres (EU) of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus* c) *S. mitis* and d) *S. sanguinis* from study participants grouped according to the level of parental education obtained (n = 16 vs 33). Raw data were plotted in each instance. Statistically significant difference for *S. mutans* specific sIgA by Mann-Whitney U test. Non-significant differences by Mann-Whitney U tests are not shown.

3.2.4.8 Salivary antimicrobial proteins according to SIMD

There were no statistically significant differences in the geometric mean concentrations of lactoferrin, calprotectin or the HNPs 1-3 in the saliva of children grouped according to dichotomised SIMD quintiles by independent samples t-tests (Table 3-13). This was supported by graphical summaries of the raw data, which did not reveal any differences in the concentrations of these antimicrobial proteins in the saliva of these children (Figure 3-27).

There was no difference in the proportion of children with or without detectable concentrations of LL37 across SIMD categories ($p = 1$ [Table 3-10]). Furthermore, no difference was identified in the concentrations of LL37 in the saliva of children with respect to SIMD quintiles ($p = 0.705$ [Figure 3-21d]).

There was a tendency for higher geometric mean titres of sIgA antibodies specific for oral streptococci in children living in the most deprived areas, although the differences were small and were not statistically significant (Table 3-13).

Similarly, graphical summaries of the raw data revealed higher median values of titres of sIgA antibodies specific for oral streptococci in children living in the most deprived areas, although the differences were not statistically significant by Mann-Whitney U tests (Figure 3-28).

Table 3-13: Analysis of salivary antimicrobial proteins with respect to dichotomised local health board SIMD quintiles (2009)

Salivary proteins by local SIMD quintiles (2009)	N	Mean	95% CIs		P
			Lower	Upper	
Lactoferrin (ng/ml)					
most deprived quintile	20	1023.5	639.7	1637.9	0.793
quintiles 2-5	24	1096.5	655.5	1833.6	
Calprotectin (ng/ml)					
most deprived quintile	20	369.7	272.0	502.7	0.399
quintiles 2-5	24	255.9	118.0	554.8	
HNPs 1-3 (ng/ml)					
most deprived quintile	20	33.0	17.2	63.0	0.890
quintiles 2-5	24	35.5	15.0	84.0	
S. mutans specific sIgA (EU)					
most deprived quintile	15	230.6	162.0	328.2	0.841
quintiles 2-5	22	188.8	147.3	242.0	
S. sobrinus specific sIgA (EU)					
most deprived quintile	15	339.6	260.8	442.0	0.321
quintiles 2-5	22	261.3	211.8	322.3	
S. mitis specific sIgA (EU)					
most deprived quintile	15	203.0	137.5	299.6	0.108
quintiles 2-5	22	176.7	139.6	223.8	
S. sanguinis specific sIgA (EU)					
most deprived quintile	15	220.0	142.5	339.6	0.500
quintiles 2-5	22	170.3	133.1	217.7	

Mean concentrations of salivary antimicrobial proteins in children grouped according to local health board SIMD quintiles (2009) were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

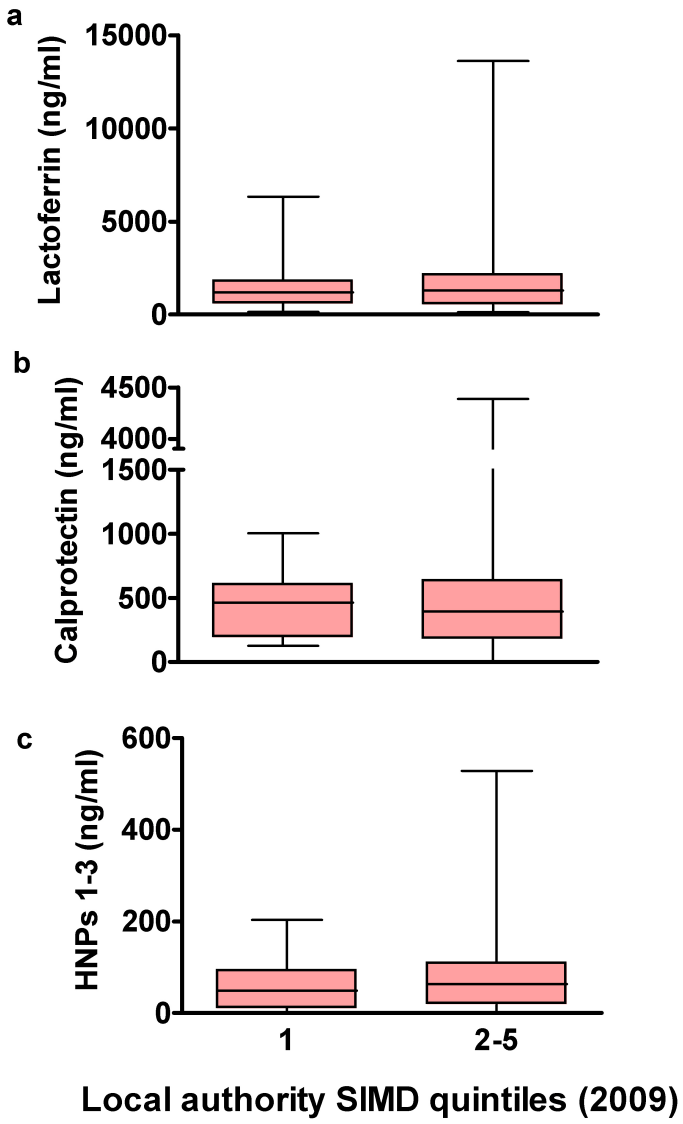


Figure 3-27: Antimicrobial proteins in saliva of children grouped according to dichotomised SIMD quintiles.

Box plots of concentrations (ng/ml) of antimicrobial proteins a) lactoferrin b) calprotectin and c) HNPs 1-3 in the saliva of study participants grouped according to dichotomised local health board SIMD quintile categories (n = 20 vs 24). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-13) or Mann-Whitney U tests.

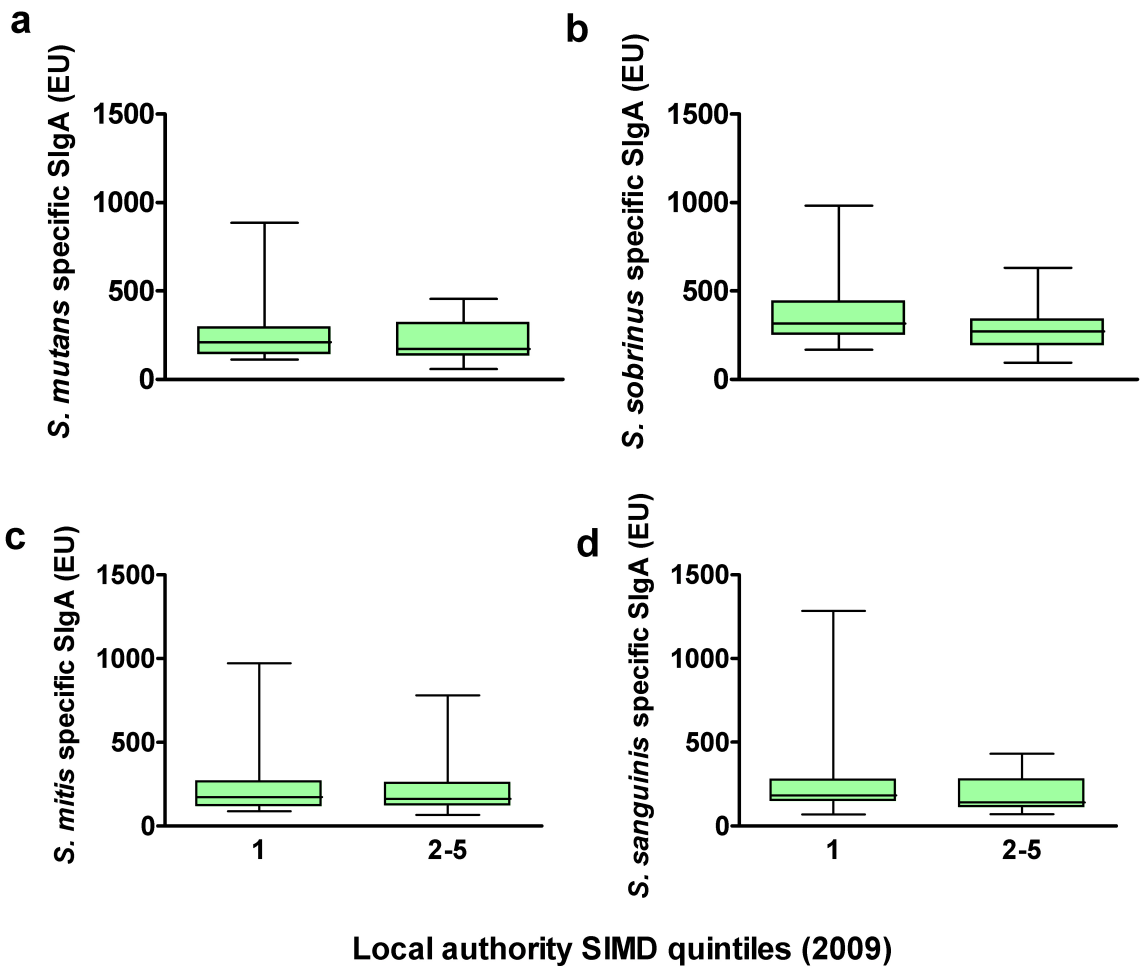


Figure 3-28: Titres of sIgA antibodies specific for oral streptococci grouped according to dichotomised SIMD quintile categories

Box plots of titres (EU) of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis* in study participants grouped according to dichotomised SIMD quintile categories (n = 15 vs 22). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-13) or Mann-Whitney U tests.

3.2.4.9 Salivary cortisol concentrations according to measures of socioeconomic status

It was hypothesised that children of lower SES may have measureable differences in the concentrations of salivary cortisol compared with higher SES children.

There were no statistically significant differences in the geometric mean concentrations of salivary cortisol in children grouped according to measures of socioeconomic status (Table 3-14).

Graphical summaries of the raw data revealed marginally higher median levels of cortisol in children from families with a total household income of below £10,000, while there were little or no differences in median cortisol levels in children according to the proportion of benefits received, parental education or SIMD quintiles (Figure 3-29).

Table 3-14: Analysis of salivary cortisol levels with respect to measures of socioeconomic status

Demographic variables	N	Mean	95% CIs		P
			Lower	Upper	
Household income					
< £10,000	21	0.15	0.12	0.19	0.346
≥ £10,000	28	0.12	0.08	0.17	
Proportion of income received as benefits					
Half – all	20	0.13	0.10	0.17	0.931
None – about a quarter	33	0.13	0.10	0.17	
Level of parental education					
Secondary school	19	0.12	0.09	0.16	0.191
6^{th} form or above	35	0.14	0.10	0.18	
Local SIMD quintiles					
Most deprived quintile	19	0.12	0.09	0.16	0.227
Quintiles 2-5	22	0.14	0.09	0.20	

Mean levels of salivary cortisol concentrations (μg/dL) in children grouped according to dichotomised measures of socioeconomic status were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

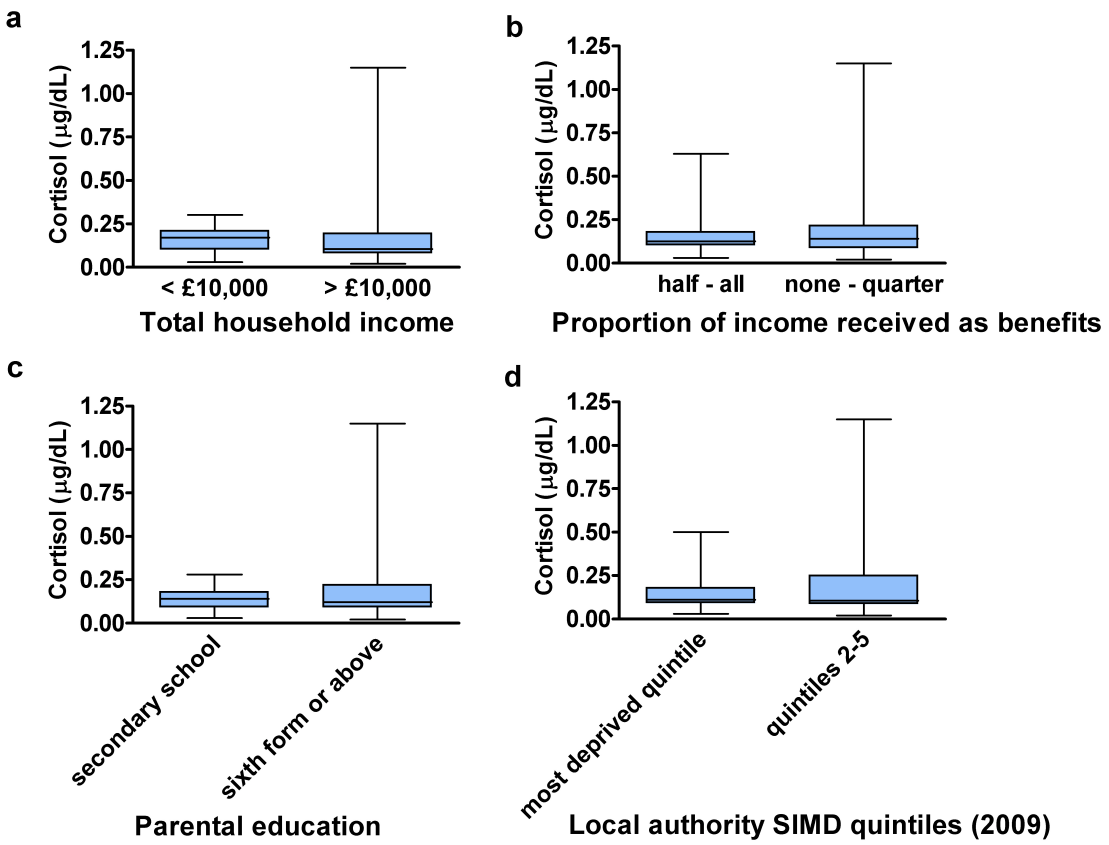


Figure 3-29: Salivary cortisol in children grouped according to dichotomised measures of socioeconomic status

Box plots of salivary cortisol concentrations (µg/dL) in study participants grouped according to dichotomised measures of socioeconomic status. a) Total household income (n = 21 vs 28), b) proportion of income received as benefits (n = 20 vs 33), c) parental education (n = 19 vs 35) and d) local SIMD quintiles (n = 19 vs 22). Raw data were plotted in each instance. Differences were not statistically significant by independent samples t-tests (Table 3-14) or Mann-Whitney U tests.

3.2.5 Do salivary cortisol concentrations influence total bacterial numbers or the detection of cariogenic bacteria?

It was hypothesised that life stresses may indirectly impact upon the bacterial load of plaque or the detection of cariogenic bacteria. Salivary cortisol was used as a surrogate measure of stress to investigate this hypothesis.

Bacterial load of plaque, estimated using total aerobic flora or total oral streptococci, showed no associations with salivary cortisol (Figure 3-30) or ranked salivary cortisol tertiles (Figure 3-31a and b). Mean Mutans streptococci (CFU/ml) were higher in the plaque of children ranked in the high cortisol tertile, than those in the low or medium tertiles, although the differences between groups were not statistically significant (Figure 3-31c). Somewhat contrary to this finding, there were three children with particularly high salivary cortisol concentrations in whom Mutans streptococci could not be detected by diagnostic culture of plaque. Overall, there was no difference by Mann-Whitney U test in salivary cortisol concentrations between the children who were culture positive for Mutans streptococci compared with children who were culture negative ($p = 0.492$ [Figure 3-32]).

Visual inspection of scatter plots of *S. mutans* and *S. sobrinus* absolute or relative numbers in saliva and plaque, respectively, with salivary cortisol did not reveal any associations (Figure 3-33). Mean numbers of salivary *S. mutans* and *S. sobrinus* were investigated further by ranked tertiles of salivary cortisol. There was a trend for increased mean salivary and plaque *S. mutans* and *S. sobrinus* with each increasing cortisol tertile, although neither the differences between groups nor the linear trends reached statistical significance by ANOVA (Figure 3-34a-d).

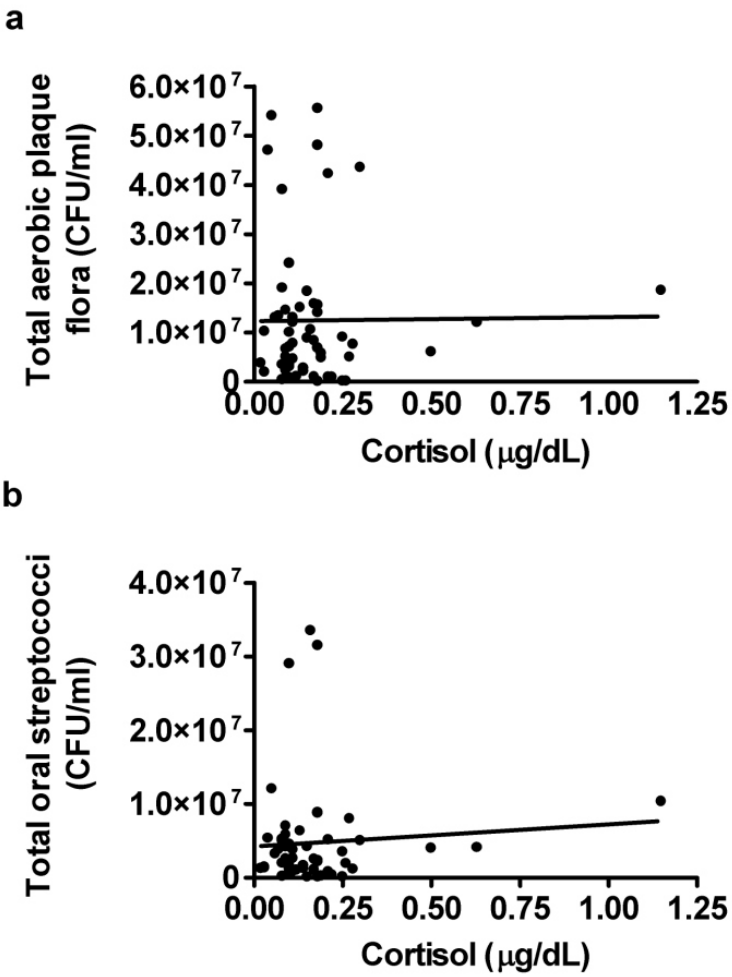


Figure 3-30: Associations between bacterial load of plaque and salivary cortisol

Scatter plots of salivary cortisol concentrations (μg/dL) and a) total aerobic flora (CFU/ml) and b) total oral streptococci (CFU/ml) from the plaque of study participants, estimated by diagnostic culture. Raw data were plotted and the line of best fit is shown for each graph.

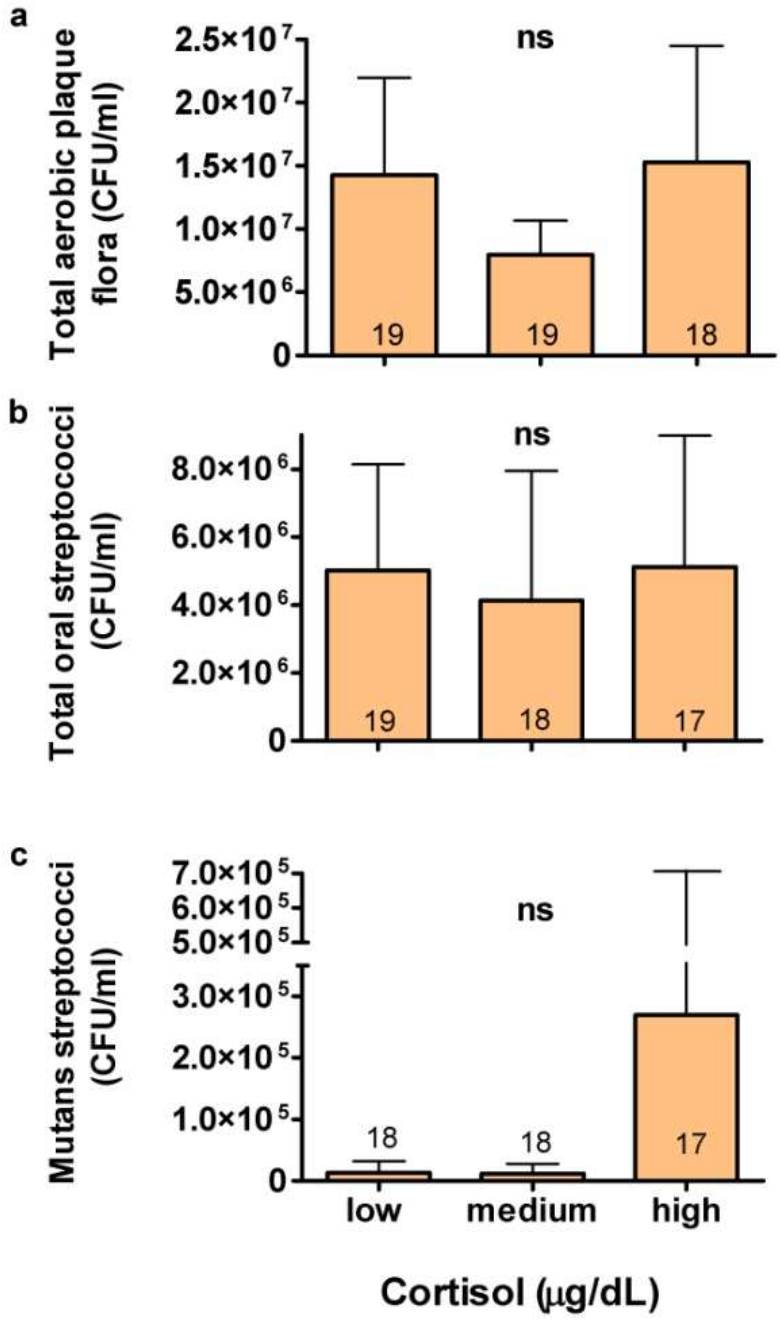


Figure 3-31: Associations of plaque bacterial counts and ranked tertiles of salivary cortisol

Data are mean and 95% confidence intervals of the mean of a) total aerobic flora, b) total oral streptococci and c) total Mutans streptococci, estimated from plaque of study participants by diagnostic culture and grouped according to ranked cortisol tertiles (n in groups are shown on graph). Raw data were plotted in each instance. Differences between groups were not statistically significant for (a & b) by ANOVA of $\log_{10}$ transformed parametric data or c) Kruskal-Wallis of raw data.

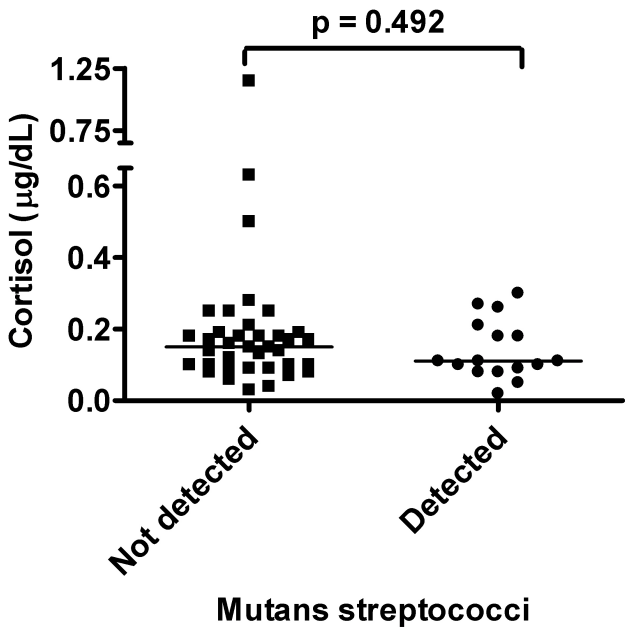


Figure 3-32: Salivary cortisol in children grouped according to detection of Mutans streptococci

Scatter plots of salivary cortisol concentrations (µg/dL) in study participants grouped according to detection of Mutans streptococci (n = 36 vs 16), estimated by diagnostic culture of plaque. Each data point represents a mean cortisol value for an individual study participant. The horizontal bars indicate the group median. Raw data were plotted in each instance. The difference was not statistically significant by Mann-Whitney U test.

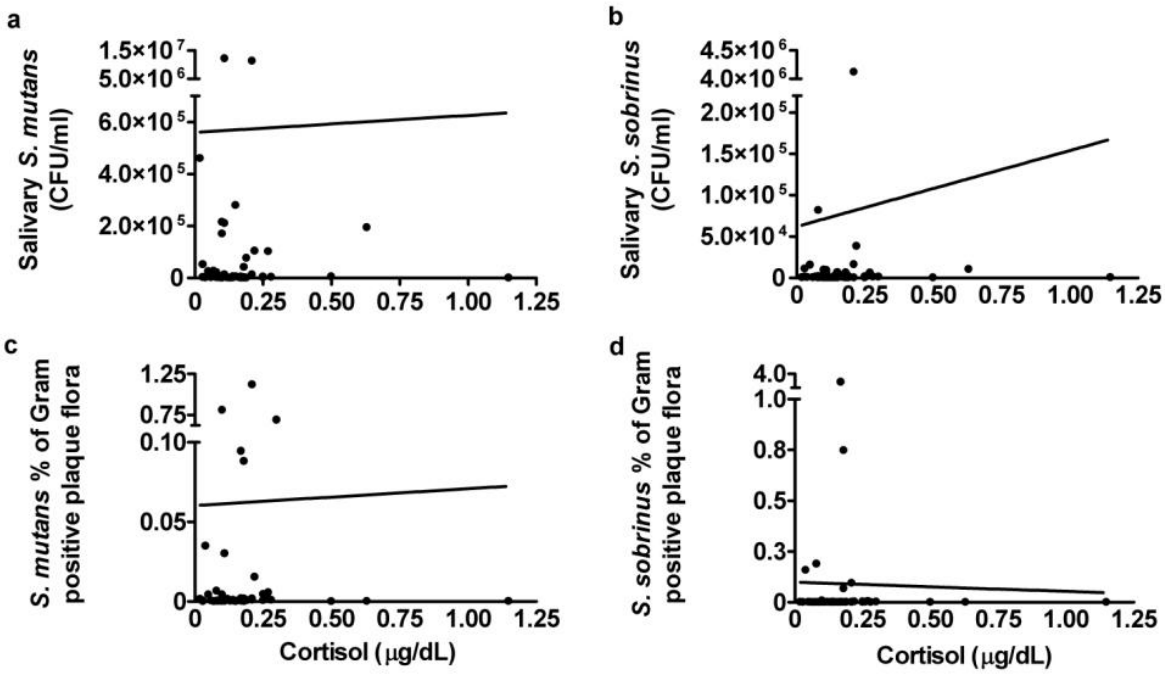


Figure 3-33: Associations between salivary cortisol and Mutans streptococci from plaque and saliva

Scatter plots of salivary cortisol concentrations (µg/dL) and a) salivary *S. mutans* (CFU/ml) b) salivary *S. sobrinus* (CFU/ml), and c) *S. mutans* (%) and d) *S. sobrinus* (%) as a proportion of Gram positive plaque flora. Raw data were plotted and the line of best fit is shown for each graph.

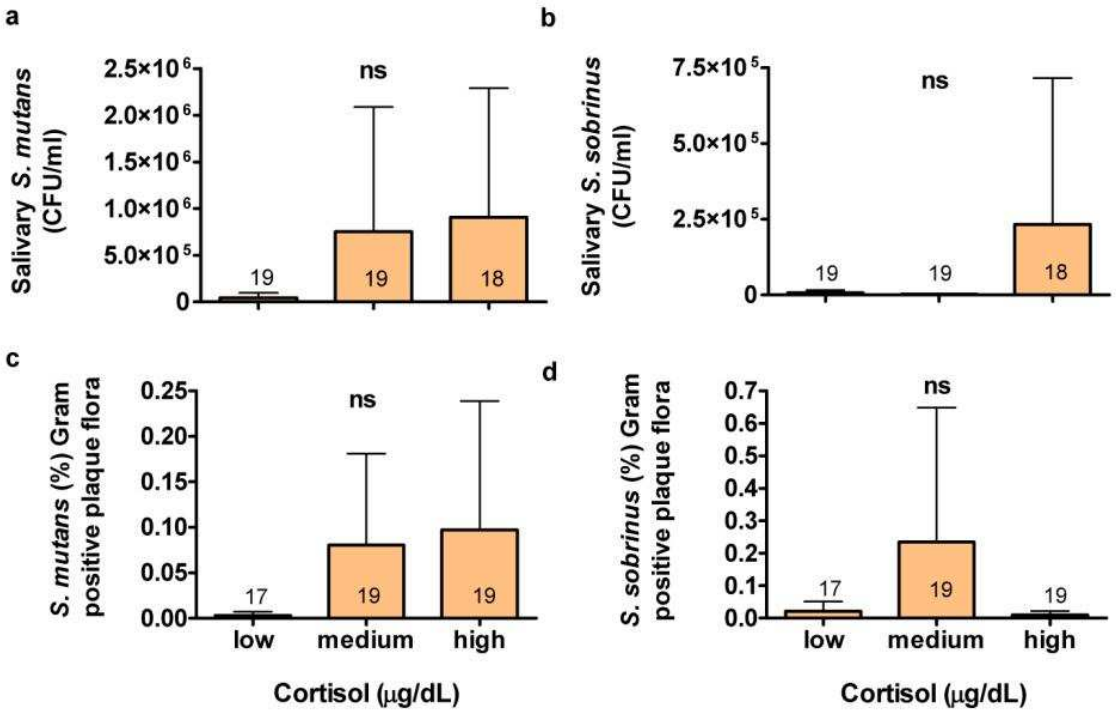


Figure 3-34: Associations of bacterial counts from plaque and saliva and ranked tertiles of salivary cortisol

Data are mean and 95% confidence intervals of the mean of a) salivary *S. mutans* (CFU/ml), b) salivary *S. sobrinus* (CFU/ml), and c) *S. mutans* (%) and d) *S. sobrinus* (%) of Gram positive plaque flora grouped according to ranked tertiles of salivary cortisol (n for each group are shown on graph). Raw data were plotted. Differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

3.2.6 Do salivary cortisol concentrations influence salivary immune responses?

It was hypothesised that life stresses may indirectly influence the concentrations of salivary proteins involved in innate and adaptive immune responses. Salivary cortisol concentrations were used as a surrogate measure of stress to investigate this hypothesis.

Visual inspection of scatter plots of salivary cortisol levels with salivary antimicrobial proteins did not reveal evidence of any associations (Figure 3-35). As LL37 was undetectable in a high number of participants, salivary cortisol concentrations were investigated with respect to the presence or absence of detectable LL37. There was no statistical difference between these two groups by Mann-Whitney U test ($p = 0.492$ [Figure 3-36]).

Further inspection of associations of salivary antimicrobial protein levels with ranked cortisol tertiles revealed marginal trends for changes in mean concentrations of antimicrobial proteins with ranked cortisol tertiles (Figure 3-37). Mean lactoferrin was highest in children ranked in the lowest cortisol tertile, and was similar across the medium and high cortisol tertiles. Mean concentrations of calprotectin were highest in children with cortisol ranked in the high tertile. Mean concentrations of the HNPs 1-3 marginally decreased with each increased cortisol tertile. Mean concentrations of LL37 increased with each increased cortisol tertile. There was a trend for increased mean titres of sIgA antibodies with increased cortisol tertiles (Figure 3-37e - h). None of the linear trends for changes in mean antimicrobial proteins or salivary antibodies with cortisol tertiles reached statistical significance by ANOVA.

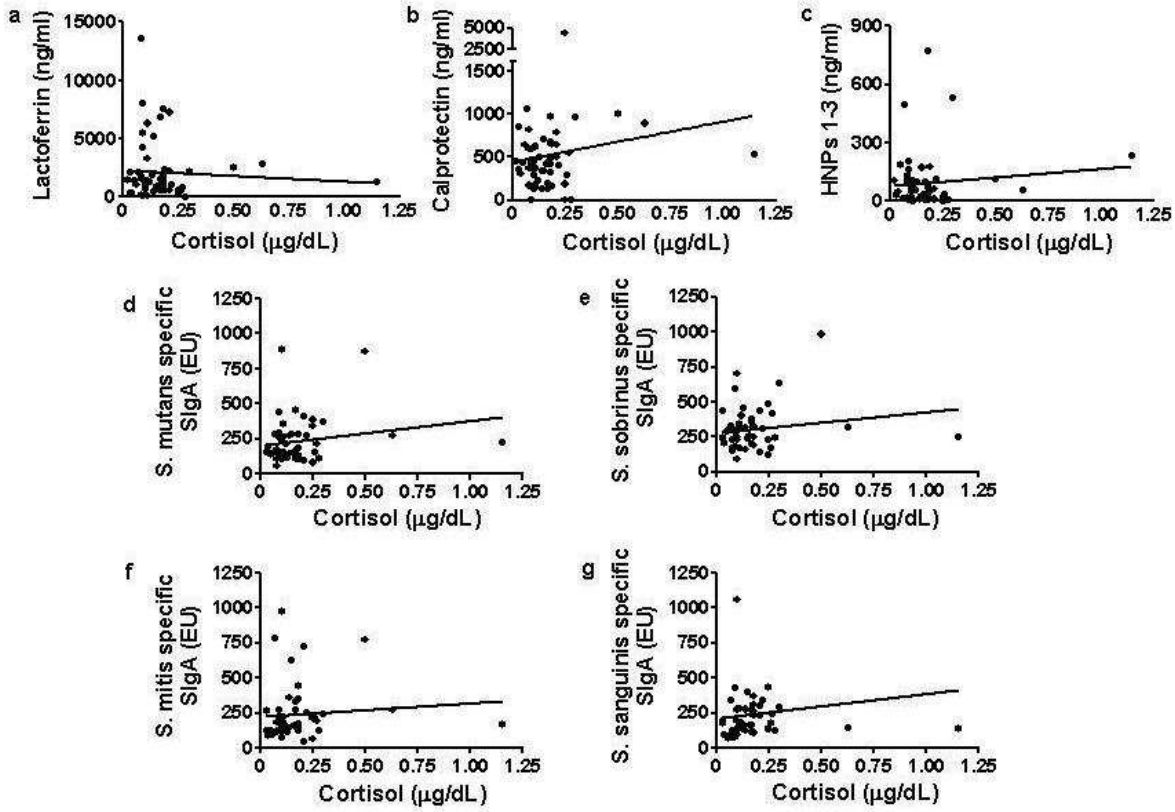


Figure 3-35: Associations between salivary antimicrobial proteins and salivary cortisol

Scatter plots of a) lactoferrin, b) calprotectin, c) HNPs 1-3, and titres (EU) of sIgA antibodies specific for d) *S. mutans* e) *S. sobrinus* f) *S. mitis* and g) *S. sanguinis*, with salivary cortisol concentrations (µg/dL). Raw data were plotted and the line of best fit is shown for each graph.

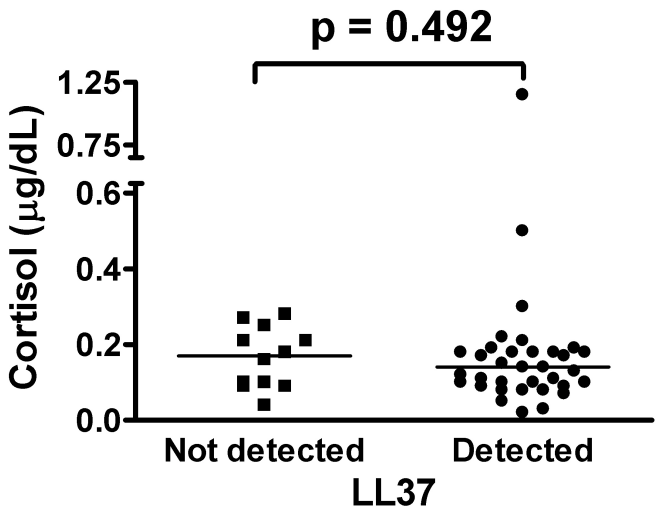


Figure 3-36: Salivary cortisol in children according to detection of LL37

Scatter plot of salivary cortisol concentrations (µg/dL) in study participants grouped according to detection of LL37 (n = 12 vs 35). Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group median. Raw data were plotted. The difference was not statistically significant by Mann-Whitney U test.

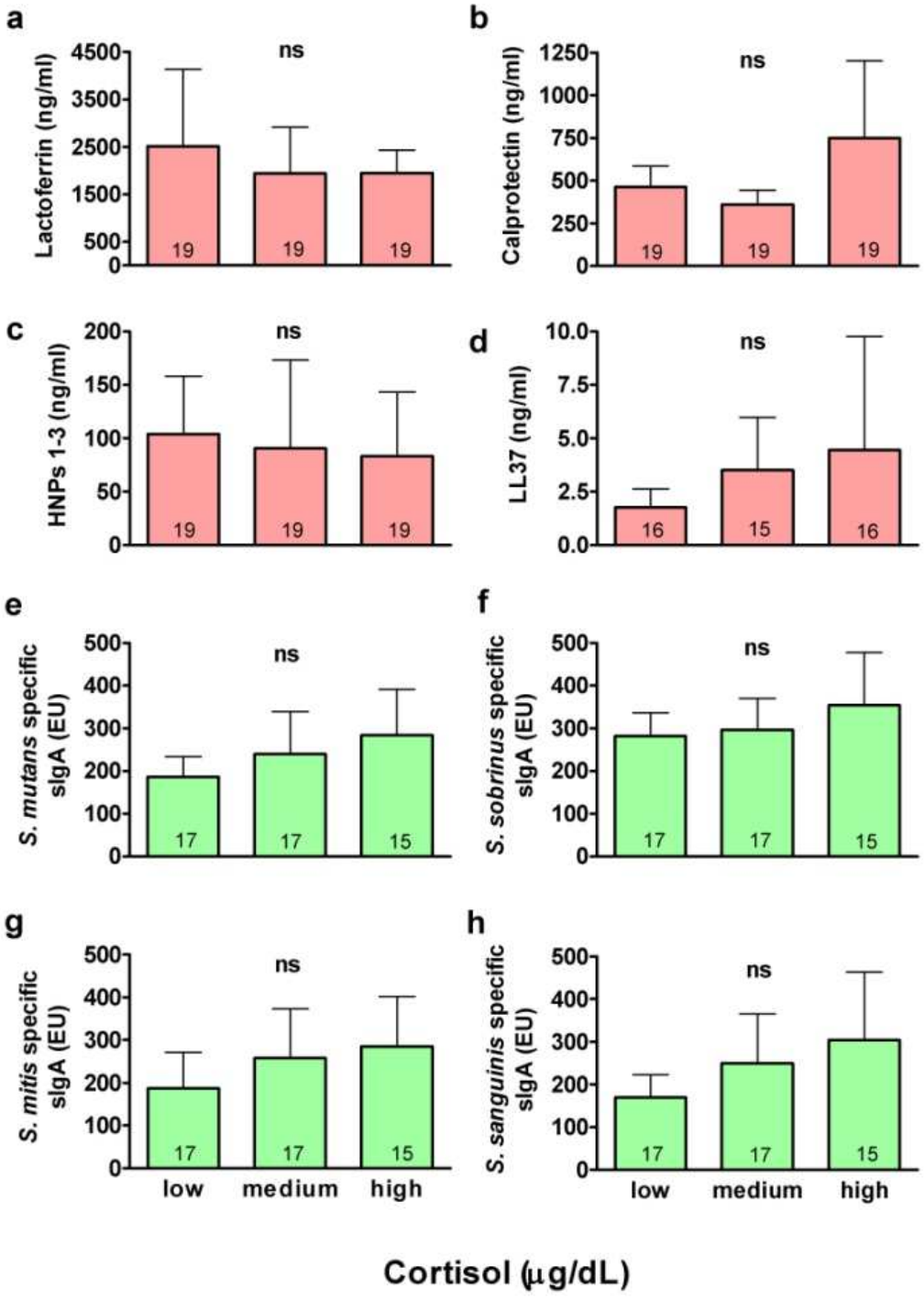


Figure 3-37: Associations of salivary antimicrobial proteins and ranked tertiles of salivary cortisol.

Data are mean and 95% confidence intervals of the mean of a) lactoferrin b) calprotectin c) HNPs 1-3 d) LL37; and titres (EU) of sIgA antibodies specific for e) *S. mutans* f) *S. sobrinus* g) *S. mitis* and h) *S. sanguinis* by ranked cortisol tertiles (n in each group indicated on graph). Raw data were plotted in each instance. Differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

3.2.7 Do salivary antimicrobial proteins impact on bacterial load or the detection of cariogenic bacteria?

It was hypothesised that differences in the concentrations of salivary antimicrobial proteins may influence the bacterial load of plaque or the detection of cariogenic bacteria.

3.2.7.1 Salivary antimicrobial proteins and cariogenic bacteria cultured from plaque?

Visual inspection of scatter plots of total aerobic plaque flora by titres of sIgA antibodies did not reveal evidence of any linear associations. However, in every instance a group of six children with the highest bacterial counts, ranging from 3.9×10^7 – 5.4×10^7 CFU/ml, had some of the lowest antibody titres (Figure 3-38). There was no association of aerobic plaque flora with ranked tertiles of sIgA antibodies specific for oral streptococci (data not shown).

There were no linear associations of total aerobic plaque flora with antimicrobial proteins (Figure 3-39). Median levels of aerobic plaque flora were statistically significantly higher by Mann-Whitney U test in children in whom LL37 could be detected ($p = 0.021$, Figure 3-40a). Median levels of oral streptococci were similar in both groups (Figure 3-40b).

Investigations of aerobic plaque flora by ranked tertiles of antimicrobial proteins revealed trends for increased mean aerobic plaque counts with increasing tertiles of lactoferrin, calprotectin, the HNPs 1-3 and LL37 (Figure 3-41). The difference in bacterial numbers between the low and high groups for the HNPs 1-3 and LL37 were statistically significant by Bonferroni *post hoc* tests following ANOVA ($p = 0.015$ and $p = 0.050$ [Figure 3-41c & d, respectively]). The linear trends for increased mean aerobic plaque flora with each increased tertile of antimicrobial proteins was statistically significant for the HNPs 1-3 ($p = 0.005$) and LL37 ($p = 0.017$) and did not reach statistical significance for lactoferrin ($p = 0.103$) or calprotectin ($p = 0.085$) by ANOVA linear.

Visual inspection of scatter plots of total oral plaque streptococci with titres of sIgA antibodies did not reveal evidence of any linear associations (Figure 3-42).

There was no association of oral streptococci with ranked tertiles of titres of sIgA antibodies (data not shown).

No associations between total oral streptococci and antimicrobial peptides were identified by scatter plot analysis (Figure 3-43). No associations were identified between total oral streptococci and ranked tertiles of antimicrobial proteins (data not shown).

A large proportion of children were culture negative for Mutans streptococci in plaque; therefore concentrations of salivary antimicrobial proteins in these children were investigated according to the presence or absence of detectable Mutans streptococci in plaque. There were no statistically significant differences in the titres of sIgA antibodies specific for oral streptococci according to detection of Mutans streptococci by diagnostic culture of plaque (Figure 3-44). Median levels of antimicrobial proteins were marginally higher in children in whom Mutans streptococci was detected by diagnostic culture of plaque (Figure 3-45). The differences were not statistically significant.

There was a trend for increased mean Mutans streptococci with each increasing lactoferrin tertile. The differences between groups were not statistically significant by Kruskal-Wallis test (Figure 3-46a). Also, the linear trend was not statistically significant as assessed by the non-parametric Jonckheere-Terpstra test (data not shown).

Mean Mutans streptococci were highest in the third tertiles for both calprotectin and LL37 but the differences between the groups and the linear trend were not statistically significant (Figure 3-46b and d, respectively). There was no association of Mutans streptococci (CFU/ml) with ranked tertiles of the HNPs 1-3 (Figure 3-46c).

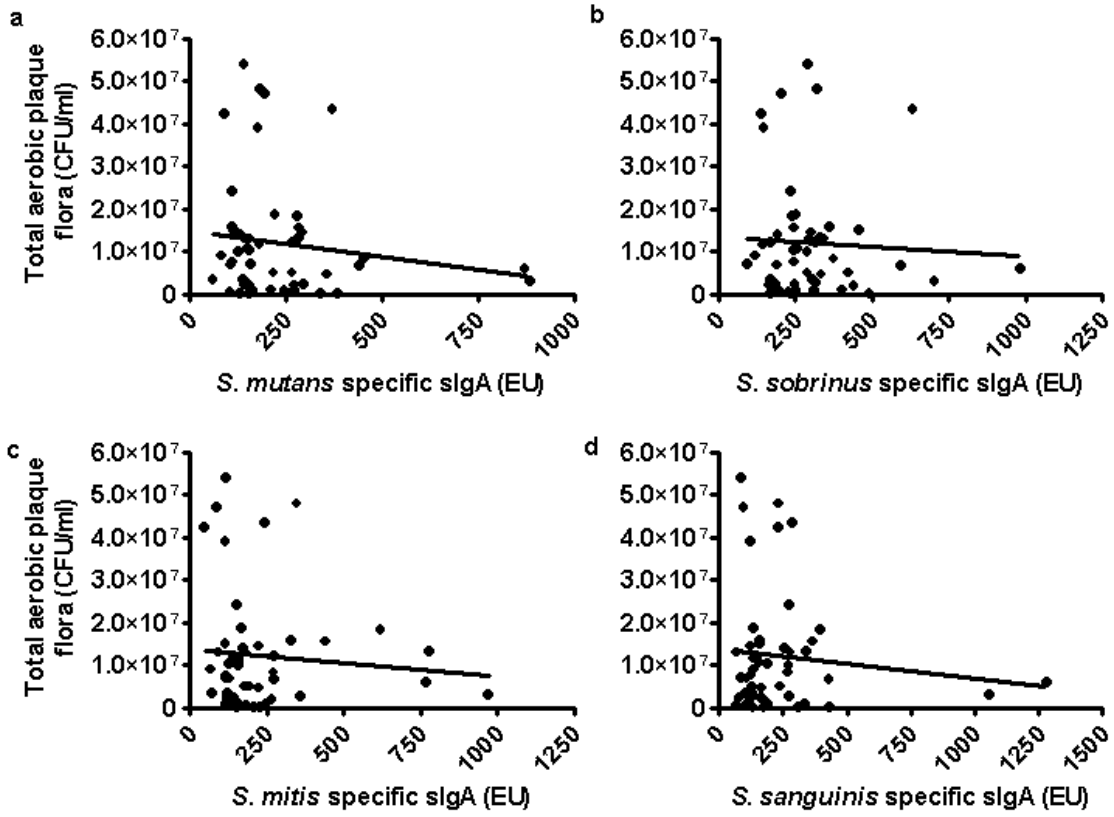


Figure 3-38: Associations between bacterial load of plaque and sIgA antibodies

Scatter plots of total aerobic plaque flora (CFU/ml) and titres (EU) of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted and the line of best fit is shown for each graph.

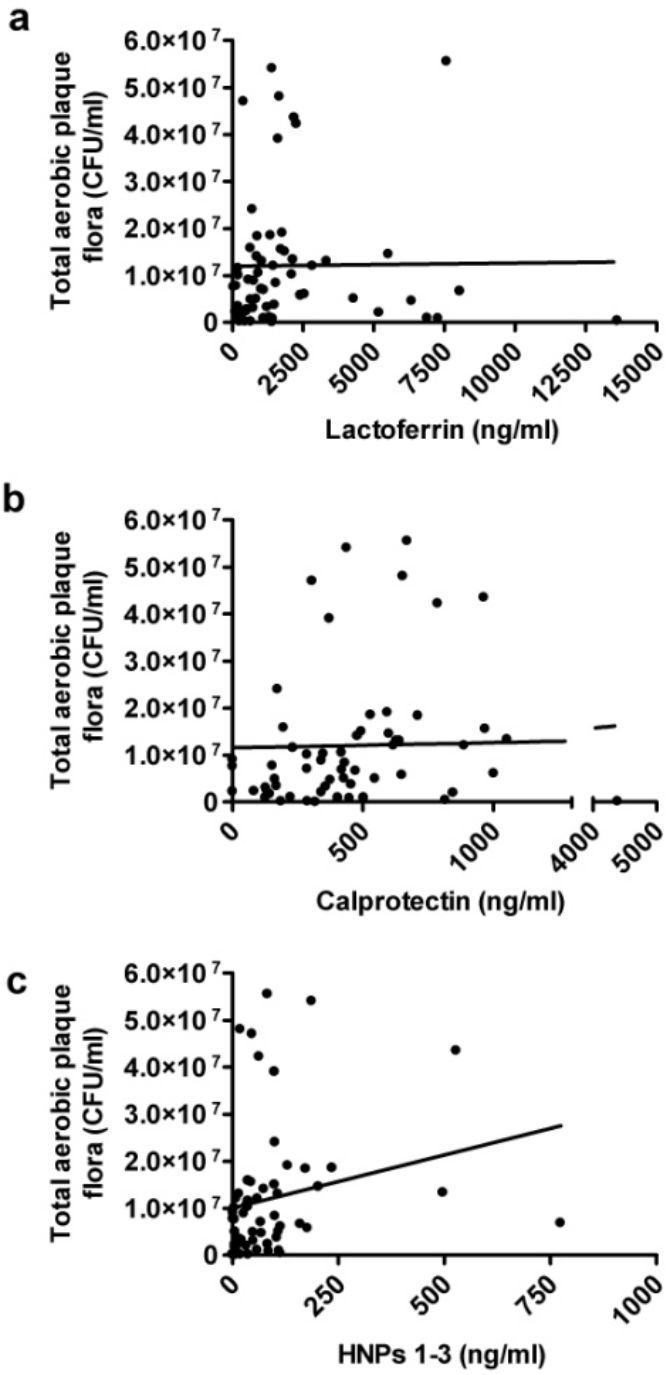


Figure 3-39: Associations between bacterial load of plaque and antimicrobial proteins

Scatter plots of total aerobic plaque flora (CFU/ml) by concentrations (ng/ml) of a) lactoferrin, b) calprotectin and c) HNPs 1-3. Raw data were plotted and the line of best fit is shown for each graph.

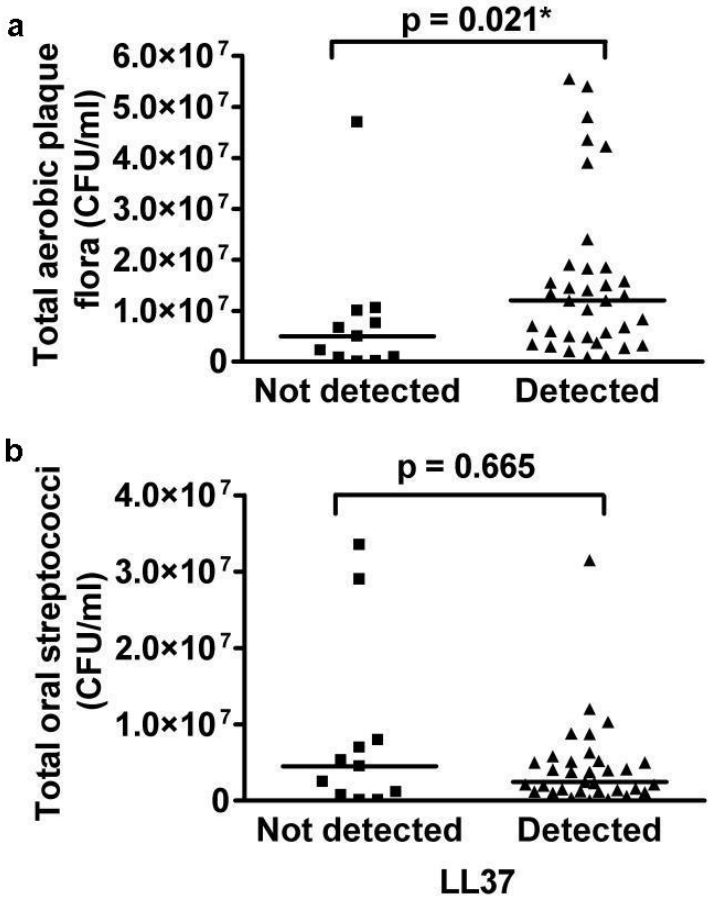


Figure 3-40: Bacterial load of plaque according to detection of LL37

Scatter plots of bacterial load, estimated by diagnostic culture of plaque of a) total aerobic flora (CFU/ml [n = 11 vs 35]) and b) total oral streptococci (CFU/ml [n = 11 vs 33]) and grouped according to detection of LL37 in the saliva of study participants. Raw data were plotted in each instance. Each data point represents a mean value for an individual participant and the horizontal bars indicate the group median. P values were generated by Mann-Whitney U tests.

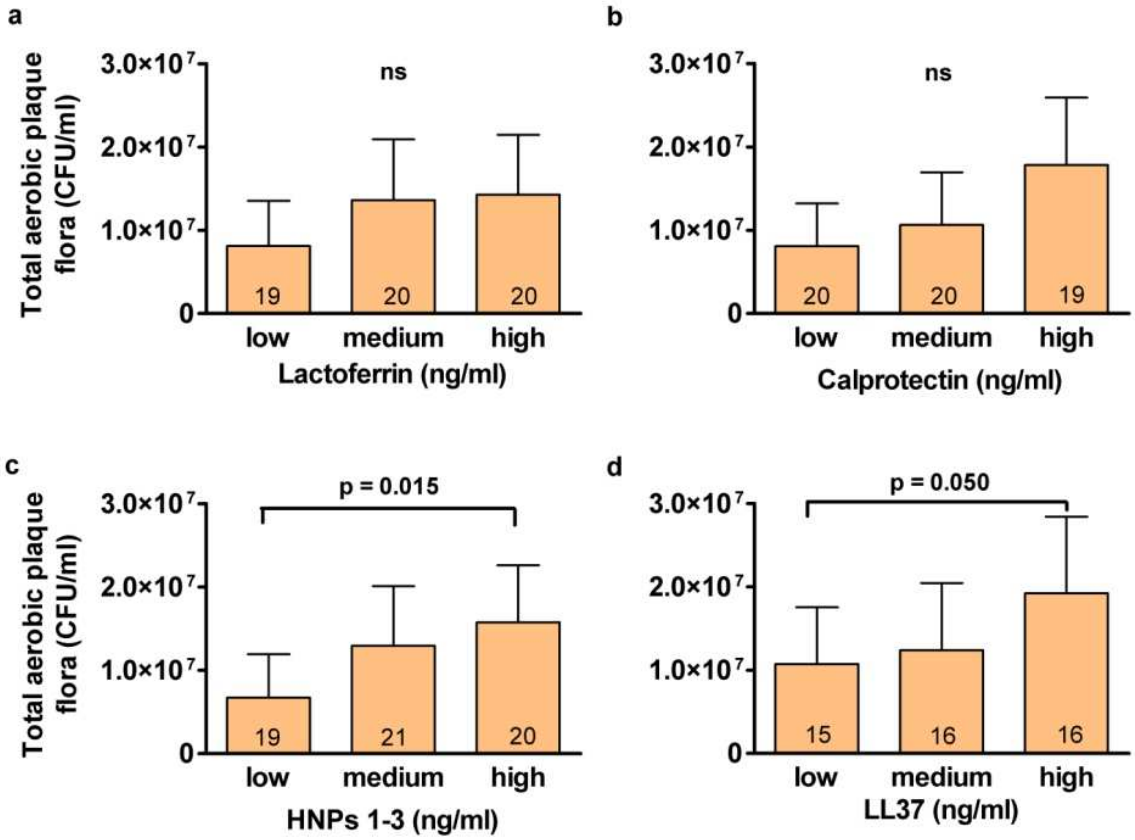


Figure 3-41: Associations of bacterial load of plaque and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of total aerobic flora (CFU/ml) estimated by diagnostic culture of plaque and grouped according to ranked tertiles of a) lactoferrin, b) calprotectin, c) HNPs 1-3 and d) LL37. Raw data were plotted in each instance (n in each group are shown on graphs). Statistically significant differences between groups as indicated were obtained using a Bonferroni *post hoc* test following ANOVA of $\log_{10}$ transformed data.

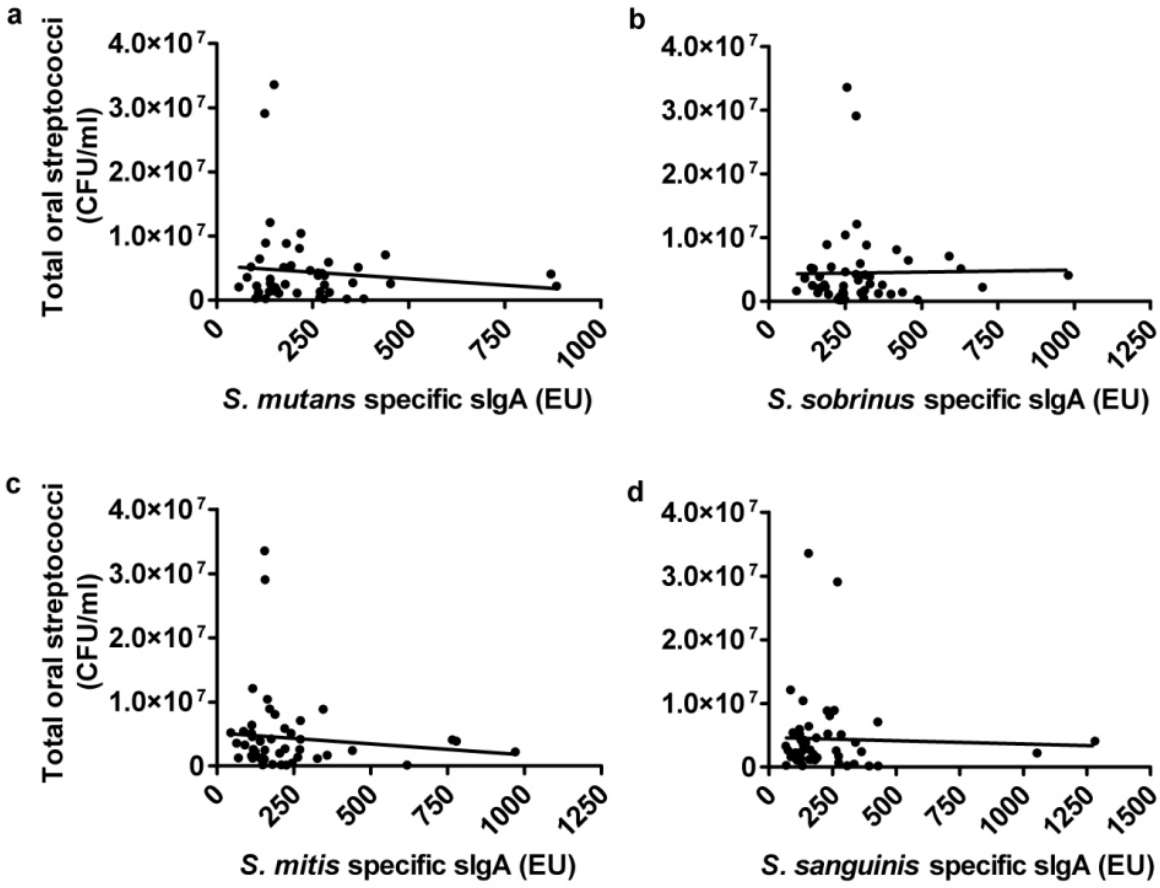


Figure 3-42: Associations of bacterial load of plaque and sIgA antibodies

Scatter plots of total oral streptococci (CFU/ml), estimated by diagnostic culture of plaque and titres (EU) of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus* c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted and the line of best fit is shown for each graph.

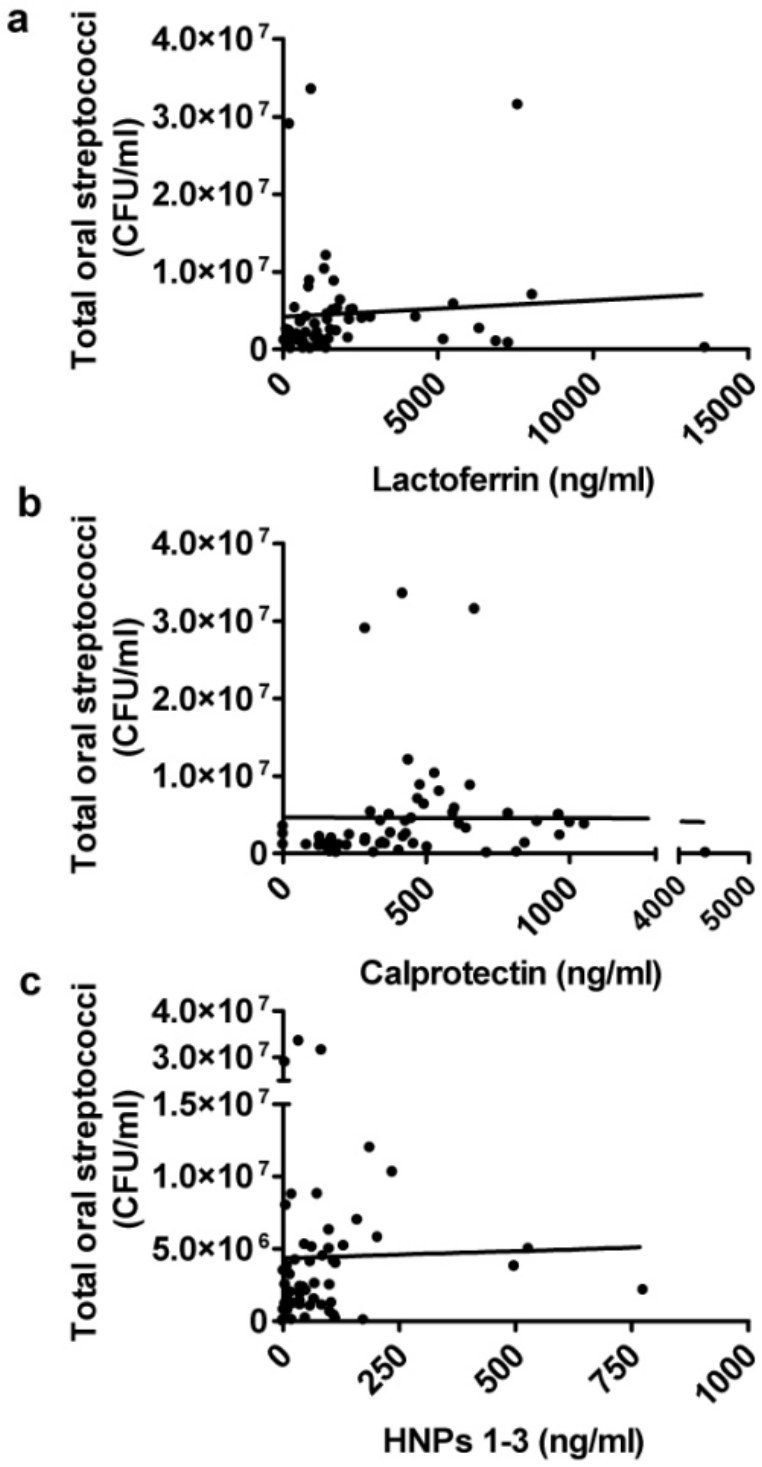


Figure 3-43: Associations of bacterial load of plaque and antimicrobial peptides

Scatter plots of total oral streptococci (CFU/ml), estimated by diagnostic culture of plaque and concentrations (ng/ml) of a) lactoferrin, b) calprotectin and c) the HNPs 1-3. Raw data were plotted and the line of best fit is shown for each graph.

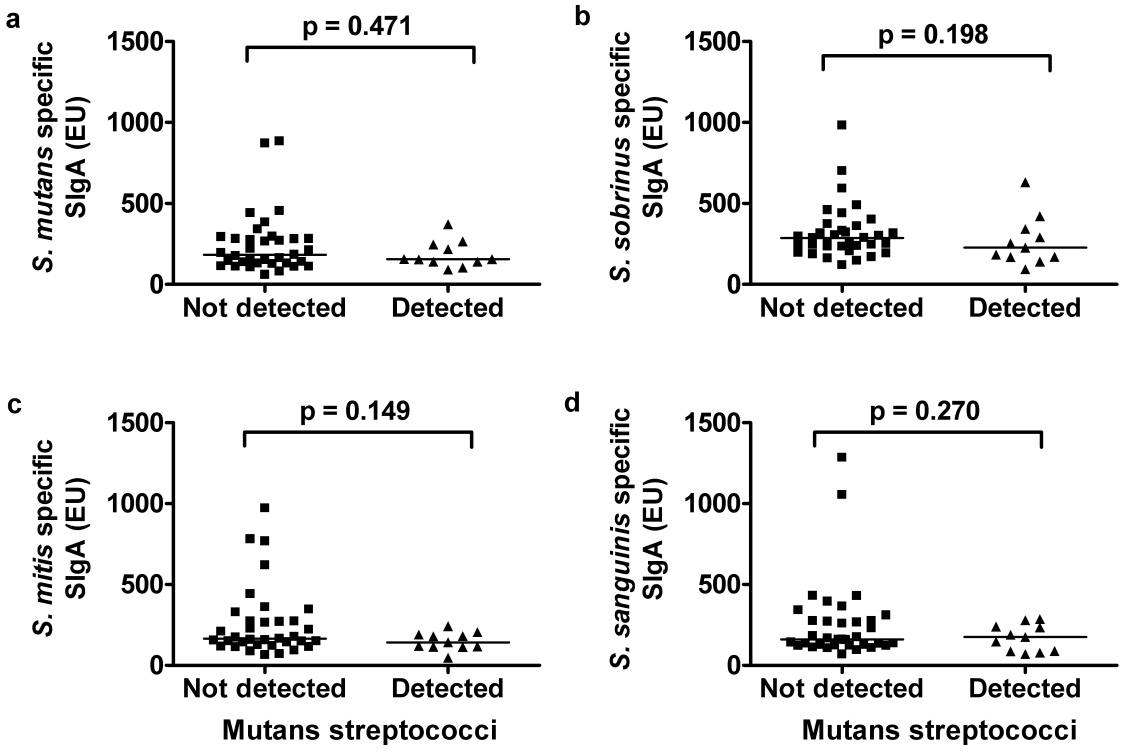


Figure 3-44: Titres of sIgA antibodies according to detection of Mutans streptococci

Scatter plots of titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis* grouped according to detection of Mutans streptococci by diagnostic culture of plaque. Raw data were plotted in each instance (n = 35 vs 11). Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group median. Differences were not statistically significant by Mann-Whitney U tests.

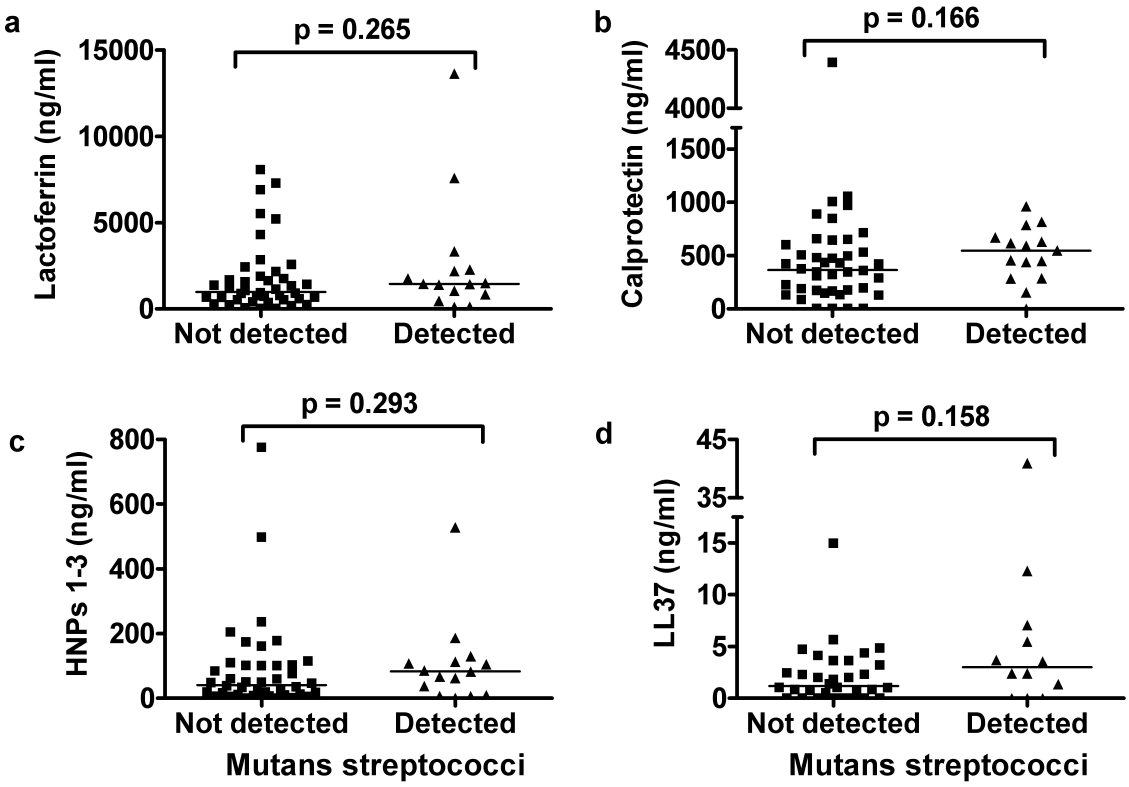


Figure 3-45: Antimicrobial proteins according to detection of Mutans streptococci

Scatter plots of a) lactoferrin (n = 42 vs 15), b) calprotectin (n = 42 vs 15), c) HNPs 1-3 (n = 42 vs 15) and d) LL37 (n = 33 vs 12) grouped according to detection of Mutans streptococci by diagnostic culture of plaque. Raw data were plotted. Each data point represents a mean value for an individual study participant and horizontal bars indicate the group median. Differences were not statistically significant by Mann-Whitney U tests.

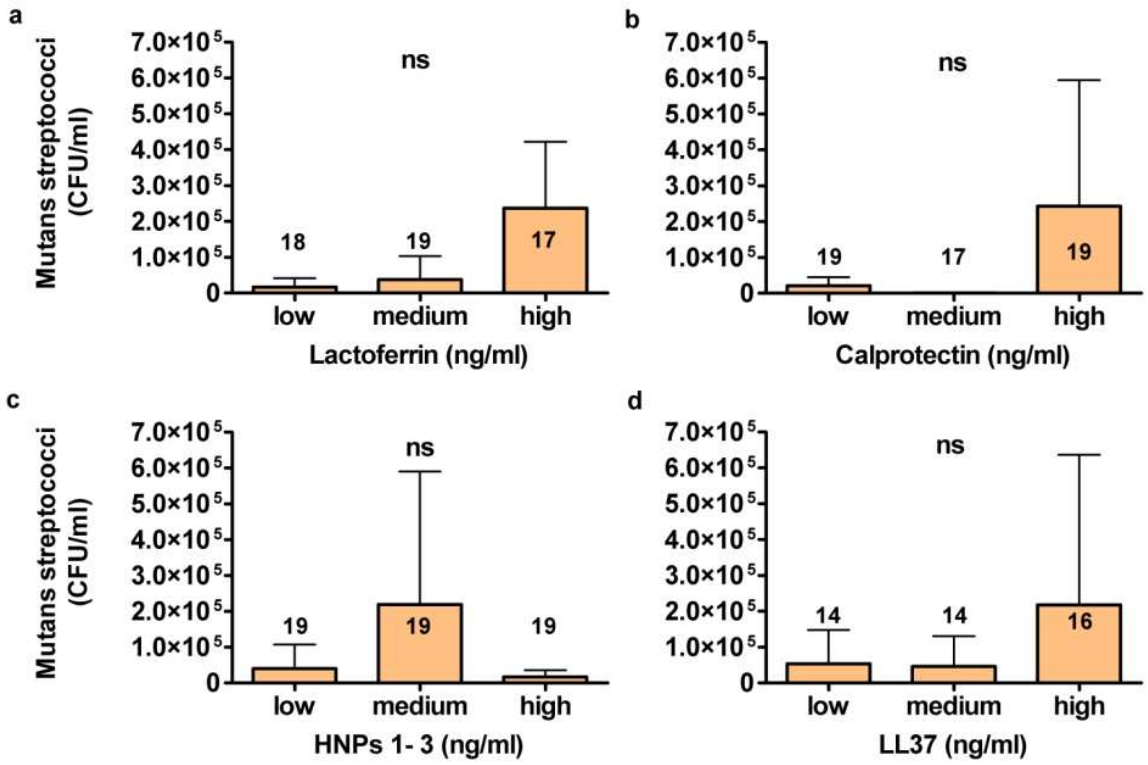


Figure 3-46: Associations of Mutans streptococci and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of Mutans streptococci (CFU/ml) estimated by diagnostic culture of plaque and grouped according to ranked tertiles of a) lactoferrin, b) calprotectin, c) HNPs 1-3 and d) LL37. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by Kruskal-Wallis test of non-parametric raw data.

3.2.7.2 Salivary antimicrobial proteins and QPCR of cariogenic bacteria from saliva

Absolute numbers of *S. mutans* (CFU/ml), estimated by TaqMan® QPCR of saliva, were not associated with titres of sIgA antibodies (Figure 3-47).

Salivary *S. mutans* was further investigated by ranked tertiles of sIgA antibodies (Figure 3-48). There was no association of mean salivary *S. mutans* with sIgA antibodies specific for *S. mutans, S. sobrinus, S. mitis* or *S. sanguinis* specific sIgA antibodies. Neither the differences between groups nor the linear trends were statistically significant by ANOVA.

Visual inspection of scatter plots of salivary *S. mutans* (CFU/ml) by antimicrobial protein concentrations did not provide evidence of linear associations (Figure 3-49).

LL37 was undetectable in a high number of participants; therefore the absolute and relative numbers of cariogenic bacteria in saliva and plaque were assessed with respect to the presence or absence of detectable LL37. Median salivary *S. mutans*, salivary *S. sobrinus* and *S. mutans* (% of Gram positive plaque flora) were slightly higher in children in whom LL37 could be detected compared to those in whom LL37 could not be detected although the differences did not reach statistical significance by Mann-Whitney U test (Figure 3-50a-c). Relative *S. sobrinus* (% of Gram positive plaque flora) was statistically significantly higher in children in whom LL37 was below the limit of detection in saliva ($p = 0.014$, Figure 3-50d).

Salivary *S. mutans* was further investigated by ranked tertiles of antimicrobial proteins (Figure 3-51). There were trends for increased mean salivary *S. mutans* CFU/ml with each increased tertile of lactoferrin, HNPs 1-3, LL37 and calprotectin. The differences between tertiles were not statistically significant by ANOVA. The linear trend was statistically significant for salivary *S. mutans* grouped by lactoferrin tertiles ($p = 0.046$) but did not reach statistical significance for calprotectin ($p = 0.095$), the HNPs 1-3 ($p = 0.132$) or LL37 ($p = 0.083$) by ANOVA linear.

Visual inspection of scatter plots of absolute numbers of salivary *S. sobrinus* (CFU/ml), estimated by TaqMan® QPCR, with titres of sIgA antibodies suggested a mild negative association with titres of sIgA antibodies specific for oral streptococci (Figure 3-52). However, there were many samples with very low counts lying on the axis that did not pass through the lines of best fit.

Salivary *S. sobrinus* counts were investigated by ranked tertiles of sIgA antibody titres (Figure 3-53). There was a trend for lower mean salivary *S. sobrinus* with each increasing tertile of *S. sobrinus, S. mutans* and *S. mitis* specific sIgA. Neither the differences between the groups, nor the linear trends were statistically significant by ANOVA or ANOVA linear respectively.

Scatter plots demonstrated no association between salivary *S. sobrinus* counts and concentrations of antimicrobial proteins from the saliva of study participants (Figure 3-54). Salivary *S. sobrinus* were investigated according to ranked tertiles of antimicrobial proteins (Figure 3-55). Mean salivary *S. sobrinus* increased with each increasing tertile of lactoferrin and calprotectin, although differences between the groups were not statistically significant. The linear trend for increased mean salivary *S. sobrinus* with each increased lactoferrin tertile was statistically significant by ANOVA linear ($p = 0.020$). Mean salivary *S. sobrinus* showed no association with tertiles of the HNPs 1-3 or LL37.

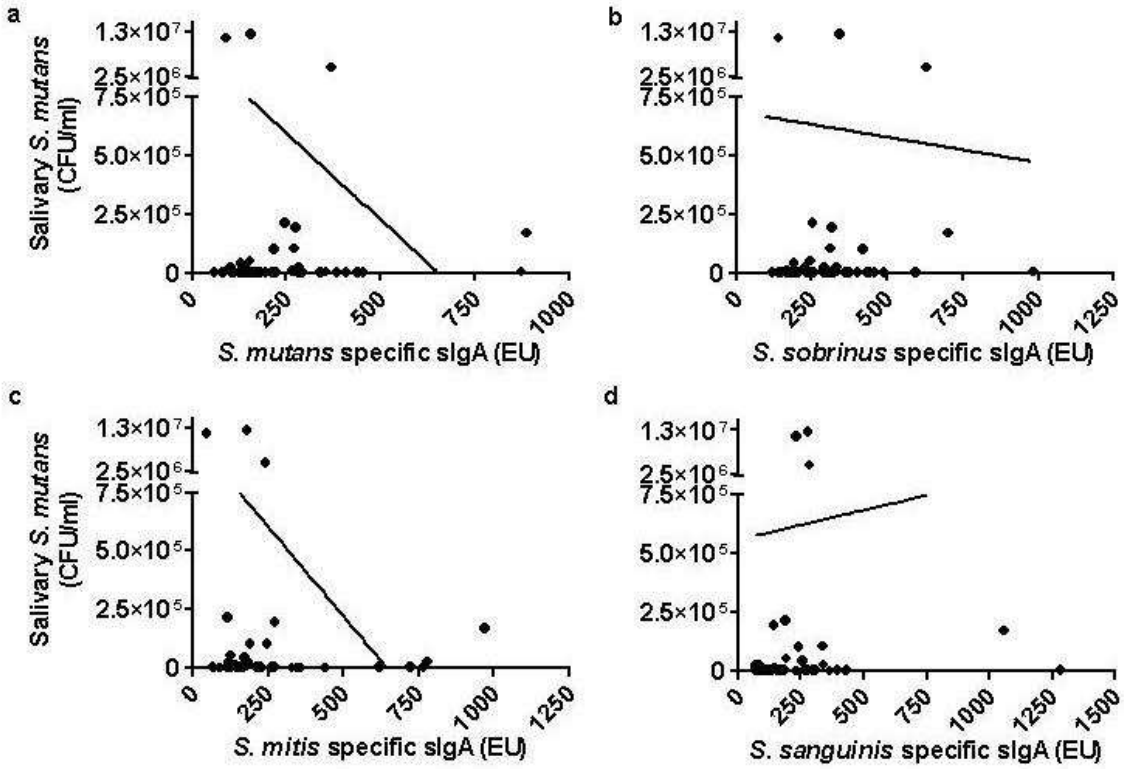


Figure 3-47: Associations between salivary *S. mutans* and sIgA antibodies

Scatter plots of *S. mutans* (CFU/ml) estimated by Taqman® QPCR of saliva and titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted and the line of best fit is shown for each graph.

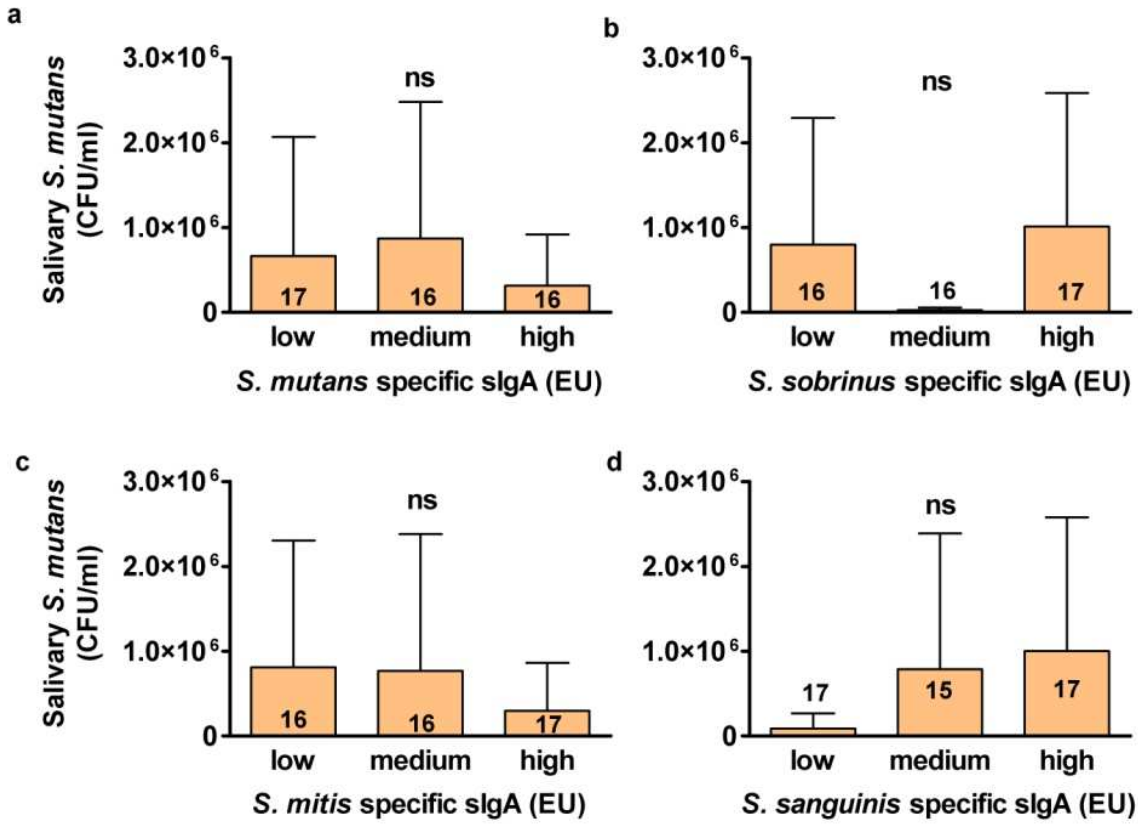


Figure 3-48: Associations of salivary *S. mutans* and ranked tertiles of sIgA antibodies

Data are mean and 95% confidence intervals of the mean of *S. mutans* (CFU/ml) estimated by TaqMan® QPCR of saliva grouped according to ranked tertiles of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

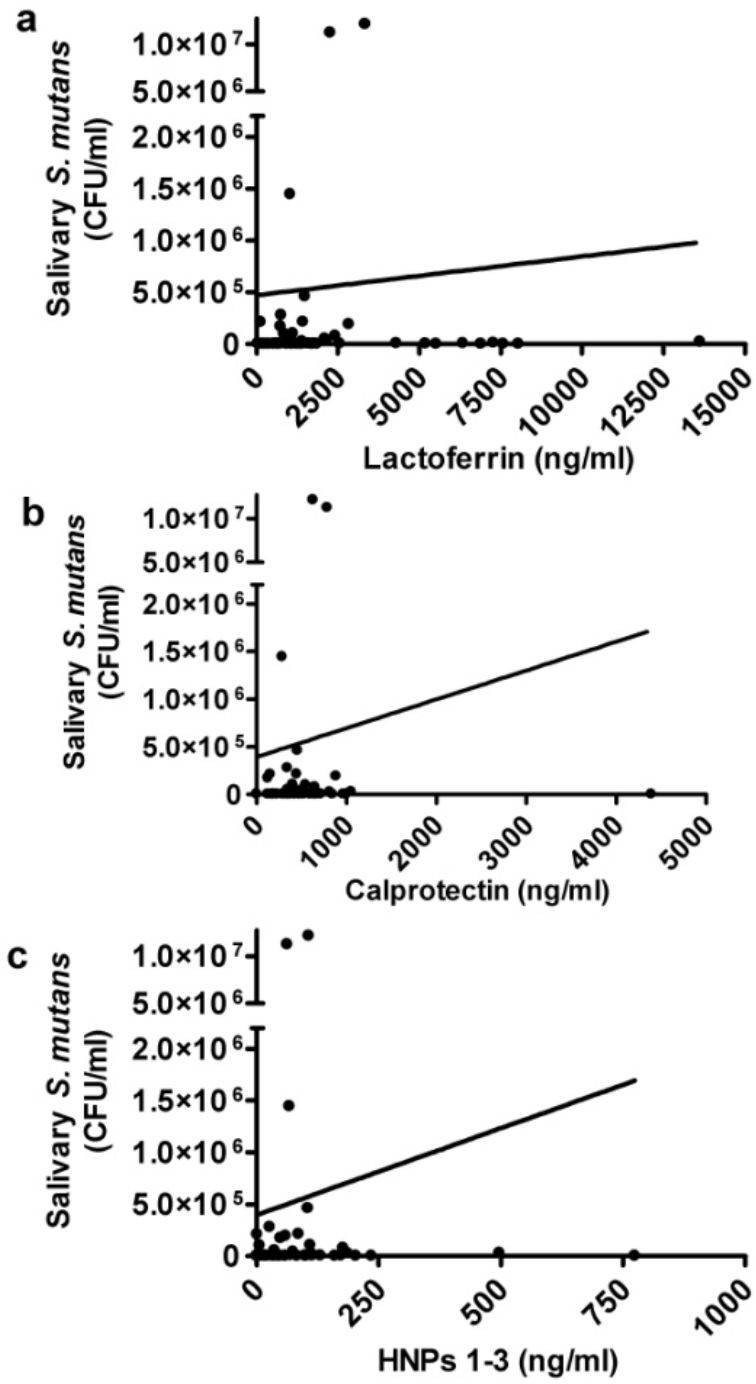


Figure 3-49: Association of salivary *S. mutans* and antimicrobial proteins

Scatter plots of *S. mutans* (CFU/ml) estimated by TaqMan® QPCR of saliva with a) lactoferrin, b) calprotectin and c) HNPs 1-3. Raw data were plotted and the line of best fit is shown for each graph.

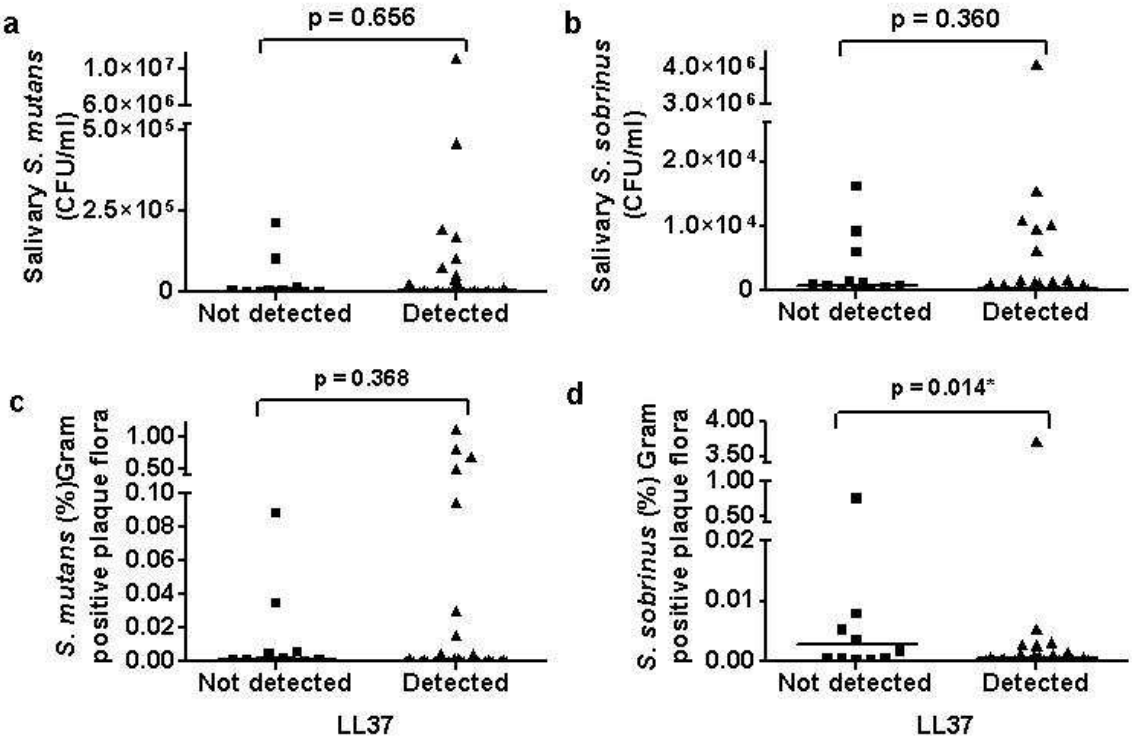


Figure 3-50: Cariogenic bacteria in plaque and saliva by detection of LL37

Scatter plots of cariogenic bacteria estimated by TaqMan® QPCR of saliva a) *S. mutans* (CFU/ml) and b) *S. sobrinus* (CFU/ml), and c) *S. mutans* and d) *S. sobrinus* as a proportion (%) of Gram positive plaque flora, grouped according to detection of LL37. Raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and the horizontal bars indicate the group median. In some cases the median values lie close to or on the x axis. P values were generated by Mann-Whitney U tests.

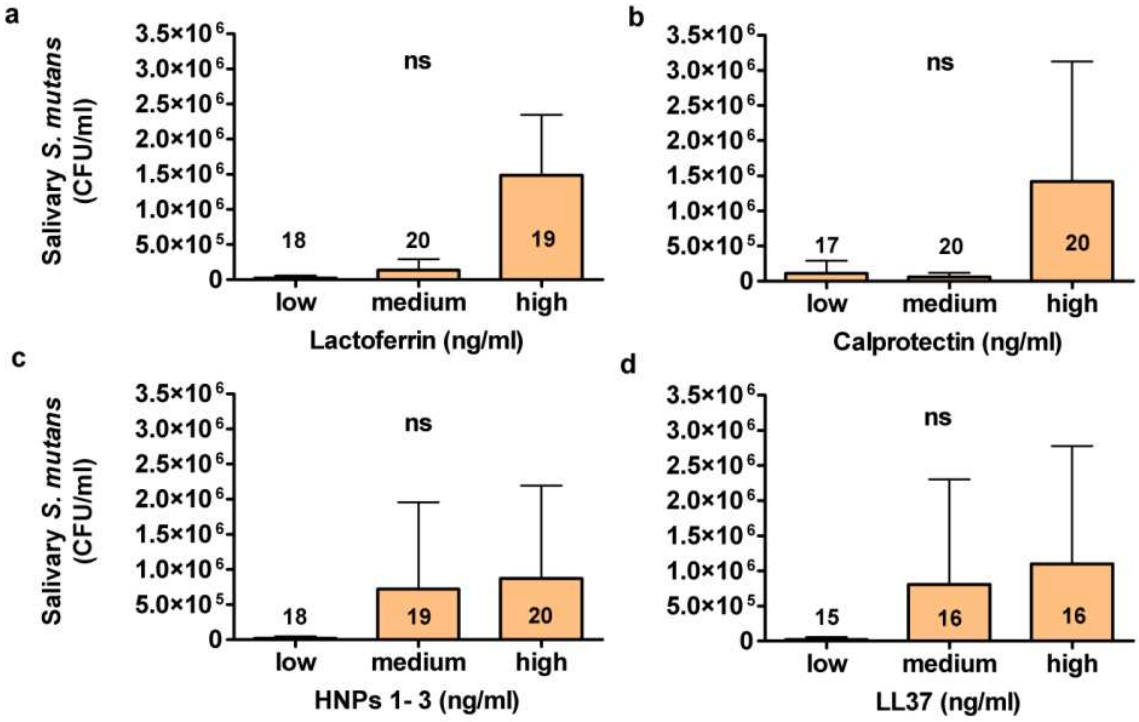


Figure 3-51: Associations of salivary *S. mutans* and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of *S. mutans* (CFU/ml), estimated by TaqMan® QPCR of saliva of study participants, grouped according to ranked tertiles of a) lactoferrin, b) calprotectin, c) HNPs 1-3 and d) LL37. Raw data were plotted in each instance. Differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data. The linear trend was statistically significant for (a) by ANOVA linear ($p = 0.046$).

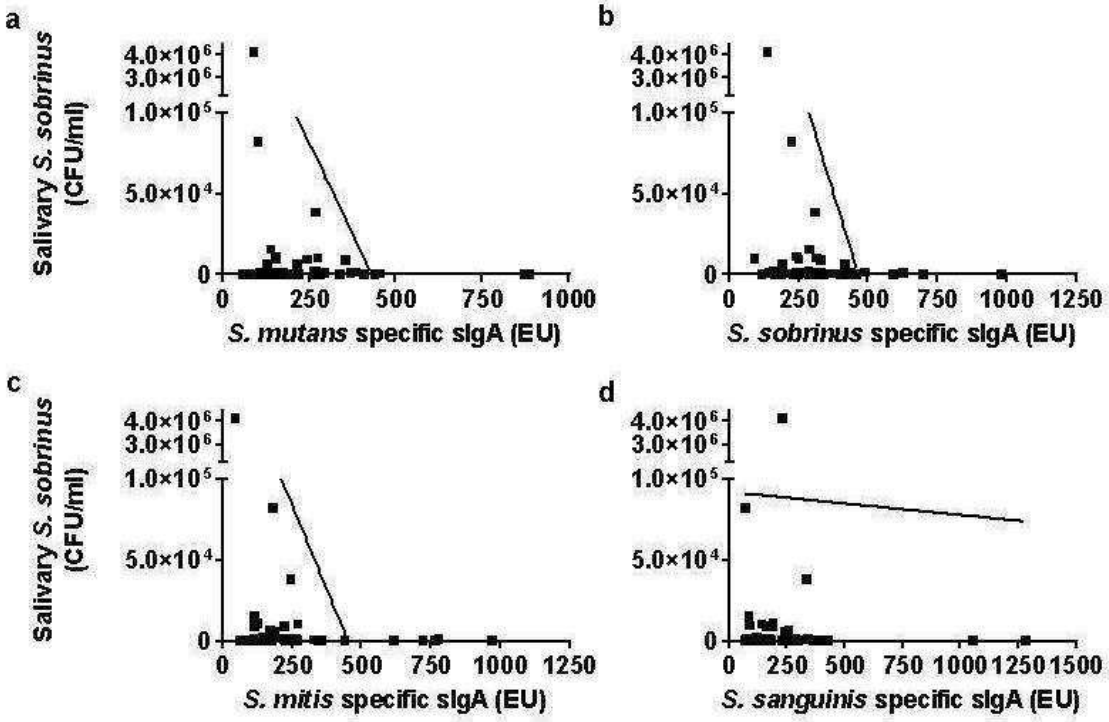


Figure 3-52: Associations of salivary *S. sobrinus* and sIgA antibodies

Scatter plots of *S. sobrinus* (CFU/ml), estimated by TaqMan® QPCR of saliva, and titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance. The lines of best fit are shown for each graph.

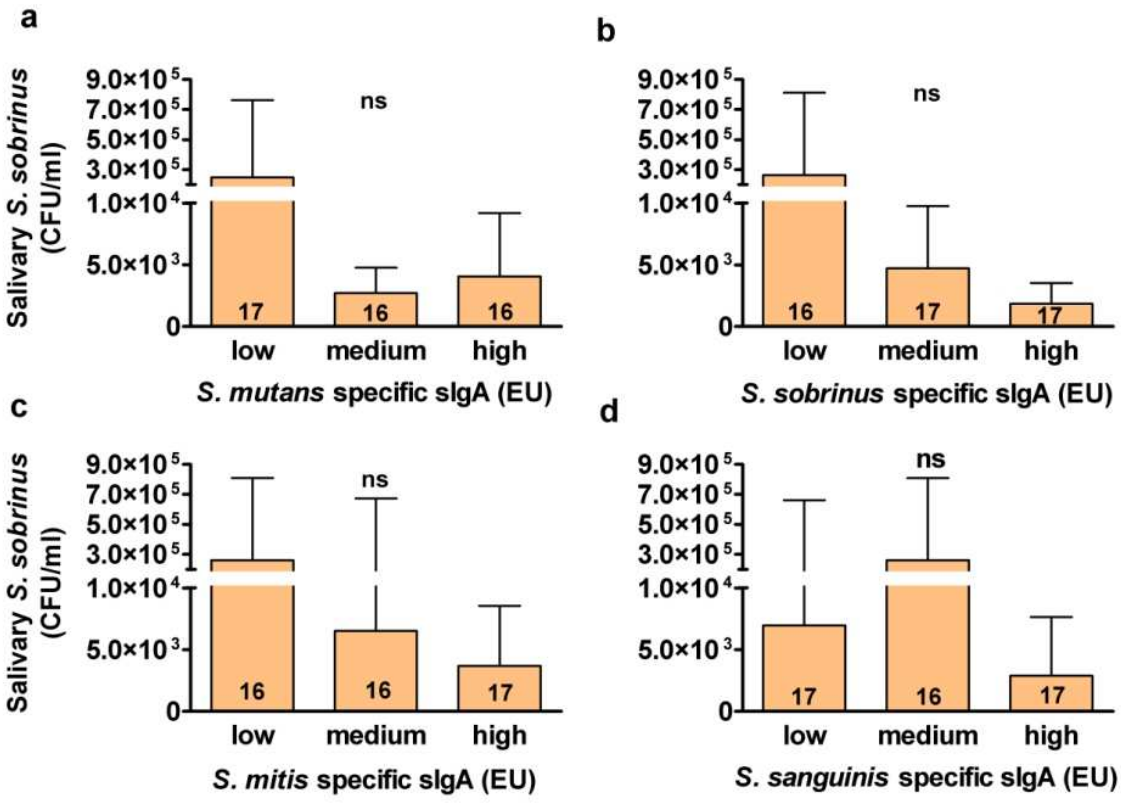


Figure 3-53: Associations of salivary *S. sobrinus* and ranked tertiles of sIgA antibodies

Data are mean and 95% confidence intervals of the mean of *S. sobrinus* (CFU/ml), estimated by TaqMan® QPCR of saliva, grouped according to ranked tertiles of titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

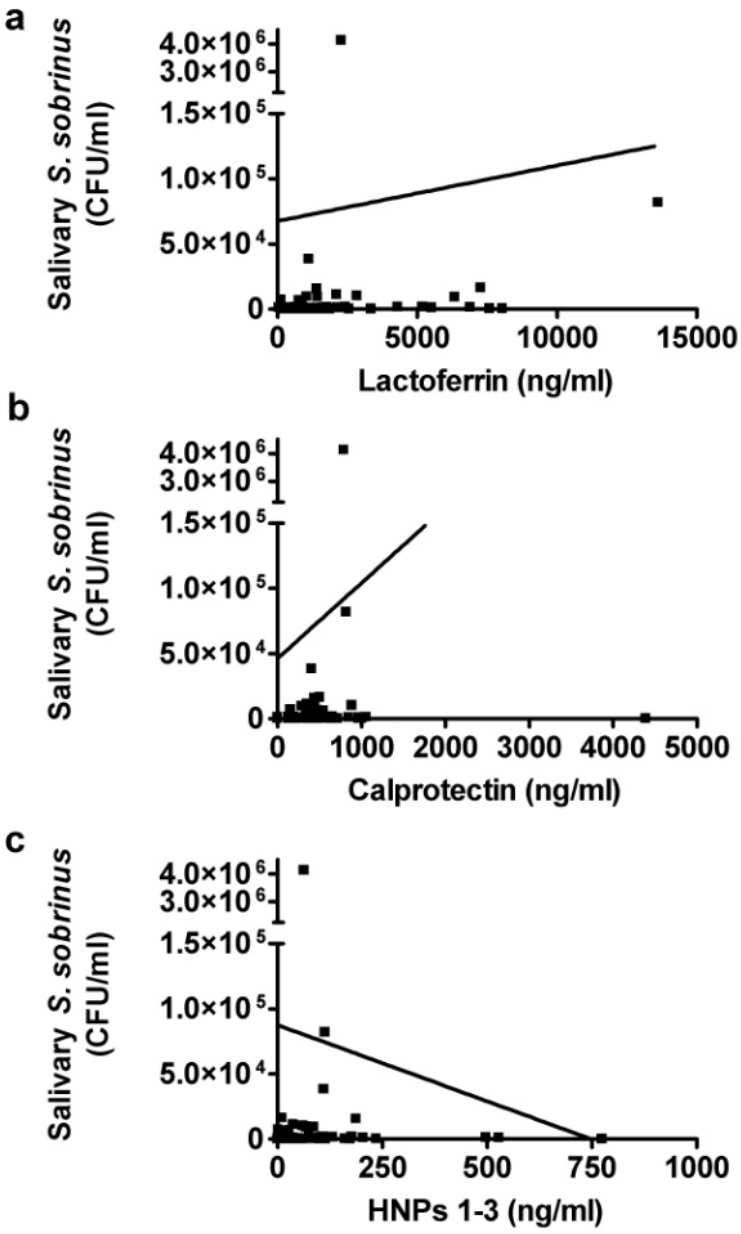


Figure 3-54: Associations of salivary *S. sobrinus* and antimicrobial proteins

Scatter plots of associations of *S. sobrinus* (CFU/ml), estimated by TaqMan® QPCR of saliva, and a) lactoferrin, b) calprotectin and c) HNPs 1-3. Raw data were plotted and the line of best fit is shown for each graph.

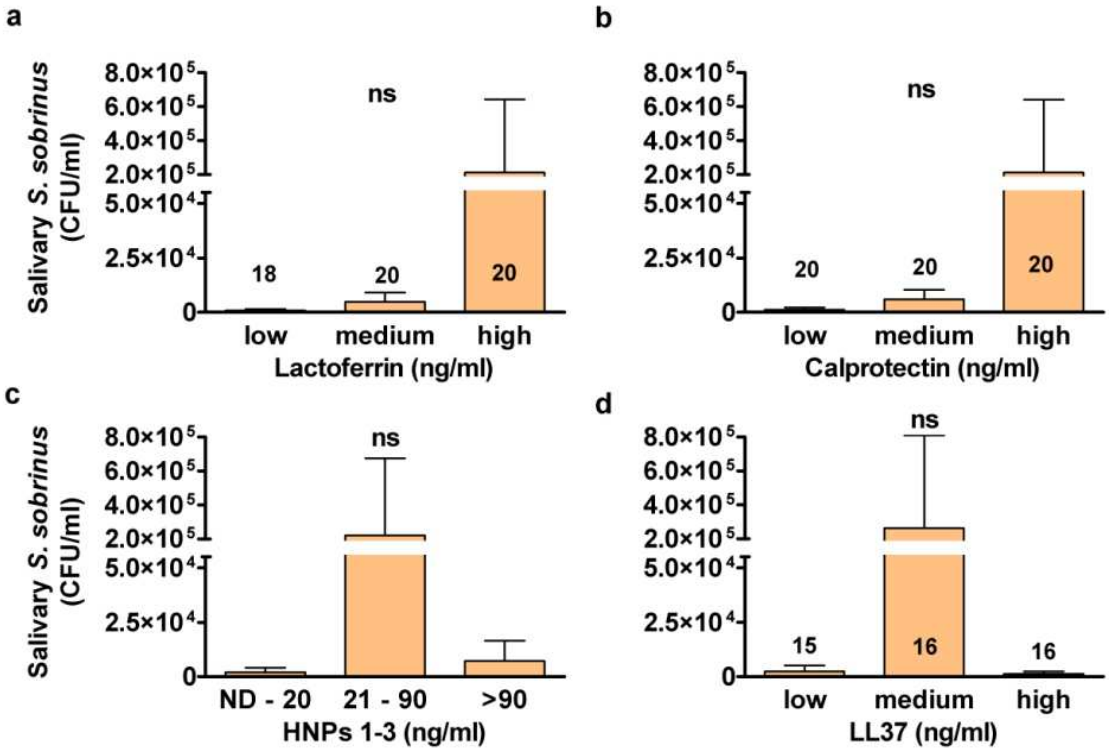


Figure 3-55: Associations of salivary *S. sobrinus* and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of *S. sobrinus* (CFU/ml), estimated by TaqMan® QPCR of saliva, grouped according to ranked tertiles of a) lactoferrin, b) calprotectin, c) HNPs 1-3 and d) LL37. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data. The linear trend for (a) was statistically significant by ANOVA linear ($p = 0.020$).

3.2.7.3 Salivary antimicrobial proteins and QPCR of cariogenic bacteria from plaque

Visual inspection of scatter plots revealed no associations of *S. mutans* (%) of Gram positive plaque flora with sIgA antibodies specific for oral streptococci (Figure 3-56). *S. mutans* (%) of Gram positive plaque flora were investigated according to ranked tertiles of sIgA antibody titres. The differences between groups and the linear trends were not statistically significant (Figure 3-57b-d and data not shown).

S. mutans (%) of Gram positive plaque flora was not associated with lactoferrin, calprotectin or the HNPs 1-3 (Figure 3-58). *S. mutans* as a proportion (% of Gram positive plaque flora) was also investigated by ranked tertiles of antimicrobial proteins. Mean *S. mutans* (% Gram positive plaque flora) increased with each increased tertile of lactoferrin, although the differences between groups and the linear trend were not statistically significant (Figure 3-59a and data not shown). There were no associations of *S. mutans* (% Gram positive plaque flora) with ranked tertiles of calprotectin, the HNPs 1-3 or LL37 and the differences between groups were not statistically significant (Figure 3-59b-d).

Visual inspection of scatter plots revealed no associations of relative *S. sobrinus* as a proportion (% of Gram positive plaque flora) with titres of sIgA antibodies (Figure 3-60). *S. sobrinus* (% of Gram positive plaque flora) was investigated for associations with ranked tertiles of titres of sIgA antibodies (Figure 3-61). Mean *S. sobrinus* (%) decreased with each increased tertile of titres of *S. mutans* specific sIgA, with a statistically significant difference between the low and high tertile ($p = 0.047$, Figure 3-61a), and statistically significant linear trend by ANOVA linear ($p = 0.016$). Conversely, mean *S. sobrinus* (% Gram positive plaque flora) increased with each increased tertile of titres of *S. sobrinus* and *S. mitis* specific sIgA, although the differences between groups and the linear trend were not statistically significant (Figure 3-61b and c). There was no association of *S. sobrinus* (% Gram positive plaque flora) with ranked tertiles of *S. sanguinis* specific sIgA antibody titres (Figure 3-61d).

Scatter plots of relative *S. sobrinus* as a proportion (%) of Gram positive plaque flora with concentrations of antimicrobial proteins did not provide any linear associations (Figure 3-62).

S. sobrinus as a proportion (%) of Gram positive plaque flora was investigated for associations with ranked tertiles of antimicrobial proteins. Mean *S. sobrinus* (%) decreased with each increased tertile of lactoferrin and calprotectin, although the differences between groups and the linear trends were not statistically significant (Figure 3-63a & b, and data not shown). There was no association of relative *S. sobrinus* (%) with ranked tertiles of the HNPs 1-3. Mean *S. sobrinus* was statistically significantly higher in children with LL37 concentrations ranked in the low tertile, compared with LL37 concentrations in the medium and high tertiles ($p = 0.004$ and $p = 0.039$, respectively Figure 3-63d). The trend for decreased mean *S. sobrinus* (%) with each increased LL37 tertile was also statistically significant ($p = 0.017$) by ANOVA linear.

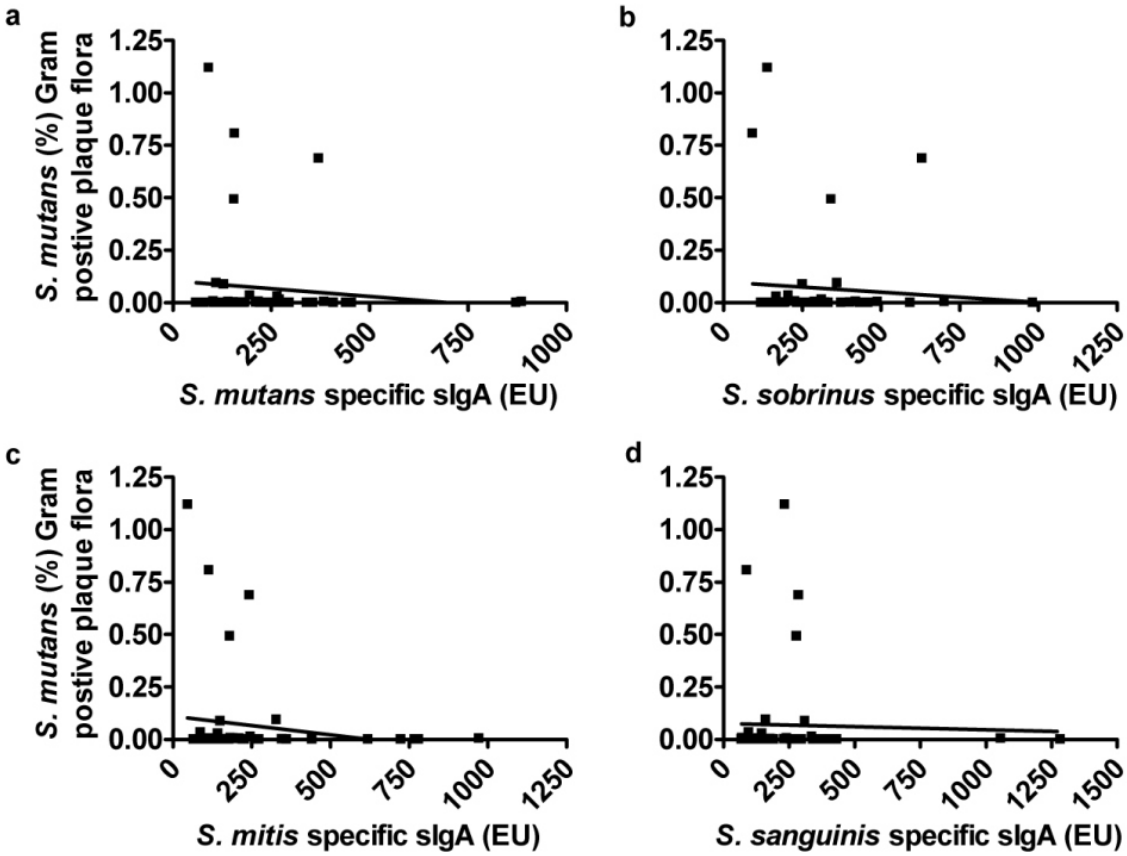


Figure 3-56: Associations between *S. mutans* as a proportion of Gram positive plaque flora and sIgA antibodies

Scatter plots of *S. mutans* (%) of Gram positive plaque flora, and titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance. The line of best fit is shown for each graph.

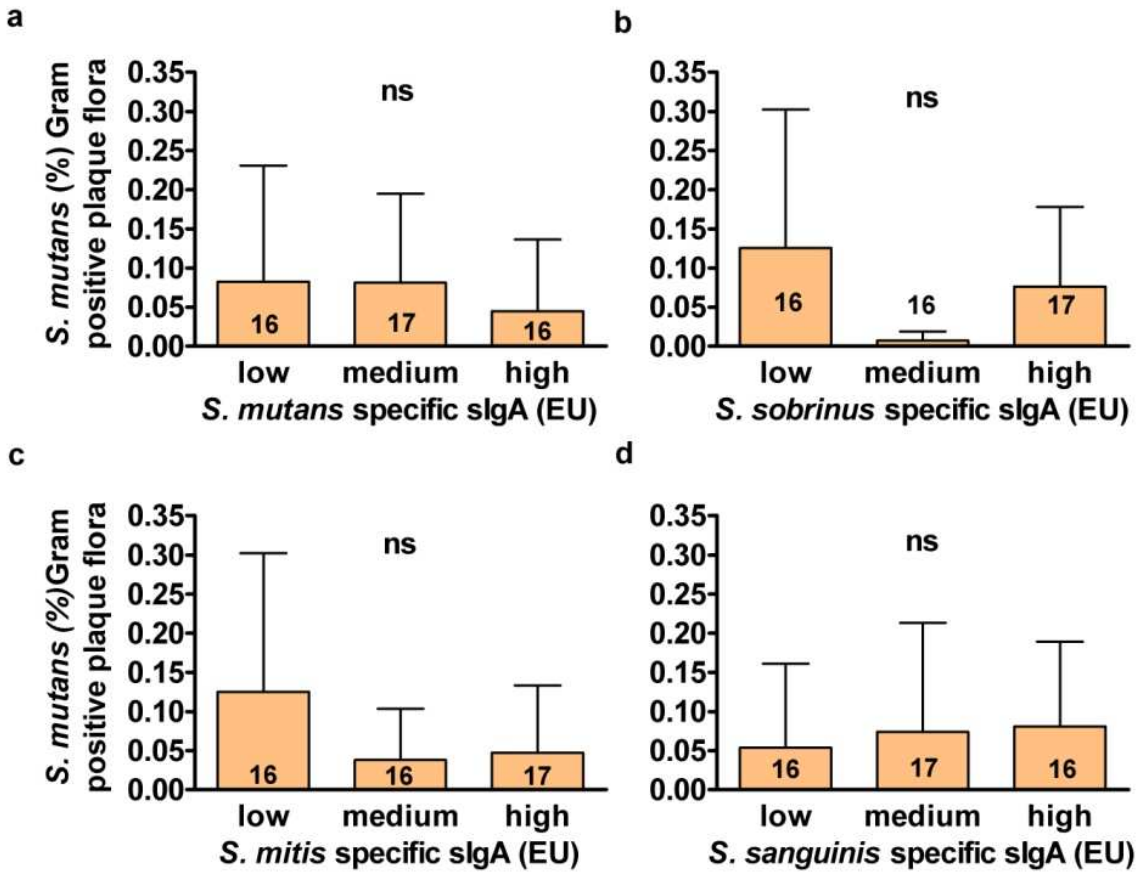


Figure 3-57: Associations of *S. mutans* as a proportion of Gram positive plaque flora and ranked tertiles of sIgA antibodies

Data are mean and 95% confidence intervals of the mean of *S. mutans* (%) of Gram positive plaque flora, grouped according to ranked tertiles of titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

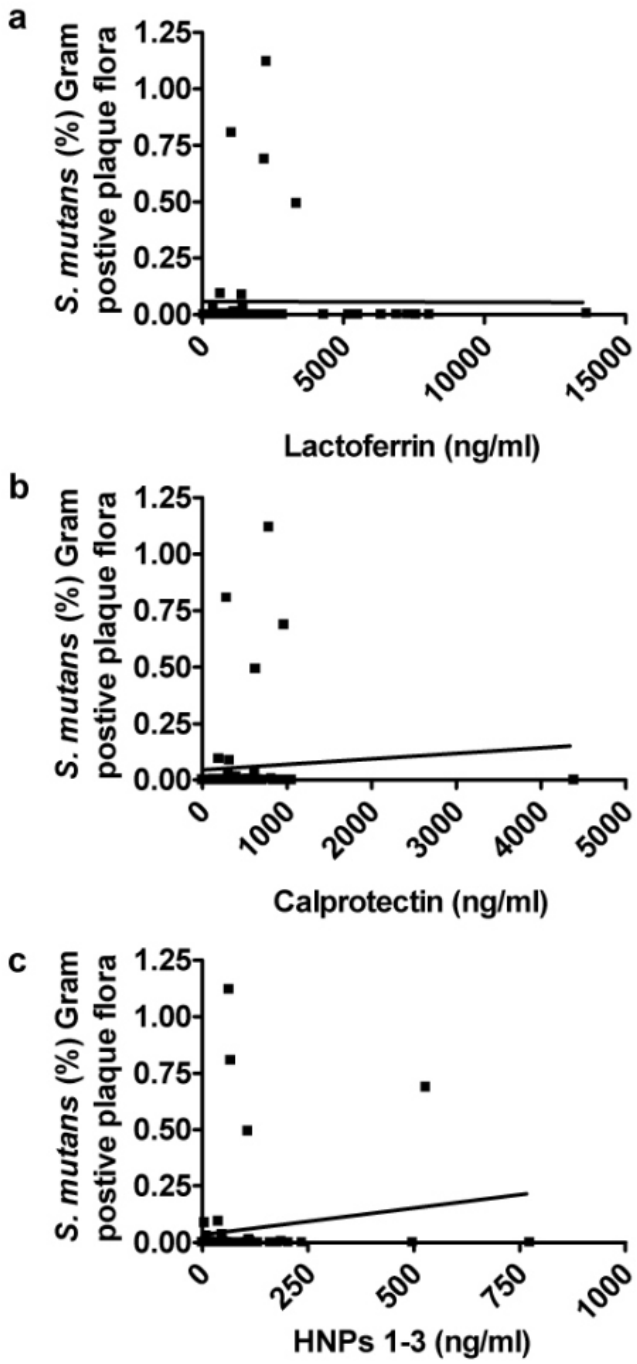


Figure 3-58: Associations between *S. mutans* as a proportion of Gram positive plaque flora and antimicrobial proteins

Scatter plots of *S. mutans* (%) of Gram positive plaque flora and a) lactoferrin, b) calprotectin and c) the HNPs 1-3. Raw data were plotted in each instance. The line of best fit is shown for each graph.

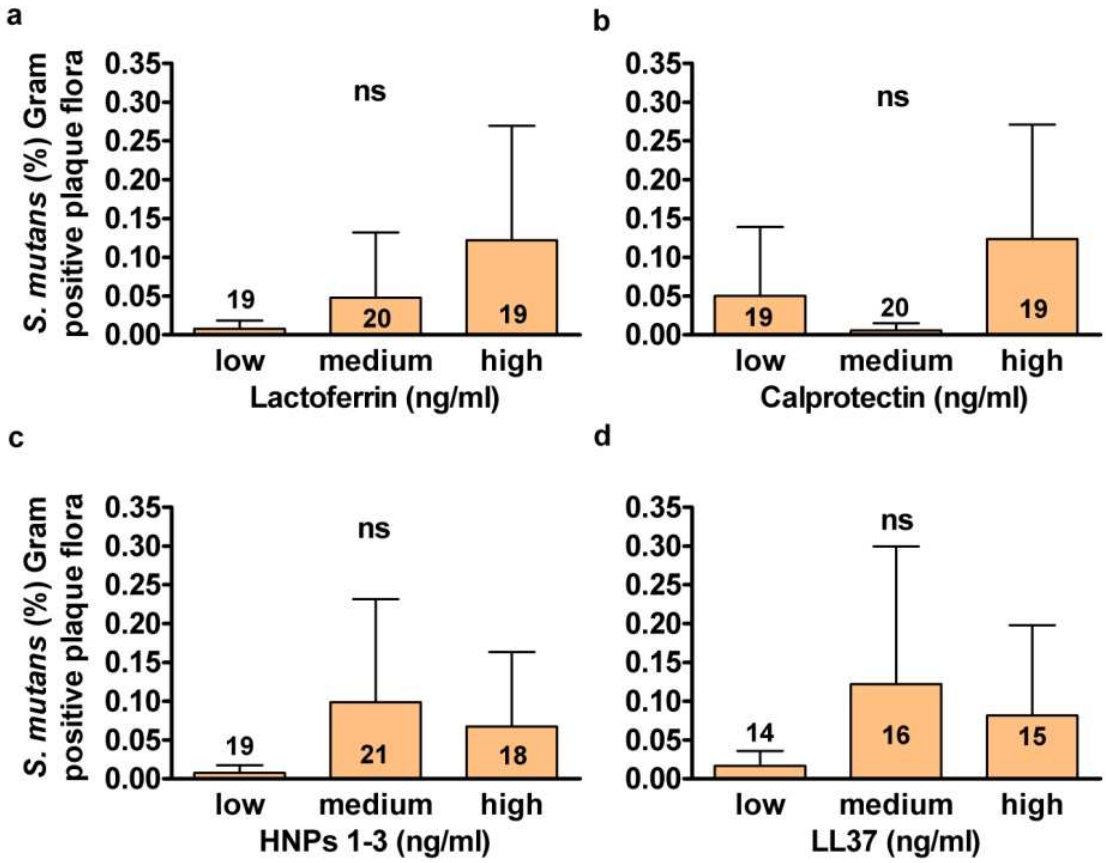


Figure 3-59: Associations of *S. mutans* as a proportion of Gram positive plaque flora and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of *S. mutans* (%) of Gram positive plaque flora, grouped according to tertiles of a) lactoferrin, b) calprotectin and c) the HNPs 1-3. Raw data were plotted in each instance (n in each group are shown on graphs). The differences between groups were not statistically significant by ANOVA of $\log_{10}$ transformed data.

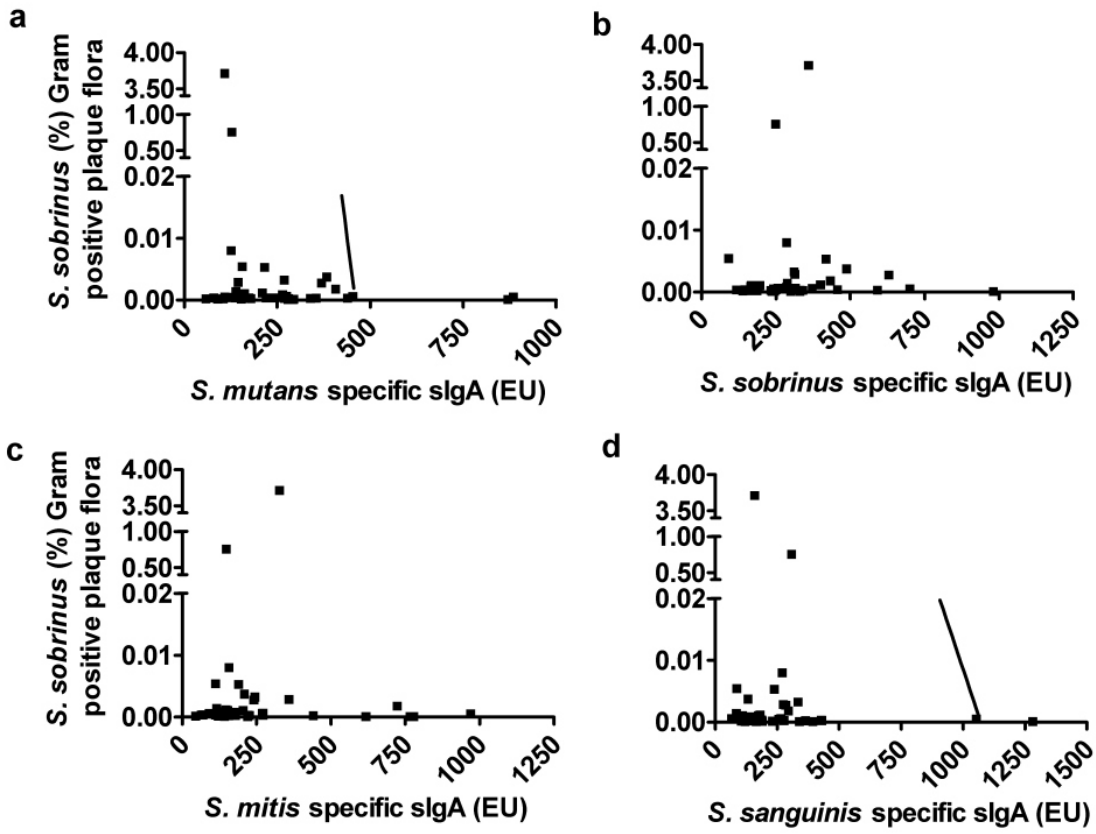


Figure 3-60: Associations of *S. sobrinus* as a proportion of Gram positive plaque flora and titres of sIgA antibodies

Scatter plots of *S. sobrinus* (%) of Gram positive plaque flora and titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted and the line of best fit is shown on each graph.

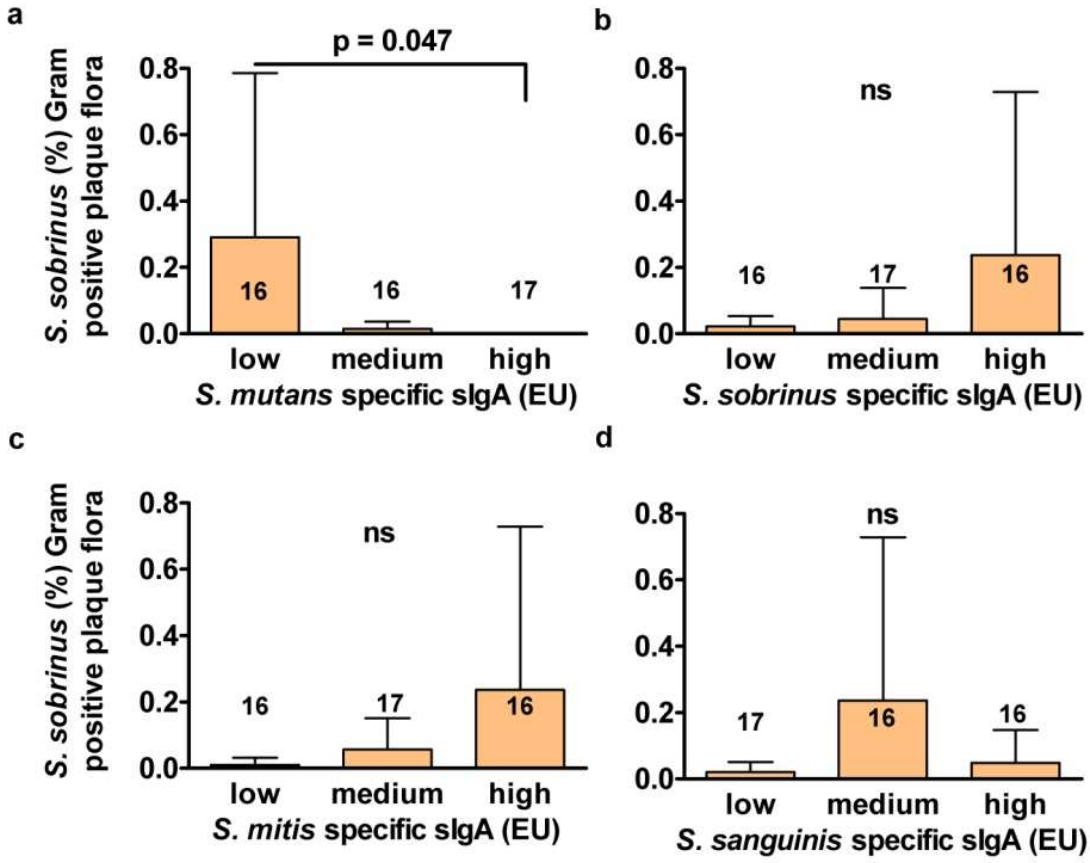


Figure 3-61: Associations of *S. sobrinus* as a proportion of Gram positive plaque flora and ranked tertiles of sIgA antibodies

Data are mean and 95% confidence intervals of the mean of *S. sobrinus* (%) of Gram positive plaque flora grouped according to ranked tertiles of titres of sIgA antibodies specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis*. Raw data were plotted in each instance (n in each group are shown on graphs). Statistically significant difference obtained from Bonferroni *post hoc* test following ANOVA of $\log_{10}$ transformed data. The linear trend for (a) was statistically significant by ANOVA linear (p = 0.016).

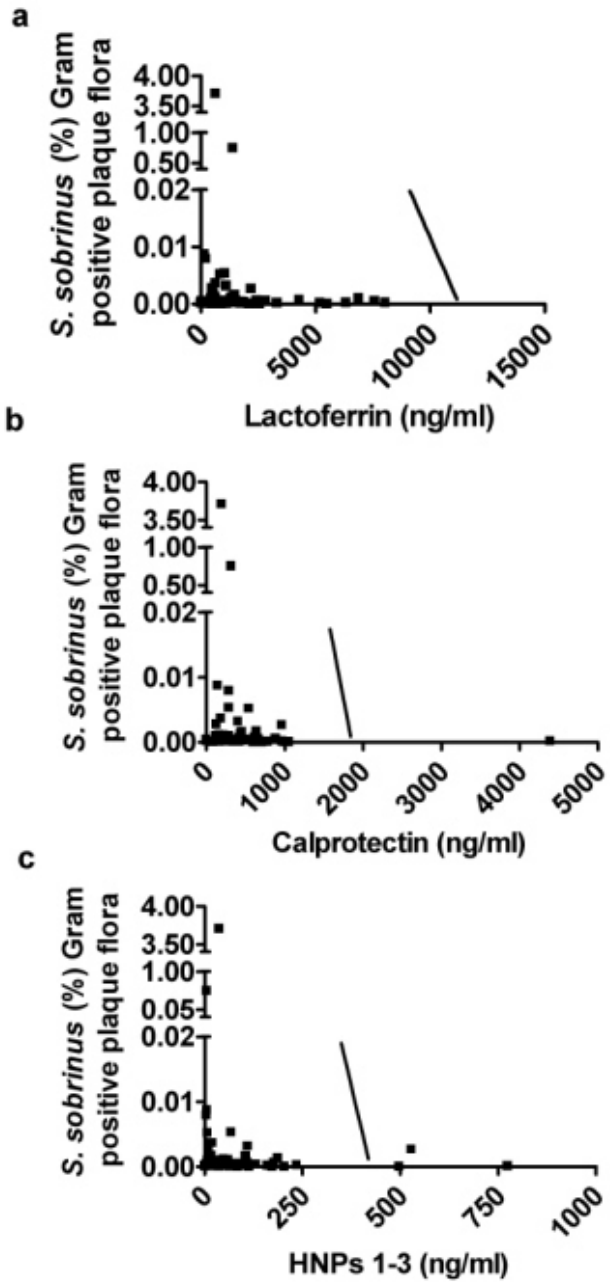


Figure 3-62: Associations of *S. sobrinus* as a proportion of Gram positive plaque flora and antimicrobial proteins

Scatter plots of *S. sobrinus* (%) of Gram positive plaque flora and a) lactoferrin, b) calprotectin and c) the HNPs 1-3. Raw data were plotted and the line of best fit is shown on each graph.

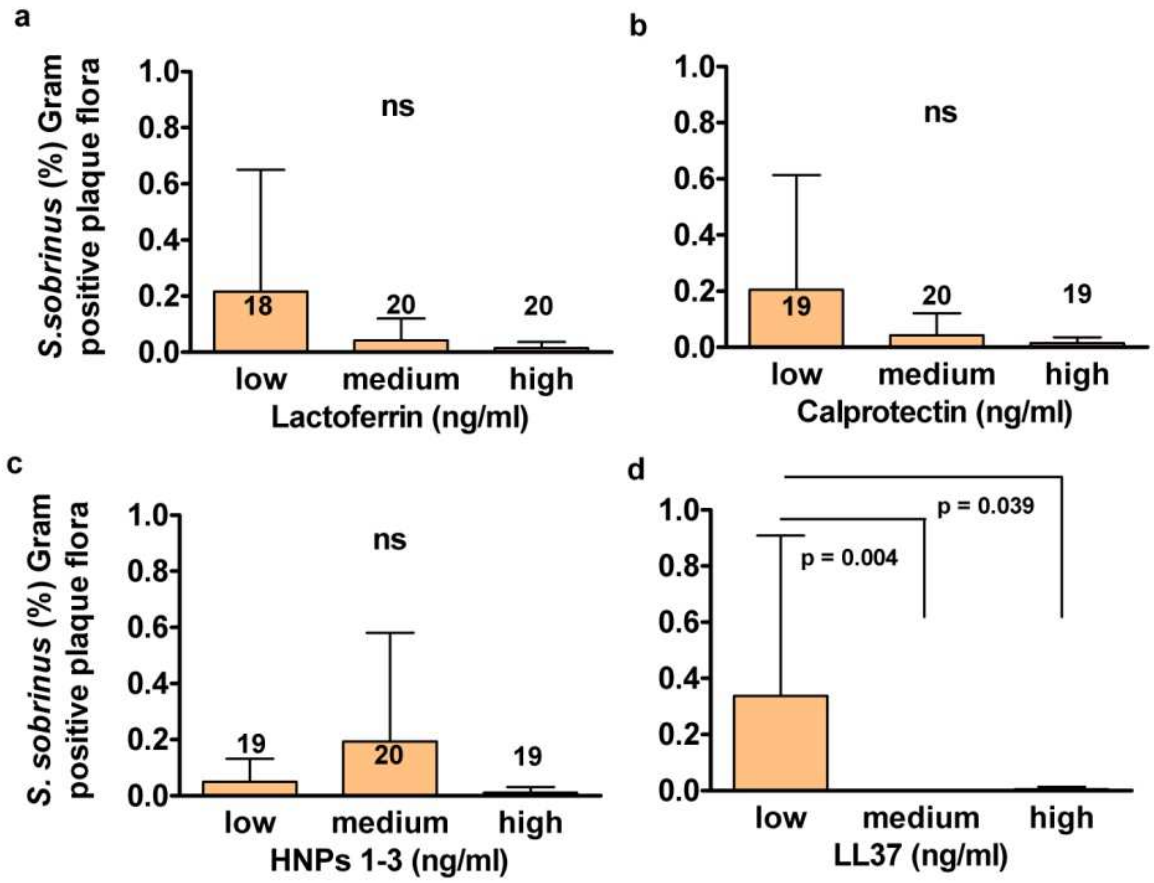


Figure 3-63: Associations of *S. sobrinus* as a proportion of Gram positive plaque flora and ranked tertiles of antimicrobial proteins

Data are mean and 95% confidence intervals of the mean of *S. sobrinus* (%) of Gram positive plaque flora, grouped according to ranked tertiles of a) lactoferrin, b) calprotectin, c) HNPs 1-3 and d) LL37. Raw data were plotted in each instance (n in each group are shown on graphs). Statistically significant differences between groups obtained by Bonferroni *post hoc* test following an ANOVA of $\log_{10}$ transformed data. The linear trend for (d) was statistically significant by ANOVA linear ($p = 0.017$).

3.3 Discussion

This study demonstrates that Childsmile's unusually young target age group combined with the multiple visit interventions integral to the program are a useful opportunity to investigate the oral biofilm coincident with the salivary immune response. Variables hypothesised to influence the development of carious disease can be collected and successfully quantified in one-year-old children. The investigations of whether socioeconomic status or life stresses influence bacterial load, the detection of cariogenic bacteria or the immune response were complex and the data interrogating the impact of the immune response on bacterial load or the detection of cariogenic bacteria reveal potentially interesting findings. This was an ambitious pilot project and one of the main limitations was the sample size.

Based on previous epidemiological data of dental caries prevalence in three-year-old children of 26%, allowing a confidence interval of 5% and a confidence level of 95% it was estimated that around 300 children would have been required to provide adequate power for the present study. However, this was primarily a pilot study with the aim of defining the distribution of the parameters under investigation in this age group. Therefore, it was hypothesised that N = 100 children would be sufficient to identify trends and possible associations within the data in the present study and would also reflect a realistic target with respect to patient recruitment, sample analysis, time frame and budget of the project.

Sixty-three children with usable samples were recruited. Childsmile dental health nurses who operated Childsmile clinics in community settings in three areas of Glasgow recruited the majority of children to this study. The refusal rate was extremely low, with only one parent who did not provide consent for their child to participate. Therefore, all children of the correct age attending clinics staffed by the two Childsmile dental nurses between April and December 2009 were recruited to this study. In an attempt to increase recruitment, 11 dental practices participating in the Childsmile programme were invited to provide samples. Those accepting were provided with training in the sample collection procedures and a small payment for each recruited child. In spite of

apparent enthusiasm from the staff of the Dental Practices, only 16 children were recruited, of which only four were accompanied with usable samples. Thus, there appeared to be significant limitations to extending this study into the General Practice setting.

The first aim of this study was to provide a detailed description of the study participants in terms of the demographic, microbiological and immunological data obtained. From these data a number of observations were made and a detailed discussion of these follows here.

Childsmile now encompasses the national NHS dental improvement programme across the whole of Scotland. At the time of the study Childsmile interventions were targeted towards children living in the most deprived communities across the West of Scotland (Macpherson et al. 2010a). It was hypothesised that this study would therefore be biased towards recruitment of children from the most deprived areas, especially since it was in these communities that Childsmile dental nurses operated Childsmile clinics. It was expected that this bias would be reflected in the demographic data collected from parents by questionnaire. The data collected regarding total household income demonstrated that participants were recruited from across the income categories. However, 40% of families received a total household income of £10,000 or less, compared with the Scottish Government's figure of £22,000 for average household income in Glasgow (The Scottish Government 2010). Furthermore, over one third (37%) of families reported that all of their household income was received as benefits and data from both the national and local SIMD quintiles demonstrated that the largest proportion of families lived in areas ranked the most deprived SIMD quintile. This data, suggest that recruitment to this study was biased towards those from the most deprived areas.

Detection of Mutans streptococci in the youngest age groups is important to identify children who are at increased risk of developing caries. Caries prevalence increases with earlier acquisition of Mutans streptococci and the presence of Mutans streptococci in both plaque and saliva has been used as an indicator of caries risk (Fujiwara et al. 1991; Parisotto et al. 2010b). However, few studies have looked for the presence of Mutans streptococci in children as young as one-year-old. Studies of this design are of added importance as it has

been demonstrated that the presence of Mutans streptococci in the saliva of one-year-old children is high risk for caries development by age three-years (Grindefjord et al. 1995).

The isolation frequency of Mutans streptococci by diagnostic culture of plaque was 26% with a minimum detection level of 10^3 CFU. These results are in broad agreement with previous studies reporting 6.3% - 20% isolation rates of Mutans streptococci in children aged 12 – 18 months (Fujiwara et al. 1991) , rising to 29.7% in children aged 18 – 24 months (Fujiwara et al. 1991). The observed variation may be due to sampling and culture differences between studies.

DNA detection methods are increasingly used to detect Mutans streptococci due to their rapidity and sensitivity compared with conventional culture techniques. In this study *S. mutans* and *S. sobrinus* were detected in the saliva of 100% of children by TaqMan® QPCR. By this method, 35% of children had *S. mutans* counts greater than 10^5 CFU per ml saliva, which is reported to be associated with higher caries risk (Leal and Mickenautsch 2010). Furthermore, by PCR *S. sobrinus* was also detected in all individuals, although 60% were found to have below 10^3 CFU per ml saliva, while 12.3% harboured numbers greater than 10^5 CFU per ml saliva. *S. mutans* counts were on average 10-fold greater than those for *S. sobrinus*. This is in agreement with previous studies using both culture and PCR (Kishi et al. 2009; Yano et al. 2002). Additionally, Pearson bivariate analysis revealed a moderate to high level of correlation ($R = 0.556$, $p < 0.001$, data not shown) between salivary *S. mutans* and *S. sobrinus* CFU per ml saliva by TaqMan® QPCR, indicating that numbers of *S. sobrinus* increased with increasing numbers of *S. mutans*. This is of clinical significance as children who harbour both *S. mutans* and *S. sobrinus* show higher caries prevalence (Okada et al. 2012; Okada et al. 2002).

To standardise *S. mutans* and *S. sobrinus* across plaque samples the results were normalised to the copy number of the 16S rRNA gene, specifically the 16S rRNA gene of Gram-positive bacteria using the comparative threshold cycle method ($\Delta\Delta Ct$). Using this assay, the proportion of *S. mutans* in plaque was found to range from 0.0012 – 1.12% and *S. sobrinus* from 0.0008 – 3.7% of Gram-positive plaque flora. Surprisingly, in one participant, *S. sobrinus* was detected at a higher proportion than *S. mutans*.

It is important to note that the relative percentages of *S. mutans* and *S. sobrinus* as a proportion of Gram positive plaque flora may be underestimated because the copy number of the 16S rRNA gene is higher than that of *gtfB* or *gtfT* (the genes used to detect *S. mutans* and *S. sobrinus*, respectively). In this study it was not logistically possible to accurately weigh the plaque. The method described here for normalising data between plaque samples is an accepted technique when the weight of plaque cannot be determined (Yoshida et al. 2003a; Yoshida et al. 2003b).

This is the first study to report *S. mutans* and *S. sobrinus* as a proportion of Gram positive plaque flora, making comparisons with other studies difficult. One study conducted in children aged 71 months or younger reported detection of *S. mutans* and *S. sobrinus* at 0.04% and 0% of total bacteria, respectively in caries free children. This rose to 0.9% for *S. mutans* and 0.3% for *S. sobrinus* in children with early childhood caries, rising again to 8% for *S. mutans* and 1.6% for *S. sobrinus* in children with severe early childhood caries (Choi et al. 2009). Caries measurements were not recorded for this study and so direct comparisons cannot be made. However these results suggest *S. mutans* comprised on average 2.98% of total bacteria and *S. sobrinus* 0.8% of total bacteria. While, this represents higher proportions than the current study, the higher rate of detection in six-year-old children likely reflects increased numbers of Mutans streptococci carriage with increasing age (Fujiwara et al. 1991).

Extensive microbiological data were collected for this pilot study which allowed for comparison of the practicalities and usefulness of microbiological analysis of plaque versus saliva by both culture and TaqMan® QPCR. Unfortunately, the volume of saliva obtained was not sufficient to allow diagnostic culture, which was performed using plaque samples only.

This study confirmed TaqMan® QPCR to be a more sensitive and specific method for detection of Mutans streptococci than traditional culture techniques and even standard PCR techniques (Okada et al. 2002). Both *S. mutans* and *S. sobrinus* were detected in 100% of plaque and saliva samples, although there were vast differences in the numbers detected across samples. Furthermore, the ability to discriminate between *S. mutans* and *S. sobrinus* and to quantify their numbers independently is advantageous. It has been reported that *S. sobrinus*

may be more cariogenic than *S. mutans*. Studies using PCR to detect *S. sobrinus*, including the present study, suggest that *S. sobrinus* may be present in higher numbers than previously indicated by culture studies (Beighton 2005).

The use of saliva to estimate the level of Mutans streptococci, although easier to perform, has been reported to have a smaller predictor value for subsequent caries development than plaque (Sanchez-Perez and Acosta-Gio 2001). Mutans streptococci have been detected in the saliva of pre-dentate infants, although it is generally agreed that Mutans streptococci primarily colonise tooth surfaces and their numbers increase in both saliva and plaque as teeth continue to erupt (Parisotto et al. 2011). It has also been reported that the bacterial species present in plaque can be detected in saliva, although their numbers in un-stimulated saliva are not representative of those in plaque. Moreover, the bacterial species present at specific sites can vary extensively and the length of time since teeth were last brushed is an important consideration (Simon-Soro et al. 2012b). In the present study Pearson bivariate analysis revealed a statistically significant positive correlation between the numbers of *S. mutans* detected in saliva and plaque by TaqMan® QPCR ($p = < 0.0001$). Thus, both these data sets provided reliable information for this study. However, it is generally agreed that plaque is the most reliable and clinically relevant specimen for detection of Mutans streptococci.

Lactobacillus spp. are acidogenic and aciduric and therefore have also been attributed to caries risk (Kanasi et al. 2010). In the current study Lactobacillus were not detected in plaque of children by diagnostic culture. This was not perhaps surprising as Lactobacilli are reported to be associated with more complex biofilms than are found in children of this age range (Badet and Thebaud 2008). One study of 40 children aged three- to four-years reported very low levels of detection of lactobacillus at 0.1×10^{-6}, rising to 1.5×10^{-6} (percent of total plaque flora) one-year later (Parisotto et al. 2011). While, another study reported lactobacilli are generally not found in the mouths of children until at least two years of age and then only transiently (Carlsson et al. 1975). The presence of Lactobacillus spp. in saliva was not assessed due to insufficient volume.

Antimicrobial proteins are innate immune factors that have broad spectrum antimicrobial activity against a wide range of microorganisms, including *S. mutans* (Chung et al. 2007). Several families of AMPs are found in saliva, including the cathelicidins, human neutrophil (alpha) peptides and calprotectin, indicating their presence in the oral cavity is important for the maintenance of oral health (Dale and Fredericks 2005). Low levels of the HNPs 1-3 (Tao et al. 2005) and LL37 (Davidopoulou et al. 2012) have been attributed to increased caries risk in children.

This is the first study to report the concentrations of antimicrobial proteins in children aged one-year. Each antimicrobial protein was detected in the saliva of all children assessed, with the exception of LL37, which was not detected in the saliva of 12 children. The high level of variability reported in these studies is in agreement with a previous study which found highly variable levels of the HNPs 1-3, LL37 and the human-beta defensin 3 in a population of children aged between 11 and 15 years (Tao et al. 2005). However, the concentrations reported were higher than found in the present study. Another study also reported higher concentrations of the HNPs 1-3 and calprotectin in the saliva of three– to five-year-old children (Toomarian et al. 2011). Together these findings support a role for the continuing development and maturation of salivary glands and immune responses during early childhood.

Concentrations of salivary proteins are often normalised to total salivary protein due to differences in stimulation or salivary flow rates. However, it has been reported that this practice may give rise to misleading results due to differences which exist in the control of secretion of salivary proteins by individual salivary glands (Brandtzaeg 2007). The peptides measured here are expressed from multiple sources in the oral cavity, including the salivary glands, oral epithelium and neutrophils (Nisapakultorn et al. 2001a; Tao et al. 2005). For this reason whole, un-stimulated saliva was collected for this study and concentrations of salivary proteins were reported per ml of saliva. Furthermore, the levels of antimicrobial proteins previously reported remained highly variable among the study populations even when normalised to total salivary protein levels (Phattarataratip et al. 2011; Tao et al. 2005).

Of interest was the finding that the concentrations of antimicrobial proteins were correlated within individuals. Pearson bivariate analysis (data not shown) using $\log_{10}$ transformed data revealed moderate correlation between concentrations of lactoferrin and calprotectin ($r = 0.50$, $p < 0.001$) and the HNPs 1-3 ($r = 0.48$, $p < 0.001$). LL37 was moderately correlated with concentrations of calprotectin ($r = 0.34$, $p < 0.05$) and the HNPs 1-3 ($r = 0.33$, $p < 0.05$). Calprotectin was also moderately correlated with concentrations of HNPs 1-3 ($r = 0.32$, $p < 0.05$). This is in agreement with findings from a previous study that also reported statistically significant correlations between the levels of antimicrobial peptides in saliva (Phattarataratip et al. 2011). Interestingly, antimicrobial proteins are known to have synergistic effects in their role of innate immune defence (Nagaoka et al. 2000). Furthermore, in addition to their direct antimicrobial activity, antimicrobial peptides have other important roles in the oral cavity, including tissue repair and bridging innate with adaptive immune responses through their chemoattractant properties (Dale and Fredericks 2005). Thus, the antimicrobial activity of saliva of children with high concentrations of multiple antimicrobial proteins is likely to be far greater than children with low concentrations. It would have been of interest to measure the antimicrobial activity of saliva samples by incubation of saliva with *S. mutans* biofilms *in vitro*. However, the volume of available saliva negated this investigation which was not a primary aim of the study.

The effect of sIgA antibody responses on initial colonisation by oral bacteria in children remains poorly understood. In the current study sIgA antibodies specific for cariogenic species *S. mutans* and *S. sobrinus* and commensal species *S. mitis* and *S. sanguinis*, were detected in the oral cavity of children aged one-year, presumably indicating previous or current exposure to these or closely related species. Similarly variable levels of sIgA antibodies were documented in a previous study of sIgA in infants aged three to 20 weeks (Smith et al. 1989).

Pearson bivariate analyses revealed strong, statistically significant correlations for the levels of sIgA antibodies specific for different oral streptococci among individuals ($r = 0.56 – 0.69$, $p < 0.001$, data not shown). This may indicate the presence of cross-reactive antibodies of low specificity as has been previously reported in children of this age (Cole et al. 1999).

The investigations of life stresses were based on the hypothesis that low SES associates with stress. Salivary cortisol concentrations were estimated to provide a surrogate biological measure of stress. Salivary sampling is a well established technique for cortisol measurement in children and is relatively stress free (Woolston et al. 1983). Cortisol expression follows a circadian rhythm; however levels rise independently of the circadian rhythm in response to stress (Miller et al. 2007). In the current study cortisol was detected in the saliva of all children and the concentrations are in broad agreement with those reported by other studies for children of this age (Watamura et al. 2004). There were no statistically significant differences in salivary cortisol concentrations with respect to gender (data not shown). This is in agreement with a study which investigated the circadian rhythm of cortisol in young healthy children of the same age (Groschl et al. 2003).

The second aim of this study was to investigate the relationship of socioeconomic status with bacterial counts, the salivary immune response and stress. Dental caries is strongly associated with low SES (Sisson 2007) and measures of SES are routinely used to identify caries risk (Reisine and Psoter 2001). In the present study it was hypothesised that children of lower SES may have measurable differences in bacterial numbers of plaque and saliva compared with children from affluent backgrounds. The relationships between bacterial numbers and SES were limited with few statistically significant differences identified between the measures assessed. One obvious reason is likely due to low numbers of study participants, which resulted in low power to detect statistically significant differences. Furthermore, because numbers of study participants were low the measures used to estimate SES were dichotomised. As a result the SES measures were broad and this likely resulted in a diluting effect.

Using the data identified in the present study it was possible to perform a power calculation to identify the sample size that would have been required to identify statistically significant differences in the carriage of *S. mutans* and the concentrations of antimicrobial proteins according to the socioeconomic position of children, using the dichotomised SIMD quintiles. In order to detect a difference of 0.25 SD in *S. mutans* or antimicrobial proteins with 80% power at the 5% significance level would require 253 individuals in each SIMD category. A

difference of 0.5 SD would require 64 individuals in each group, 0.75 would require 29 in each group and 1 SD would require 17 individuals in each group.

Occupational information was also collected from parents by demographic questionnaire and could have been used to assign a socioeconomic position to each family using the National Statistics standard occupational classification (www.ons.gov.uk 2010). However, this classification system consists of nine major groups and would have given rise to broad measures with low numbers of participants in each group. The decision was taken not to include this analysis in the current study.

In contrast to the original hypothesis there was a trend for higher numbers of *S. mutans* in the saliva and plaque of children from more affluent backgrounds compared to those from relatively more deprived backgrounds. The difference was statistically significant for salivary *S. mutans* according to the proportion of benefits received. However, *post hoc* tests were not performed during this exploratory pilot study and it is likely this relationship would not remain statistically significant following these corrections. Thus, the low power of this study was insufficient to provide reliable evidence to indicate a relationship between SES and the carriage of *S. mutans*.

Given that dental caries is so routinely and strongly associated with low SES, even in young children it was surprising that no evidence for an association with caries risk factors was identified here. One reason may be due to the fact that these children are enrolled within an oral health improvement programme and samples were collected during the child's Childsmile appointment. As a result oral health behaviours, such as regular tooth-brushing may have been modified. Indeed, the aim of Childsmile is to improve oral health related behaviours and there is evidence to suggest that Childsmile is associated with an improvement in the oral health of children. In a recently published paper dental decay experience of three-year-olds in Glasgow was reduced from 26% in 2006/7 to 17% in 2009/10. Moreover, this improvement in dental health was evident across the SES spectrum (McMahon et al. 2011). If parents had ensured children brushed their teeth prior to their Childsmile appointment to demonstrate compliance with the programme this would inevitably limit the microbiological findings

reported here. It would have been unethical to discourage tooth-brushing under any circumstances in this study group.

Dental caries is an infectious disease and as such the host response may play an important role in defining caries susceptibility. It was hypothesised that immune responses may be altered in children from lower SES backgrounds and that this may ultimately influence caries susceptibility. Associations of salivary antimicrobial proteins with measures of SES were limited. No statistically significant relationships were identified with regard to the concentrations of antimicrobial peptides in the saliva of children grouped according to measures of SES.

Children of parents who received a relatively higher level of education were found to have statistically higher levels of *S. mutans* specific sIgA than children of parents who received a lower level of education. This finding may be a response to the higher numbers of salivary *S. mutans* that were found in these same children. Conversely, sIgA titres for oral streptococci tended to be higher in children from the relatively more deprived category for the remaining SES measures, which may reflect higher or more frequent exposure to these bacterial species.

In the present study salivary cortisol concentrations were assessed to investigate differences in children according to their relative SES. Although not apparent in this study, associations between low SES and increased salivary cortisol levels in children and adults have been demonstrated in other studies (Boyce et al. 2010; Chen et al. 2010; Cohen et al. 2006). Additionally, positive associations have been reported between the concentrations of salivary cortisol and the increased prevalence of dental caries in children aged five- to six-years (Boyce et al. 2010) and the severity of periodontal disease in adults (Genco et al. 1998; Hilgert et al. 2006; Rosania et al. 2009).

In very young children circadian regulation of the HPA-axis is still undergoing maturation (Watamura et al. 2004). It may be that the children assessed here were too young to respond to psychological stressors in the same way as older children or adults. Additionally, results reported here are likely limited by potential biases associated with collection of salivary cortisol at a single time

point. Accurate quantification of cortisol would usually involve numerous measurements taken throughout the day to determine the circadian rhythm and to establish a basal cortisol measurement. Ideally, this would be repeated over consecutive days. Basal cortisol levels would then be used to identify differences among the study population. Moreover, the time of sample collection is important. The circadian rhythm of cortisol secretion dictates that concentrations differ significantly in children according to the time of day samples are collected (Groschl et al. 2003). The majority of samples were collected from children in the morning. However, it was not possible in this study to collect saliva on numerous occasions over consecutive days or to accurately define the time of sample collection. However, future studies should ideally incorporate multiple saliva collections at specified intervals into the study design to allow more accurate quantification of salivary cortisol concentrations.

The third aim of this study was to investigate if salivary cortisol concentrations influenced the numbers of cariogenic bacteria detected in plaque or saliva or the concentrations of salivary antimicrobial proteins. Numerous pathogenic bacteria possess the ability to recognise and respond to host hormones (Lyte 1993). It was hypothesised that differences in salivary cortisol concentrations may influence the composition of the oral biofilm. There were trends for increased mean numbers of *S. mutans* cultured from plaque or detected in plaque and saliva with increasing cortisol tertiles. These trends were not statistically significant, but are interesting. A previous study reported differing growth responses by oral bacteria, including *S. mutans* in the presence of adrenaline and noradrenalin *in vitro* (Roberts et al. 2002). This, study provided evidence to suggest that stress hormones may have the potential to directly modulate the composition of the oral biofilm. Further investigations are required to determine if higher concentrations of salivary cortisol can influence the carriage of *S. mutans* in dental plaque.

Cortisol is an anti-inflammatory molecule involved in the regulation of innate and adaptive immune responses to bacterial and viral infections (Raison et al. 2006; Webster Marketon and Glaser 2008). Glucocorticoid regulation of antimicrobial peptides in response to psychological stress has been documented in a murine model. Increased severity of *Streptococcus pyogenes* infection was

accompanied by increased expression of glucocorticoids and down regulation of antimicrobial peptide expression (Aberg et al. 2007). In the present study no associations of salivary cortisol with concentrations of innate salivary antimicrobial proteins were identified. Future investigations using basal cortisol concentrations will be required to elucidate this relationship.

Evidence has shown that chronic stress can result in lower levels of detection of antigen-specific sIgA antibodies following vaccination (Cohen et al. 2001). Synthesis and secretion of sIgA is regulated by antigenic stimulation and is under neuroendocrine control (Cohen et al. 2001; Teeuw et al. 2004). It therefore follows that biochemical changes in neuroendocrine regulation can alter sIgA levels within the oral cavity. Intriguingly, the gene encoding the secretory component of sIgA, a protein responsible for its translocation across the epithelium, contains a putative androgen/glucocorticoid response element (Verrijdt et al. 1997). This suggests that cortisol may play a role in the regulation of expression of secretory component and therefore indirectly modulate levels of sIgA in the oral cavity by regulating its translocation across the epithelium. In the present study, there was a statistically significant trend for increased mean titres of *S. sanguinis* specific sIgA antibodies with each increased cortisol tertile ($p = 0.018$). This trend was also evident for sIgA antibody titres specific for *S. mutans*, *S. sobrinus* and *S. mitis* although they did not reach statistical significance. Whether these differences reflect regulation by cortisol remains unclear.

The fourth aim of this study was to investigate the relationship between the cariogenic oral biofilm and the concentrations of salivary antimicrobial proteins. Data reported here suggest that concentrations of innate antimicrobial proteins increased non-specifically with increased numbers of oral bacteria. This is consistent with their role for innate immune defence and inducible expression in response to local inflammatory stimuli.

Several studies have indicated that the presence of *S. mutans* specific antibody can modify the course of carious disease. In one study the presence of *S. mutans* specific sIgA in the saliva of 12– to 18-month-old children were negatively correlated with *S. mutans* infection (Nogueira et al. 2007). While other studies have reported a positive correlation (Smith and Taubman 1995) or no correlation

(Camling and Kohler 1987). Data reported here found no association between numbers of *S. mutans* in plaque or saliva with specific sIgA antibody titres in young children. This was consistent with findings reported from a previous study (Koga-Ito et al. 2004) which additionally reported a correlation between increased *S. mutans* specific sIgA and caries-free status in young adults but not among children.

Experimental studies conducted in animal models have demonstrated that infection with *S. mutans* can give rise to salivary antibodies directed towards mutans specific virulence proteins (Nogueira et al. 2008; Smith et al. 1998). Furthermore, clinical studies conducted in young children have shown that the ability to mount a broad immune response to specific Mutans streptococcal antigens, such as glucan binding protein B are important for caries resistance (Parisotto et al. 2011).

Difficulties arise in the interpretation of the sIgA antibody responses to oral bacteria, particularly with regard to cross-sectional studies since the stage of development of the salivary immune response to antigenic challenge at the time of sampling is not known. Moreover, the levels of sIgA antibodies within individuals vary over time (Gahnberg and Krasse 1981) and it cannot be determined whether the presence of specific antibody reflects present or past exposure to bacterial antigens. The saliva of children recently colonised by *S. mutans* was reported to contain higher levels of specific sIgA antibody than saliva from children who had been colonised with *S. mutans* for greater than 24 months (Camling and Kohler 1987), suggesting that the salivary antibody response is influenced by age and the length of exposure to bacterial antigens. Longitudinal studies are better placed to investigate the development of the salivary antibody response coincident with development of the oral biofilm and clinical disease scores.

In this study antibody specificities to whole bacterial antigens were assessed. It is likely that the use of intact whole bacteria to investigate the contribution of sIgA to Mutans streptococcal colonisation is a less reliable method than the use of purified antigens. However, further investigations of sIgA antibody specificities to purified antigens were not possible due to an insufficient volume of saliva.

The high correlation of antibody titres within individuals suggests the presence of cross-reactive antibodies of low specificity. It would perhaps have been of interest to also investigate the avidity of sIgA antibodies to oral streptococci in order to identify antibodies that strongly bind to their antigen and as a result may provide a higher degree of protection against cariogenic bacteria. Once again the volume of saliva was a limiting factor for additional investigations. Moreover, it has been reported that the amount of *S. mutans* specific sIgA antibody and not the presence of high avidity antibodies is important for caries resistance (Lehtonen et al. 1984).

Serum IgG enters the oral cavity through gingival crevicular fluid (Challacombe et al. 1978) and there is evidence to show that high titres of serum IgG antibodies specific for Mutans streptococci are associated with protection against caries in children aged 2.6 – 4.9 yrs (Aaltonen et al. 1987). However, while the synthesis of sIgA antibodies in young children is equivalent to the concentrations produced in adults by one-year of age, serum IgG concentrations remain low in children up to three-years of age (Luo et al. 1988). For this reason, serum IgG antibodies specific for cariogenic bacteria were not investigated in the present study.

3.4 Conclusion

This work demonstrated that variables hypothesised to influence the development of carious disease can be collected and successfully quantified in one-year-old children. The collection of saliva is an easy, relatively stress free and reliable collection technique for use in young children and is a highly valuable specimen from which numerous investigations can be performed. It would have been advantageous to collect a larger volume of saliva from the children to allow for additional investigations. However, this would be difficult in children of this age and would likely involve multiple collections which in itself would lead to difficulties. Careful consideration should be given to decisions regarding the salivary assays to perform in order to achieve the highest quality analysis with the resources available.

The major limitation for this pilot clinical study was the small sample size. Childsmile can provide an important platform from which to recruit young children to clinical studies. However, the difficulties associated with the recruitment of children of this age should not be underestimated. Clearly, the current incentive for dental practice participation was not sufficient to engage their full support. However, in future studies dental practice participation will likely be required if sufficient numbers of children are to be recruited. This would require the current incentives to be addressed and would likely involve a more rigorous training programme regarding the sample collection procedures.

The data did not provide evidence of an association of low SES with the detection of cariogenic bacteria or salivary immune responses. Moreover, the use of salivary cortisol did not represent a reliable surrogate measure of stress in children of this age. However, basal cortisol levels may provide a more reliable biological measure of stress. The suggestion that increased numbers of cariogenic bacteria may be associated with increased concentrations of salivary cortisol warrants further investigation. The concentrations of innate antimicrobial proteins were associated with increased numbers of oral bacteria, although it remains unclear whether the levels of innate antimicrobial proteins are associated with numbers of cariogenic bacteria. It remains unclear the extent to which specific sIgA antibodies provide protection against colonisation with Mutans streptococci. Longitudinal analyses are required to identify how oral biofilm development coincides with the development of the salivary immune response.

Chapter 4: Longitudinal analysis of caries-associated risk factors in three-year-old children

4.1 Introduction

The previous study documented cross-sectional analysis of caries-associated biological risk factors. The present longitudinal study sought to address a fundamental gap in current knowledge of how the immune system deals with an evolving oral biofilm in young children and to identify factors that may naturally protect against dental caries.

There is a scarcity of studies, particularly longitudinal studies, conducted in very young children to investigate the biological risk factors for caries. Young children are difficult to recruit as they do not regularly attend for dental examinations, with only around 30% of Scottish children aged zero- to two-years registered with a dentist, rising to 70% in three- to five-year-olds (Shaw et al. 2009). Thus, young children are not readily accessible for examination. On a practical basis the collection of samples and dental examinations can prove difficult. In order to better understand the caries process and to identify factors which may naturally protect against caries it is crucial that studies are targeted towards the youngest age groups at the time when *S. mutans* colonisation coincides with the development of a naive immune system. Lifestyle behaviours are established at a young age (Shaw et al. 2009) and adult oral health can be predicted by childhood oral health (Thomson et al. 2004). These phenomena further highlight the importance of strategies targeted towards the youngest age groups if significant improvements in dental health are to be made.

Previous studies have demonstrated that colonisation by the indigenous oral flora gives rise to secretory immunity (Cole et al. 1999; Nogueira et al. 2008). However, these responses are heterogeneous and their contribution to caries resistance remains unclear. Some studies have reported that the presence of anti-*S. mutans* antibodies are associated with increased incidence of dental caries (Koga-Ito et al. 2004), while others have suggested that they are protective (Kirtaniya et al. 2009). Furthermore, salivary antimicrobial proteins, such as antimicrobial peptides are known to be important for oral health and there is evidence to suggest they may influence the course of carious disease (Dale and Fredericks 2005). However, little is known about the concentrations of

antimicrobial proteins or their relationship with the oral biofilm in young children.

This study therefore sought to collect, process and analyse biological samples, socioeconomic data, dietary information and dental health status in a longitudinal clinical study of Childsmile participants. The overarching aim of this study was to investigate the acquisition of cariogenic bacteria coincident with the development of salivary immune responses in young children.

The research objectives for this study were as follows:

1. To determine whether the children who returned for follow-up were representative of the study group at baseline.

2. To provide a descriptive analysis of the total plaque bacteria, carriage of *S. mutans* and *S. sobrinus* in plaque and saliva, the concentrations of salivary lactoferrin, LL37, calprotectin, the HNPs 1-3 and sIgA antibodies specific for a panel of oral streptococci, dietary and dental disease scores of three-year-old children at follow-up.

3. To investigate effect of non-milk extrinsic sugar consumption on the detection of cariogenic bacteria in plaque and saliva of three-year-old children.

4. To investigate how the cariogenic oral biofilm and concentrations of salivary antimicrobial proteins change over time.

5. To investigate the evolution of the cariogenic oral biofilm coincident with changes in the concentrations of salivary antimicrobial proteins.

6. To investigate if salivary antimicrobial proteins and/or the presence of cariogenic bacteria relate to clinical disease scores.

7. To investigate if socioeconomic inequalities and salivary cortisol concentrations influence changes in the concentrations of salivary antimicrobial proteins and the carriage of cariogenic bacteria.

4.2 Results

Twenty-three of the 63 children (37%) who provided samples at baseline participated at follow-up between January and April 2011 for the present study. Recall appointments were arranged for 63 children who participated at baseline. Contact details proved incorrect or out of date for 17 participants. Appointments were made for children to attend local health centres and in excess of 60 appointments were made in three different health centres over four different days. Of the appointments offered only 17 participants attended. In an attempt to collect samples from children who missed health centre appointments, domiciliary appointments were made for seven children, of whom six were available on the day of the visit. The study's ethical approval was amended from the original application to include a food frequency questionnaire. The amendment was approved from the West of Scotland Research Ethics Committee and NHS Greater Glasgow and Clyde R&D Management (08/S0703/139). Parental consent was obtained for each study participant.

4.2.1 Comparison of baseline data for children who were followed-up versus those who were lost to follow-up

Prior to longitudinal analysis, data obtained from the children who returned for follow-up was assessed to investigate if these children were representative of the original study group. Baseline demographic and biological data from the children who returned for follow-up were compared with data from the children who were lost to follow-up. There were no differences with respect to age, level of parental education or area of relative deprivation for the 23 children who participated at follow-up compared with the 40 children who were lost to follow-up (

Table 4-1). A higher proportion of boys (n = 14) than girls (n = 8) were followed up, although this was not considered significant as there were no differences in the biological measurements between girls and boys at baseline (data not shown). Additionally, there were no statistically significant differences between baseline counts of bacteria in plaque or saliva (Figure 4-1 and Figure 4-2), baseline concentrations of salivary antibodies and antimicrobial proteins (Figure

4-3 and Figure 4-4) or salivary cortisol (Figure 4-5). However, a proportion of children with the highest baseline concentrations of lactoferrin were lost to follow-up. This difference was of borderline statistical significance ($p = 0.061$, Figure 4-3). Taken together these data suggest that baseline data from children who were followed up were representative of baseline data from original study group.

Table 4-1: Demographic descriptive analysis of children who returned for follow-up sample collection versus children who were lost to follow-up

Demographics	Study participants followed-up	Study participants lost to follow-up
Participants		
N	**23**	**40**
Age (months) of child		
median, min, max,	**35,** 26, 45	**35,** 31, 41
Q1, Q3	*32, 38*	*31, 38*
missing **N**	**0**	**0**
Gender of child		
female **N** (%)	**9** (39.1)	**20** (50)
male **N** (%)	**14** (60.9)	**20** (50)
missing **N**	**0**	**0**
Level of parental education		
secondary school **N** (%)	**8** (36.4)	**13** (34.2)
sixth form or college **N** (%)	**14** (63.6)	**25** (65.8)
missing **N**	**1**	**2**
Local authority SIMD quintiles		
most deprived quintile **N** (%)	**10** (47.6)	**10** (37)
quintiles 2-5 **N** (%)	**11** (52.4)	**17** (63)
missing **N**	**2**	**13**

Age (months) is the age of children at the time of each child's allocated follow-up appointment.

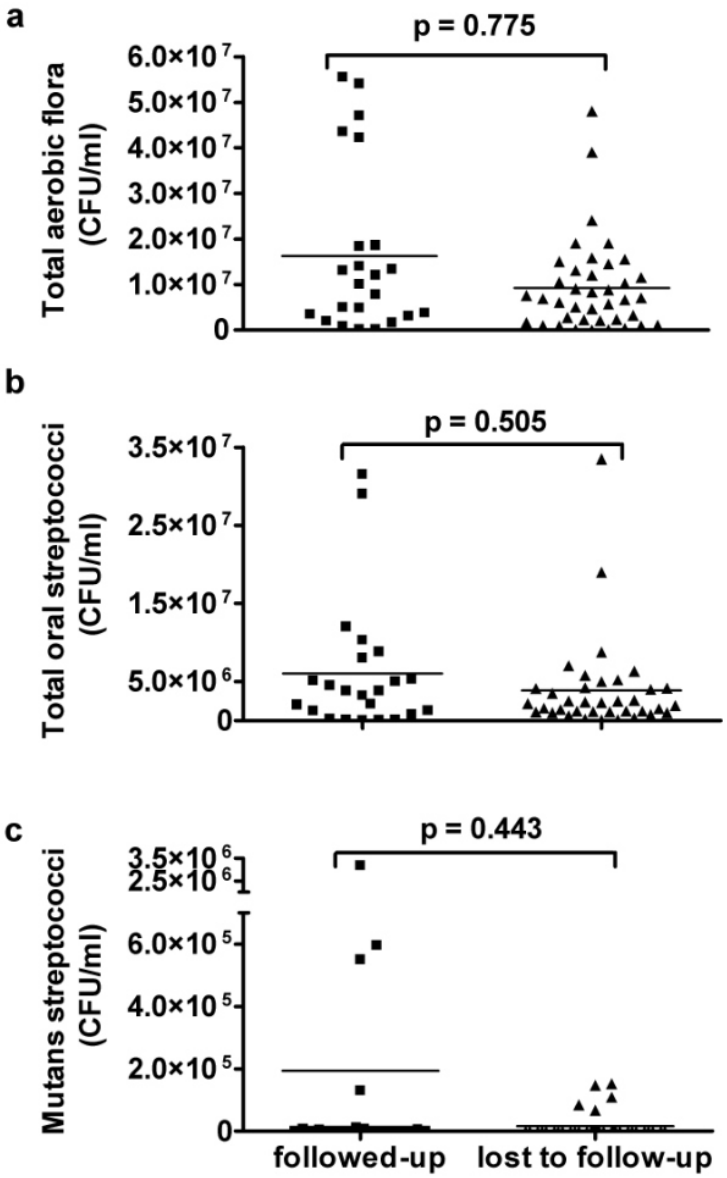


Figure 4-1: Baseline plaque bacterial counts according to follow-up status of children

Scatter plots of baseline bacterial counts (CFU/ml) of a) total aerobic flora, b) total oral streptococci and c) Mutans streptococci, estimated by diagnostic culture of plaque and grouped according to follow-up status of children. Raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group mean. Differences were not statistically significant by a) and b) independent samples t-tests of $\log_{10}$ transformed data or c) Mann-Whitney U test of raw data.

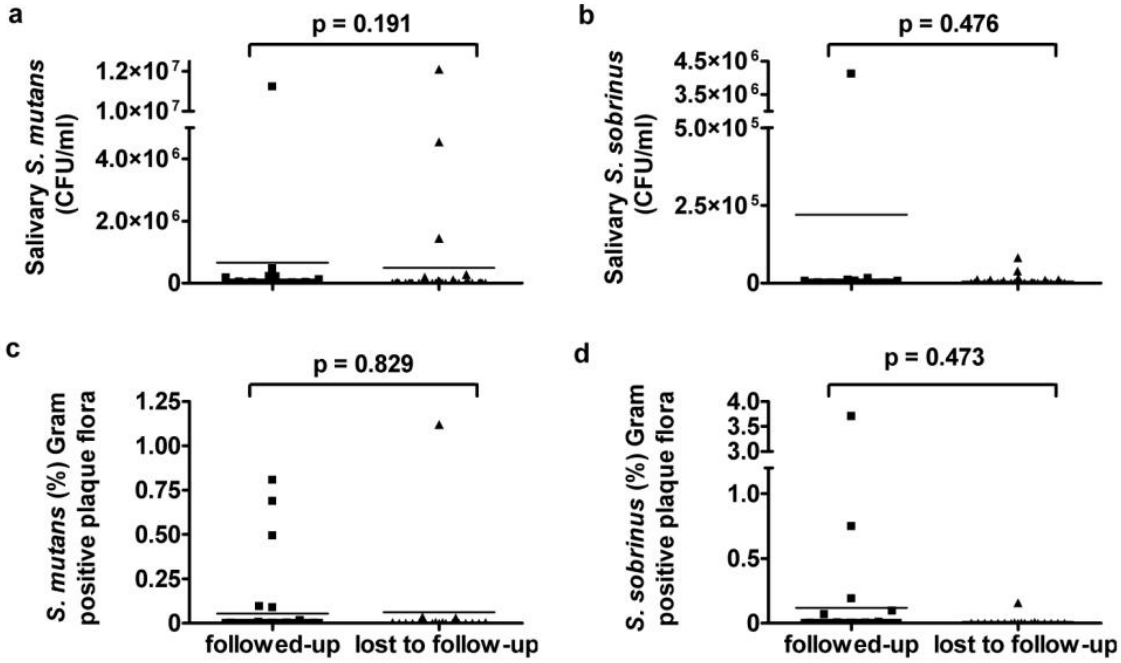


Figure 4-2: Baseline Mutans streptococcal counts from plaque and saliva according to follow-up status of children

Scatter plots of baseline bacterial counts of a) salivary *S. mutans* (CFU/ml), b) salivary *S. sobrinus* (CFU/ml), and c) *S. mutans* (%) and d) S. sobrinus (%) of Gram positive plaque flora estimated by TaqMan® QPCR and grouped according to follow-up status of children. Raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and the horizontal line indicates the group mean. Differences were not statistically significant by independent samples t-tests of $\log_{10}$ transformed data.

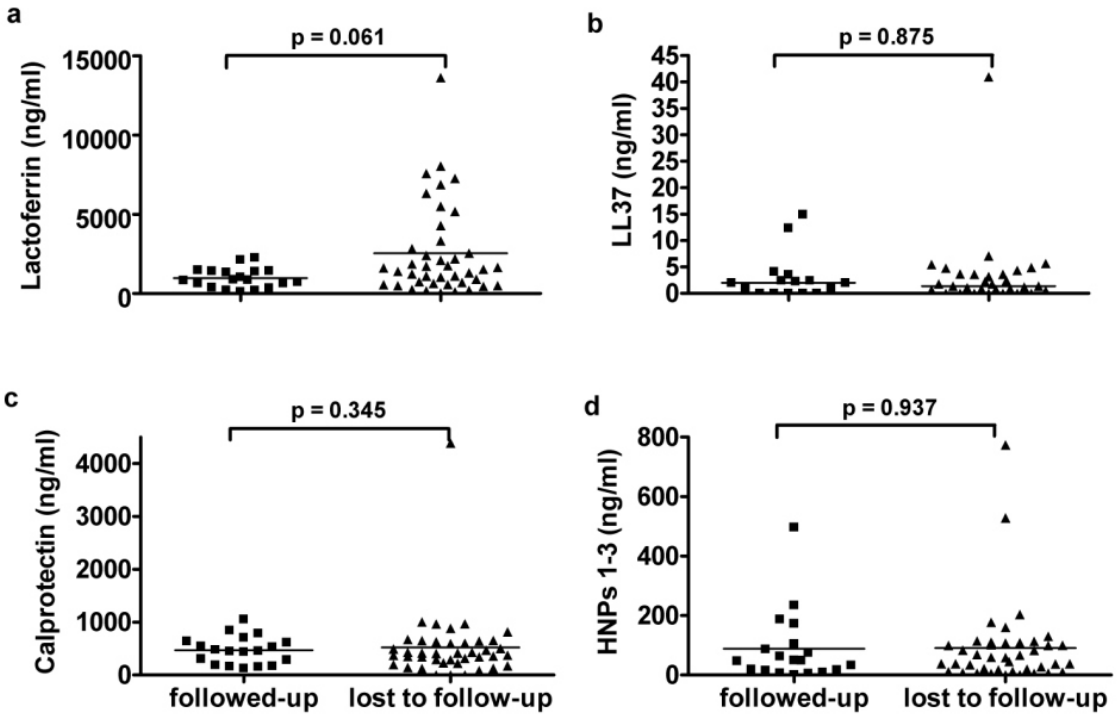


Figure 4-3: Baseline concentrations of salivary antimicrobial proteins according to follow-up status of children

Scatter plots of baseline concentrations (ng/ml) of a) lactoferrin, b) LL37, c) calprotectin and d) the HNPs 1-3 grouped according to follow-up status of children. Raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and the horizontal line indicates the group mean for parametric data (a, c & d) or the group median for non-parametric data (b). Differences were not statistically significant for (a, c & d) by independent samples t-tests of $\log_{10}$ transformed data or for (b) by Mann-Whitney U test of raw data.

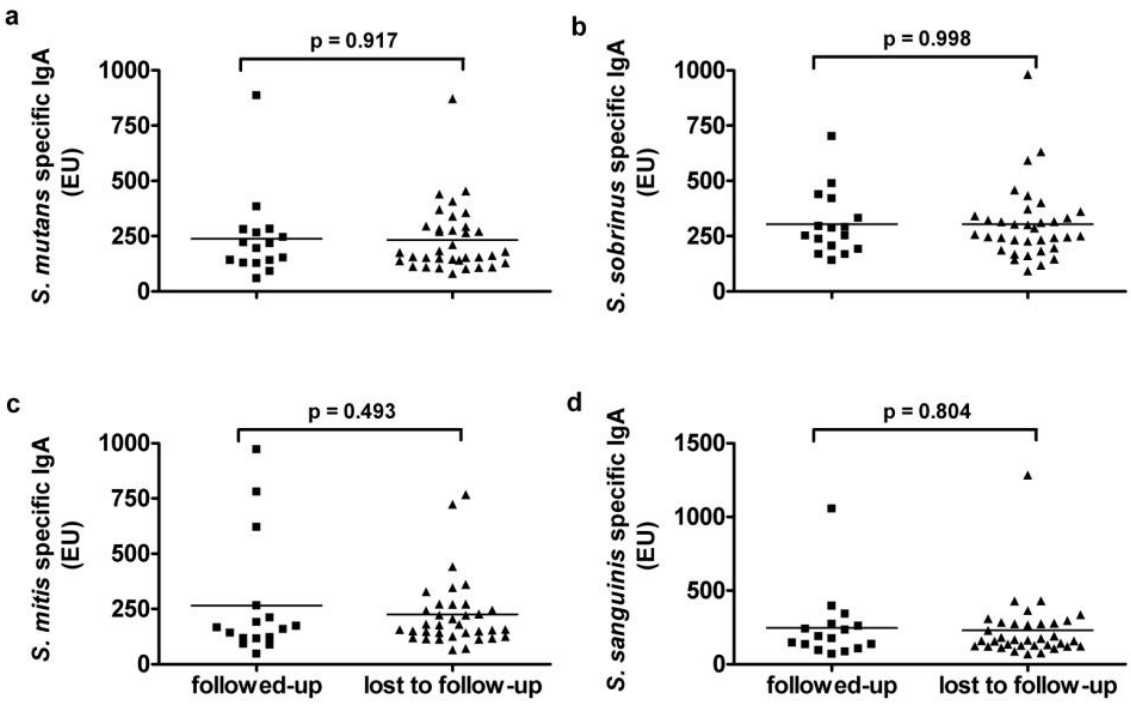


Figure 4-4: Baseline sIgA antibody titres specific for oral streptococci according to follow-up status of children

Scatter plots of baseline sIgA antibody titres (EU) specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis* grouped according to follow-up status of children. Raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and horizontal lines indicate the group mean. Differences were not statistically significant by independent samples t-tests of $\log_{10}$ transformed data.

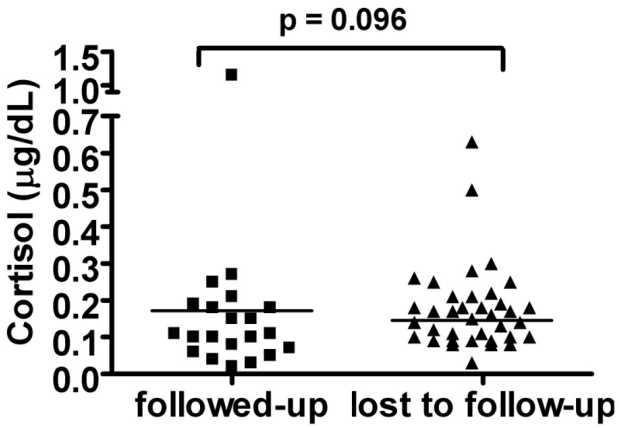


Figure 4-5: Baseline salivary cortisol concentrations according to follow-up status of children

Scatter plots of baseline salivary cortisol concentrations (µg/dL) grouped according to follow-up status of children. Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group means. Raw data are plotted. The difference was not statistically significant by independent samples t-test using $\log_{10}$ transformed data.

4.2.2 Descriptive analysis of biological data collected from plaque and saliva of three-year-old children

4.2.2.1 Bacterial counts from plaque and saliva of three-year-olds

Assessment of plaque by microbial culture revealed the study group had a geometric mean aerobic bacterial count of 1.2 x 10^7 CFU/ml, of which on average 4.0 x 10^6 CFU/ml were oral streptococcal spp, representing around 33.3% of the aerobic plaque flora. Mutans streptococci were below the limit of detection by culture in the plaque of over 52% (n = 12/23) of children. In children whose plaque was culture positive (n = 11/23) for Mutans streptococci, numbers ranged from 3.8 x 10^3 to 1.0 x 10^6 CFU/ml with a geometric mean count of 4.0 x 10^4 CFU/ml and comprised on average 1% of the total oral streptococcal flora (Table 4-2a). Lactobacillus spp were cultured from the plaque of only three children with a mean count of 5.6 x 10^3 CFU/ml.

By TaqMan® QPCR, *S. mutans* and *S. sobrinus* were assessed as a proportion of Gram positive bacteria in the plaque of 22 of 23 children with usable samples (no DNA was obtained from the plaque of one child). The relative percentages ranged from 0.0001 to 3.8% with a geometric mean of 0.006% for *S. mutans* and from 0.0002 to 0.9% with a geometric mean of 0.0005% for *S. sobrinus* (Figure 4-1a). In saliva, *S. mutans* DNA was detected in all 23 children, while *S. sobrinus* DNA was detected in the saliva of 21 of 23 children. The absolute number of *S. mutans* in saliva of children ranged from 34 to 3.0 x 10^6 CFU/ml with a geometric mean of 8.3 x 10^3 CFU/ml. Numbers of *S. sobrinus* ranged from not detected to 1.4 x 10^4 CFU/ml with a geometric mean of 112 CFU/ml (Table 4-2b).

Table 4-2a: Descriptive analysis of microbiological data collected from plaque of three-year-old children

Microbiological measurements	Statistics
Diagnostic culture of plaque (CFU/ml)	
Total aerobic flora **N** (missing)	**23** (0)
not detected **N** (%)	**0** (0)
detected **N** (%)	**23** (100)
mean, min, max	**1.2×10^7**, 2.3×10^6, 5.3×10^7
95% CIs	8.5×10^6, 1.7×10^7
Total oral streptococci **N** (missing)	**23** (0)
not detected **N** (%)	**0** (0)
detected **N** (%)	**23** (100)
mean, min, max	**4.0×10^6**, 6.2×10^5, 5.3×10^7
95% CIs	2.5×10^6, 6.2×10^6
Mutans streptococci **N** (missing)	**23** (0)
not detected **N** (%)	**12** (52.2)
detected **N** (%)	**11** (47.8)
mean, min, max	**4.0×10^4**, 3.8×10^3, 1.0×10^6
95% CIs	7.9×10^3, 2.1×10^5
Relative quantitation from plaque by QPCR (% of Gram positive flora)	
S. mutans **N** (missing)	**22** (1)
not detected **N** (%)	**0** (0)
detected **N** (%)	**22** (100)
mean, min, max	**0.0058**, 0.0001, 3.77
95% CIs	0.0012, 0.028
S. sobrinus **N** (missing)	**22** (1)
not detected **N** (%)	**0** (0)
detected **N** (%)	**22** (100)
mean, min max	**0.0005**, 0.0002, 0.87
95% CIs	0.0002, 0.0012

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

Table 1-2b: Descriptive analysis of microbiological data collected from saliva of three-year-old children

Microbiological measurements	Statistics
Absolute quantitation from saliva by QPCR (CFU/ml)	
S. mutans **N** (missing)	**23** (0)
not detected **N** (%)	**0** (0)
detected **N** (%)	**23** (100)
mean, min, max	**8.3×10^3,** 34, 3.0×10^6
95% CIs	2.5×10^3, 2.8×10^4
S. sobrinus **N** (missing)	**23** (0)
not detected N (%)	**2** (9)
detected N (%)	**21** (81)
mean, min, max	**112**, ND, 1.4×10^4
95% CIs	30, 418

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown. ND: not detected.

4.2.2.2 Mutans streptococci in plaque and saliva of three-year-old children according to the location of recall visit

As an incidental finding, marked differences were observed in the microbiology of children attending health centre appointments compared with those seen by domiciliary visit. Analysis of the numbers of Mutans streptococci in plaque and saliva of children grouped according to the location of their recall visit revealed stark differences. Mean numbers of Mutans streptococci cultured from plaque and *S. mutans* as a proportion of Gram positive plaque flora were over six-fold higher in plaque samples collected from children at domiciliary visits compared with those collected at health centre appointments. Additionally, salivary *S. mutans* (CFU/ml) was two-fold higher in children from the domiciliary visit group compared with those recruited through health centre appointments (Table 4-3). These differences were not statistically significant (Figure 4-6) but suggest that the domiciliary visits are an important recruitment method for children who failed to attend health centre appointments.

Table 4-3: *S. mutans* counts from plaque and saliva of three-year-olds according to location of recall visit

Bacterial count	N	Mean	95% CIs of the mean		Median	Quartiles	
			lower	upper		Q1	Q3
Diagnostic culture of plaque							
S. mutans (CFU/ml)							
HC	17	4.5×10^4	-1.6×10^4	1.1×10^5	ND	ND	4.5×10^4
domiciliary	6	2.9×10^5	-1.8×10^5	7.6×10^5	1.2×10^4	503	7.9×10^5
Relative quantitation from plaque							
S. mutans (%) Gram positive flora							
HC	16	0.0034	0.0005	0.0222	0.0010	0.0002	0.0943
domiciliary	6	0.0234	0.0005	1.0406	0.0128	0.0007	1.3586
Absolute quantitation from saliva							
S. mutans (CFU/ml)							
HC	17	6.9×10^3	1.6×10^3	2.9×10^4	6.4×10^3	1.3×10^3	1.7×10^4
domiciliary	6	1.4×10^4	619	3.3×10^5	1.3×10^4	893	3.2×10^5

S. mutans counts from plaque and saliva of three-year-olds according to the location of recall visit (health centre or domiciliary). *S. mutans* (CFU/ml) by diagnostic culture of plaque are raw data. *S. mutans* (% of Gram positive plaque flora) and (CFU/ml) in saliva (relative and absolute quantitation, respectively) are geometric data generated from back transformations of $\log_{10}$ transformed data. In some instances the large 95% confidence intervals of the mean have resulted in negative values.

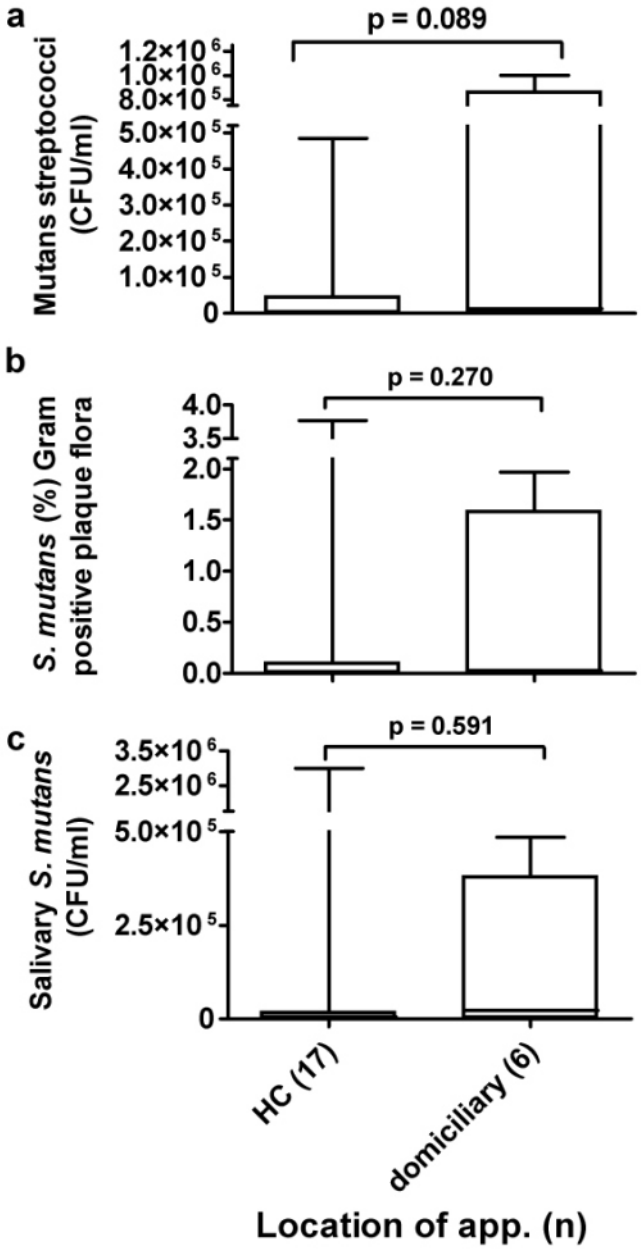


Figure 4-6: *S. mutans* in plaque and saliva of three-year-olds according to the location of recall visit

Box plots of a) Mutans streptococci (CFU/ml), estimated by diagnostic culture of plaque, b) *S. mutans* (%) of Gram positive plaque flora and c) salivary *S. mutans* (CFU/ml), estimated by TaqMan® QPCR and grouped according to the location of recall visit (health centre or domiciliary). Differences were not statistically significant by a) Mann-Whitney U test of raw data, or b) and c) independent samples T-test of $\log_{10}$ transformed data.

4.2.2.3 Salivary protein concentrations in three-year-old children

Lactoferrin was detected in the saliva of all children (n = 23). There was a high degree of variability in the concentrations detected between children, which ranged from 200 to 5129 ng/ml, with a geometric mean concentration of 856.1 ng/ml (Table 3-3).

LL37 was below the limit of detection in 61% (n = 14) of children. In children in whom LL37 was detected the geometric mean was 6.3 ng/ml, ranging from 0.43 to 95.5 ng/ml (Table 3-3).

Calprotectin and the HNPs 1-3 were detected in the saliva of all children (n = 23). Calprotectin concentrations were less variable between children, ranging from 224 to 1122 ng/ml with a geometric mean of 612 ng/ml. The concentrations of HNPs 1-3 were highly variable between children, ranging from 11 to 1513 ng/ml with a geometric mean of 299 ng/ml (Table 3-3).

Titres of sIgA antibodies specific for a panel of oral streptococci were assessed. Salivary IgA antibodies specific for *S. mutans*, *S. sobrinus*, *S. mitis* and *S. sanguinis* were detected in the saliva of all children (n = 23). The titres were variable among the study group, although the geometric mean titres for *S. mutans*, *S. sobrinus* and *S. sanguinis* specific sIgA were similar, while titres of *S. mitis* sIgA antibodies were less variable with a higher geometric mean titre than was observed for *S. mutans*, *S. sobrinus* and *S. sanguinis* specific sIgA (Table 3-3).

Salivary cortisol was detected in all children (n = 23). Concentrations ranged from 0.08 to 3.98 µg/dL, with a geometric mean concentration of 0.22 µg/dL (Table 3-3).

Table 4-4: Descriptive analysis of salivary protein concentrations in three-year-old children

Salivary proteins	Statistics
Lactoferrin (ng/ml) **N** (missing)	**23** (0)
mean, min, max	**856.1**, 199.5, 5128.6
95% CIs	579.7, 1264.2
LL37 (ng/ml) **N** (missing)	**23** (0)
not detected **N** (%)	**14** (60.9)
detected **N** (%)	**9** (39.1)
mean, min, max	**6.3,** 0.43, 95.5
95% CIs	1.6, 24.4
Calprotectin (ng/ml) **N** (missing)	**23** (0)
mean, min, max	**615.7**, 223.9, 1122.0
95% CIs	503.2, 753.5
HNPs 1-3 (ng/ml) **N** (missing)	**23** (0)
mean, min, max	**299.2**, 11.0, 1513.6
95% CIs	169.0, 529.9
S. mutans specific sIgA (EU) **N** (missing)	**23** (0)
mean, min, max	**339.0**, 141.3, 1412.5
95% CIs	259.1, 443.5
S. sobrinus specific sIgA (EU) **N** (missing)	**23** (0)
mean, min, max	**347.5**, 134.9, 1949.8
95% CIs	261.6, 461.7
S. mitis specific sIgA **N** (EU) (missing)	**23** (0)
mean, min, max	**551.3,** 204.2, 831.7
95% CIs	466.3, 651.9
S. sanguinis specific sIgA (EU) **N** (missing)	**23** (0)
mean, min, max	**355.3**, 120.2, 2041.7
95% CIs	249.0, 506.9
Cortisol (µg/dL) **N** (missing)	**23** (0)
mean, min, max	**0.22**, 0.08, 3.98
95 % CIs	0.17, 3.64

Geometric data generated from back transformations of $\log_{10}$ transformed data are shown.

4.2.2.4 Salivary antimicrobial protein concentrations according to the presence of Mutans streptococci in plaque

Baseline cross-sectional analysis revealed trends for increased concentrations of antimicrobial proteins with increased bacterial load of plaque and increased numbers of *S. mutans* in plaque and saliva (Section 3.2.7).

Pearson bivariate correlation revealed moderate (r 0.3 – 0.4) to moderately strong (r 0.5 – 0.8) positive correlations between the concentrations of salivary antimicrobial proteins with bacterial counts from plaque and saliva of three-year-olds (Table 4-5). Graphical summaries of the association of *S. mutans* (% Gram positive plaque flora) with the concentrations of salivary antimicrobial proteins are shown (Figure 4-7). The moderate correlations between *S. mutans* (% Gram positive plaque flora) with the concentrations of the HNPs 1-3 and calprotectin appear to be independent of the numbers of total plaque flora since there appears to be no correlation of total plaque bacteria with the HNPs 1-3 or calprotectin. Conversely, the correlations between *S. mutans* (% Gram positive plaque flora) with concentrations of lactoferrin and LL37 may be partly mediated by the correlations with the total numbers of aerobic plaque flora. However, it is noteworthy that the correlation between the detectable concentrations of LL37 with cariogenic bacteria in plaque and saliva were stronger than the correlations observed with the total number of aerobic plaque flora. This appears to suggest that LL37 concentrations in saliva are influenced by the numbers of *S. mutans* in plaque and saliva (Table 4-5).

Furthermore, concentrations of the HNPs 1-3 and LL37 were found to be statistically significantly higher in the saliva of children in whom Mutans streptococci could be cultured from plaque, compared with children who were culture negative ($p = 0.017$ and $p = 0.006$, respectively [Figure 4-8c and d]). Differences in the concentrations of lactoferrin and calprotectin were of borderline statistical significance ($p = 0.066$ and $p = 0.055$, respectively [Figure 4-8a and b]).

Table 4-5: Associations of salivary antimicrobial proteins with bacterial counts in three-year-old children

Microbiological measurement	lactoferrin	calprotectin	HNPs 1-3	LL37 (detected only)
Diagnostic culture of plaque (CFU/ml)				
aerobic flora **N**	**23**	**23**	**23**	**9**
r	**0.370**	**-0.011**	**-0.093**	**0.569**
R^2	0.137	>0.001	0.009	0.323
oral streptococci **N**	**23**	**23**	**23**	**9**
r	**0.005**	**0.153**	**0.079**	**0.039**
R^2	0.074	-0.392	-0.281	-0.196
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive flora)				
S. mutans **N**	**22**	**22**	**22**	**9**
r	**0.369**	**0.366**	**0.454**	**0.798**
R^2	0.136	0.134	0.206	0.637
S. sobrinus **N**	**22**	**22**	**22**	**9**
r	**0.292**	**0.321**	**0.226**	**0.819**
R^2	0.085	0.103	0.051	0.670
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)				
S. mutans **N**	**23**	**23**	**23**	**9**
r	**0.365**	**0.268**	**0.312**	**0.702**
R^2	0.133	0.072	0.097	0.493
S. sobrinus **N**	**23**	**23**	**23**	**9**
r	**-0.183**	**0.057**	**0.128**	**0.740**
R^2	0.033	0.003	0.016	0.548

Pearson correlation coefficient (r) of the relationship between log_{10} transformed bacterial counts with log_{10} transformed salivary antimicrobial proteins, R^2 is also shown. LL37 associations include only those children in whom LL37 was detected in saliva. Grey shading indicates mild to moderate associations.

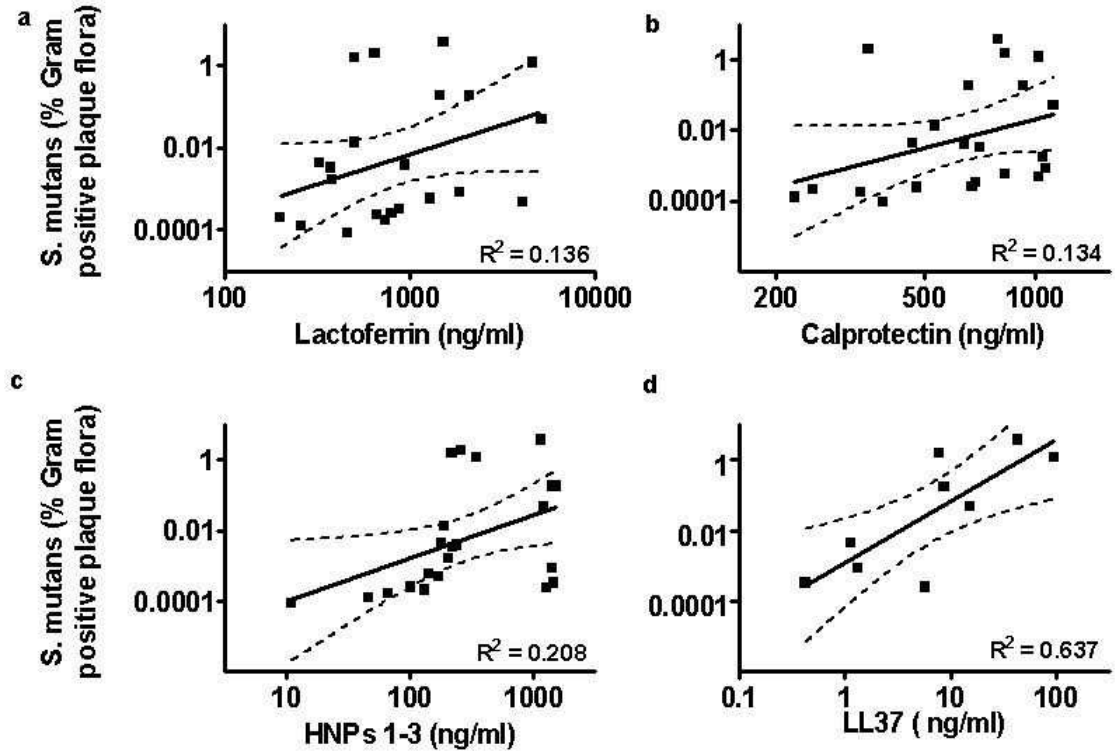


Figure 4-7: Associations of *S. mutans* in plaque with salivary antimicrobial proteins

Scatter plots of the association of $\log_{10}$ transformed *S. mutans* (% Gram positive plaque flora) with $\log_{10}$ transformed concentrations (ng/ml) of salivary a) lactoferrin (n = 23), b) calprotectin (n = 23), c) the HNPs 1-3 (n = 23) and d) LL37 (detected only [n = 9]). Each data point represents an individual study participant. The regression line (solid black line), 95% confidence intervals (dotted line) and R^2 are shown for each graph.

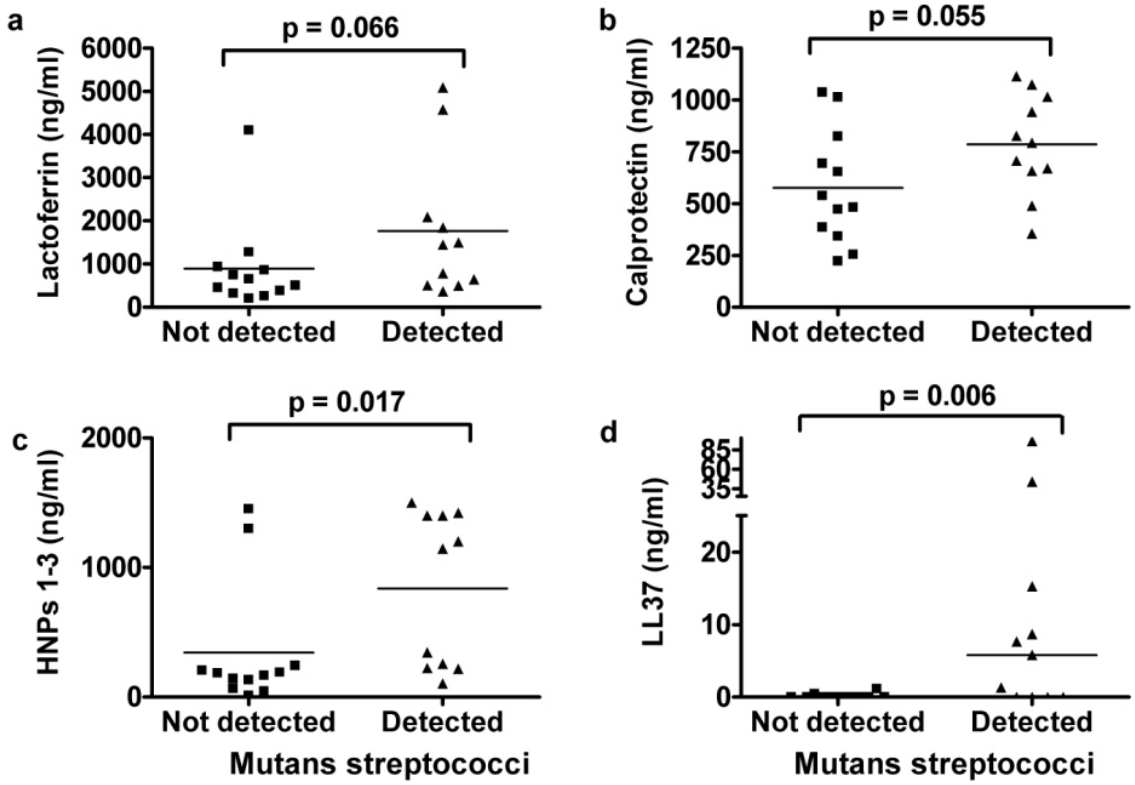


Figure 4-8: Salivary antimicrobial proteins according to culture of Mutans streptococci

Scatter plots comparing the concentrations (ng/ml) of a) lactoferrin, b) calprotectin, c) the HNPs 1-3 and d) LL37 in the saliva of children grouped according to the detection of mutans streptococci from plaque: not detected (n = 12), detected (n = 11). Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group mean. Raw data are plotted in each instance. Differences were compared by independent samples t-tests of $\log_{10}$ transformed data.

4.2.2.5 Frequency of NMES consumption in three-year-old children

Successfully completed food frequency questionnaires were available for 96% of the study group (n = 22/23). Analysis of the data revealed large variability with respect to the consumption of non-milk extrinsic sugars (NMES). The mean intake of NMES was 62.2 g/day, which on average comprised 15.2% of total food calories (Table 4-6).

4.2.2.6 Dental disease scores in three-year-old children

The dmft (decayed, missing and filled teeth) was recorded for each study participant at the time of sample collection by a national dental inspection calibrated dentist according to BASCD standards. Measurement of dental caries in a non-clinical setting, without the tools required to clean and dry teeth, does not allow for the detection of white spot enamel lesions. As a result, using only the BASCD criteria, only dentine caries is recorded and thus the true extent of caries experience in the population is likely to be underestimated. Ninety-one percent (n = 21/23) of three-year-old children were recorded as caries free (dmft = 0). Two children were recorded as having evidence of caries, with a dmft of two and five, respectively.

Table 4-6: Descriptive analysis of NMES consumption in three-year-old children

Sugar	Statistics
NMES (g/day)	
N (missing)	**22** (1)
mean, min, max	**62.2**, 16.8, 183.2
95% CIs	44.9, 79.5
NMES (% daily food calories)	
N (%)	**22** (1)
mean, min, max	**15.2**, 4.6, 43.6
95% CIs	11.7, 18.5

Average sugar consumption of three-year-old children. Data was collected from parents of children by use of a validated food frequency questionnaire. NMES: non-milk extrinsic sugars.

4.2.3 Does NMES consumption influence the detection of cariogenic bacteria?

There were no associations for the consumption (g/day) of NMES with counts of cariogenic bacteria in plaque or saliva of three-year-old children. There were mild associations of *S. sobrinus* (% Gram positive plaque flora) and *S. sobrinus* (CFU/ml) in saliva with NMES (% daily food calories, R^2 = 0.106 and 0.147 [Table 4-7]). There was insufficient evidence that the consumption of NMES influenced the detection of cariogenic bacteria the plaque or saliva of this group of three-year-old children.

Table 4-7: Association of NMES consumption with the detection of cariogenic bacteria in three-year-old children

Cariogenic bacteria	NMES (g/day)		NMES (% food calories)	
	N	R^2	N	R^2
Diagnostic culture of plaque (CFU/ml)				
mutans streptococci	22	<0.001	22	0.001
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive flora)				
S. mutans	21	>0.001	21	0.039
S. sobrinus	21	0.011	21	0.106
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)				
S. mutans	22	0.001	22	0.013
S. sobrinus	22	0.002	22	0.147

Linear regression analysis (R^2) of NMES (non-milk extrinsic sugars) consumption (g/day and % of daily food calories) with $\log_{10}$ transformed cariogenic bacterial counts from plaque and saliva. Grey shading indicates mild associations.

4.2.4 How does the cariogenic oral biofilm and concentrations of salivary antimicrobial proteins change over time?

4.2.4.1 Mutans streptococci counts at baseline and follow-up according to age groups of study participants

The time points for sample collection for this longitudinal study were chosen to coincide with reported increased colonisation of Mutans streptococci on tooth surfaces. Therefore, it was of interest to investigate if the numbers of Mutans streptococci increased in children with increased age.

At baseline the age of children ranged from 12 to 24 months and at follow-up from 26 to 45 months. Children at each time point were grouped according to age and the mean *S. mutans* counts (estimated by both culture and TaqMan® QPCR) in each group were compared. There was a trend for increased mean numbers of Mutans streptococci cultured from plaque with increasing age of children at baseline. However, the differences between groups did not reach statistical significance (p = 0.086 [Figure 4-9a]). This trend was not evident from culture data of children at follow-up (p = 0.662 [Figure 4-9b]).

There was a statistically significant linear trend for increased mean numbers of *S. mutans* as a proportion of Gram positive plaque flora with increased age of children at baseline (p = 0.045) by ANOVA linear. However, the differences between groups were not statistically significant (p = 0.098) [Figure 4-9c]). At follow-up the mean numbers of *S. mutans* as a proportion of Gram positive plaque flora, by TaqMan® QPCR increased with age but neither the linear trend nor the differences between groups (p = 0.219) reached statistical significance (Figure 4-9d).

There were trends for increased numbers of salivary *S. mutans* in children with increasing age (Figure 4-9e and f). The differences between baseline age groups were statistically significant (p = 0.015). A Bonferroni *post hoc* test identified the statistically significant difference was between the youngest and oldest age groups at baseline (p = 0.011). Additionally, the baseline linear trend was statistically significant (p = 0.007) by ANOVA linear. Neither the linear trend nor the differences between groups reached statistical significance at follow-up.

Taken together this data support the hypothesis of increased colonisation by Mutans streptococci with increased age of children.

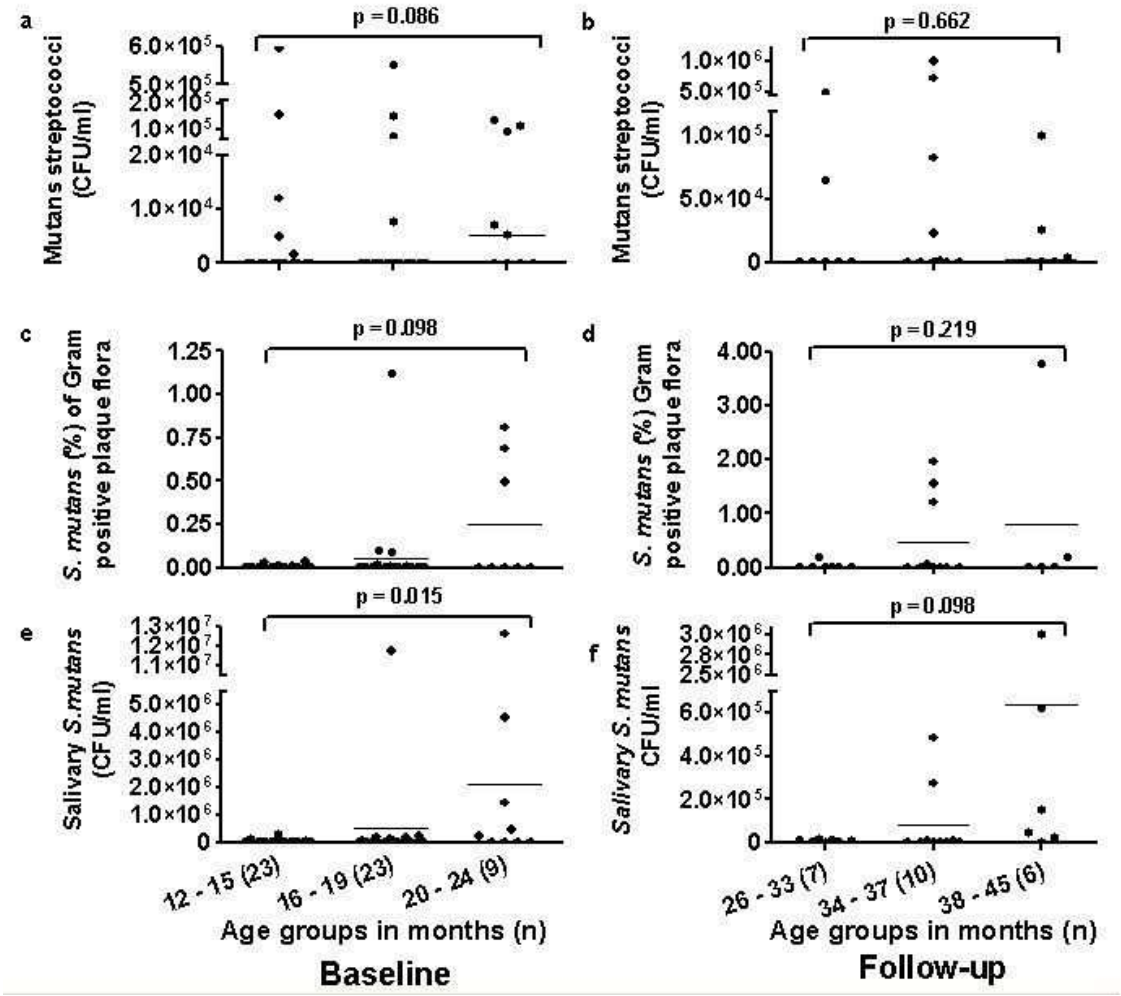


Figure 4-9: *S. mutans* counts at baseline and at follow-up according to age group of study participants

Scatter plots of baseline and follow-up bacterial counts: (a & b) Mutans streptococci (CFU/ml) estimated by diagnostic culture of plaque, (c & d) *S. mutans* (%) of Gram positive plaque flora and (e & f) salivary *S. mutans* (CFU/ml), estimated by TaqMan® QPCR and grouped according to age group of children (months). N in each group is shown on axis and raw data were plotted in each instance. Each data point represents a mean value for an individual study participant and the horizontal lines indicate the group median (a & b) for non-parametric raw data, statistically analysed using Kruskal-Wallis test, and the group mean (c – f) of log_{10} transformed parametric data, statistically analysed using ANOVA. P values shown are the overall differences between groups. The linear trend was statistically significant for (c & d [p = 0.045 & p = 0.007, respectively]) by ANOVA linear.

4.2.4.2 Longitudinal changes of bacterial counts from plaque and saliva

To investigate how the oral biofilm changed over time within individuals, baseline bacterial counts were compared with follow-up bacterial counts. There were 22 children with culture counts from both time points (Table 4-8). There were no statistically significant differences in aerobic or oral streptococci cultured from plaque over time (p = 0.884 and 0.456 [Figure 4-10a and b, respectively]).

Changes in Mutans streptococci cultured from plaque were variable, numbers increased in some children, while in others numbers decreased. Overall there was no statistically significant difference in the numbers of Mutans streptococci cultured from plaque at baseline compared with follow-up (p = 0.778 [Figure 4-10]). Due to the small numbers of participants in this study, there was insufficient evidence for changes in Mutans streptococci cultured from plaque over time.

There were 21 children with longitudinal data for both salivary *S. mutans* and salivary *S. sobrinus* (CFU/ml) by TaqMan® QPCR. Mean numbers of salivary *S. mutans* and *S. sobrinus* (CFU/ml) were higher in children at baseline, compared with follow-up (Table 4-8). Similarly, the median value for salivary *S. mutans* was eight-fold higher at baseline compared with follow-up. Changes in salivary *S. mutans* and *S. sobrinus* over time were not statistically significant (p = 0.339 and 0.289 [Figure 4-11a and b, respectively]).

There were 21 children with longitudinal data for the proportion of *S. mutans* quantified from plaque. The proportion of *S. mutans* detected in plaque of children by TaqMan® QPCR at follow-up increased in most children. Mean *S. mutans* (% Gram positive plaque flora) was over 1000-fold greater at follow-up compared with baseline (Table 4-8). The difference was highly statistically significant (p = < 0.001 [Figure 4-11c]).

There were 20 children with longitudinal data for the proportion of *S. sobrinus* quantified from plaque. Mean *S. sobrinus* (%) was higher at baseline compared with follow-up (Table 4-8). The difference was not statistically significant (p = 0.896, Figure 4-11d).

Of the 23 children recalled at follow-up, only two children had clinical evidence of caries. Therefore, no meaningful statistical analysis could be performed on such a small number. However, a number of observations of the longitudinal changes in the oral biofilm of these two children could be made.

There was a large decrease in the numbers of aerobic plaque flora at follow-up compared with baseline in both children with measurable caries (Figure 4-10a). There was little or no change in the oral streptococci cultured from the plaque of either of the children with caries (Figure 4-10b). Longitudinal changes in Mutans streptococci cultured from plaque were variable. The child with a dmft of two had fewer Mutans streptococci cultured from plaque at follow-up compared with baseline, conversely numbers increased at follow-up in the child with a dmft of five (Figure 4-10c).

There was a 10-fold and 1000-fold decrease in the numbers of salivary *S. mutans* in the children with dmft of two and five, respectively. Incidentally, these two children had the highest baseline salivary *S. mutans* counts of all the children who were followed-up. The dramatic decrease observed in these two children likely accounts for the decrease in mean salivary *S. mutans* at follow-up. There was also a dramatic decrease in the numbers of *S. sobrinus* in saliva for the child with a dmft of two, which was the highest baseline salivary *S. sobrinus* count of all the children who were followed-up. While numbers of *S. sobrinus* in the saliva of the child with dmft of five increased 10-fold at follow-up compared with baseline (Figure 4-11a and b).

Longitudinal changes in the proportion of *S. mutans* detected in the plaque of children with caries mirrored the changes in Mutans streptococci cultured from plaque. Interestingly, both children with caries had the highest proportion of *S. mutans* detected in plaque at baseline of all the children who were followed-up (Figure 4-11c). The proportion of *S. sobrinus* detected in plaque increased dramatically in the child with a dmft of five (Figure 4-11d).

Table 4-8: Longitudinal changes of bacterial counts from plaque and saliva of young children

Bacterial count	N	Mean	95% CIs of the mean		Median	Quartiles	
			lower	upper		Q1	Q3
Diagnostic culture of plaque (CFU/ml)							
Aerobic flora							
time 1	22	1.7×10^7	8.8×10^6	2.5×10^7	1.1×10^7	3.3×10^6	2.4×10^7
time 2	22	1.5×10^7	9.5×10^6	2.1×10^7	1.1×10^7	5.7×10^6	1.9×10^7
Oral streptococci							
time 1	22	6.3×10^6	2.5×10^6	1.0×10^7	3.8×10^6	1.1×10^6	8.2×10^6
time 2	22	6.9×10^6	2.1×10^6	1.2×10^7	4.2×10^6	1.7×10^6	7.3×10^6
Mutans streptococci							
time 1	22	2.0×10^5	-9.9×10^4	5.1×10^5	ND	ND	8.8×10^3
time 2	22	9.2×10^4	-2.1×10^4	2.0×10^5	ND	ND	3.5×10^5
Absolute quantitation from saliva (CFU/ml)							
S. mutans							
time 1	21	8.1×10^5	-3.6×10^5	2.0×10^6	1.3×10^4	1.5×10^3	1.9×10^5
time 2	21	2.2×10^5	-7.8×10^4	5.2×10^5	1.6×10^3	1.6×10^3	9.6×10^4
S. sobrinus							
time 1	21	2.0×10^5	-2.1×10^5	6.1×10^5	368	186	6114
time 2	21	1.7×10^3	45	3.3×10^3	261	34	838
Relative quantitation from plaque (%)							
S. mutans							
time 1	21	0.0015	-0.0369	0.2173	0.0017	0.0002	0.0050
time 2	21	2.6613	-0.0090	0.8592	1.0077	1.0006	1.5170
S. sobrinus							
time 1	20	0.0091	-0.0074	0.0256	0.0005	0.0001	0.0015
time 2	20	0.0059	-0.0032	0.0150	0.0004	0.0001	0.0011

Comparisons of mean and median bacterial counts estimated from the plaque and saliva of young children at baseline and follow-up. In some instances the large 95% confidence intervals have resulted in negative values. ND: not detected.

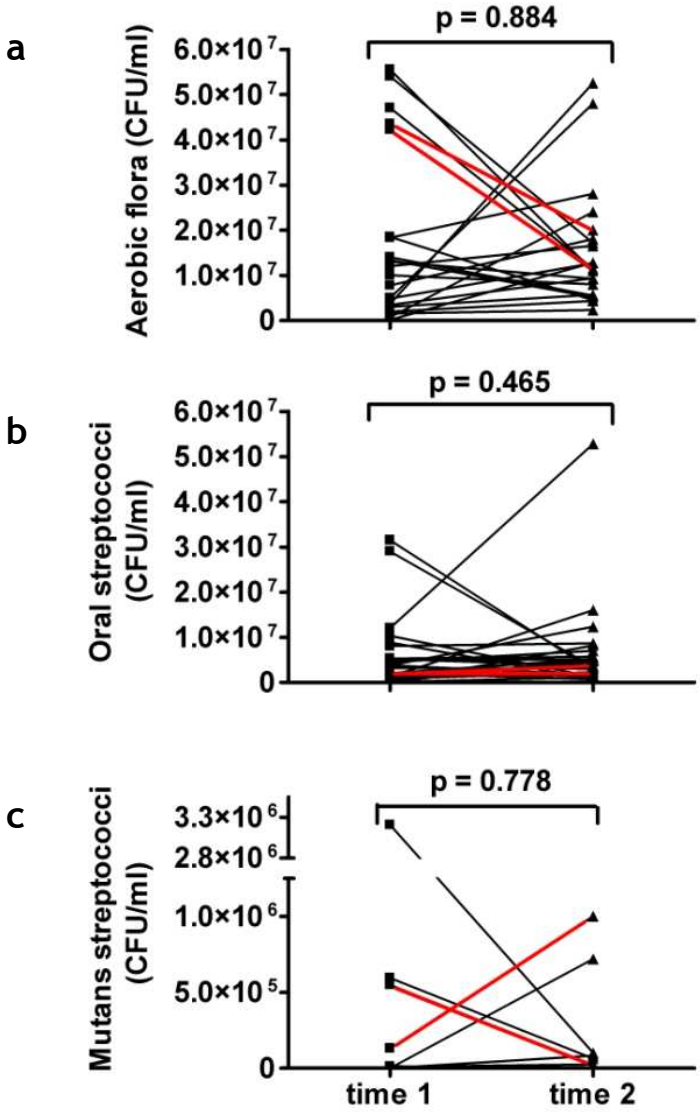


Figure 4-10: Longitudinal changes of bacterial counts from plaque of young children

Scatter plots of bacterial counts (CFU/ml) of a) total aerobic plaque flora (n = 22), b) total plaque streptococci (n = 22) and c) Mutans streptococci (n = 22, including eight children with no detectable Mutans streptococci at both time points), estimated by diagnostic culture of plaque collected from children at baseline and follow-up. Each data point represents a mean value for an individual study participant and connecting lines show the change over time for each participant. Red lines indicate children with detectable caries. Raw data are shown in each instance. Differences were compared using Wilcoxon signed ranks tests.

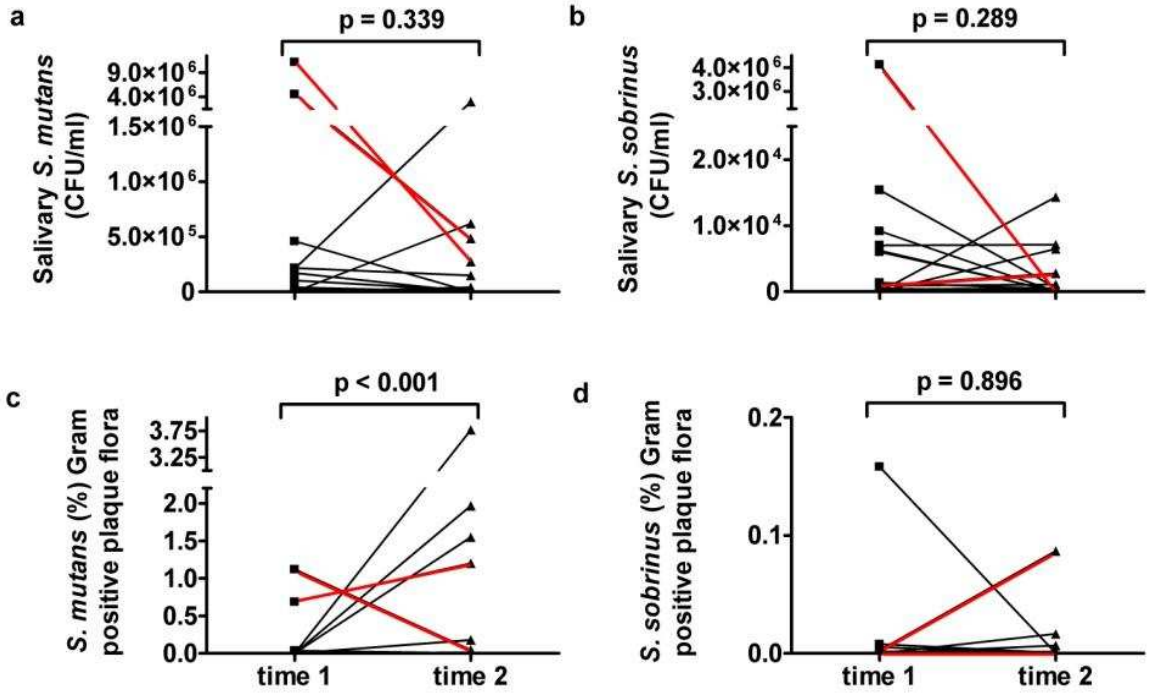


Figure 4-11: Longitudinal changes of Mutans streptococci counts from plaque and saliva of young children

Scatter plots of a) salivary *S. mutans* (CFU/ml [n = 21]), b) salivary *S. sobrinus* (CFU/ml [n = 21]) and c) *S. mutans* (% [n = 21]) and d) *S. sobrinus* (% [n = 20]) of Gram positive plaque flora, estimated by TaqMan® QPCR of plaque and saliva collected from children at baseline and follow-up. Each data point represents a mean value for an individual study participant and the connecting lines show the change over time for each participant. Red lines indicate children with detectable caries. Raw data are plotted in each instance. Differences were compared by Wilcoxon signed ranks test.

4.2.4.3 Longitudinal changes in the concentrations of salivary proteins

There were 21 children with longitudinal data for lactoferrin, calprotectin, the HNPs 1-3 and 18 children for LL37 (Table 4-9). There was no difference in the mean concentrations of lactoferrin of LL37 in the saliva of children at baseline compared with follow-up. Longitudinal increases in the mean concentrations of salivary proteins were statistically significant for calprotectin and the HNPs 1-3 ($p = 0.03$ and $p = 0.008$ [Figure 4-12]).

There were 17 children with longitudinal data for sIgA antibody titres specific for oral streptococci. Salivary IgA antibody titres specific for oral streptococci either remained constant or increased longitudinally in most children. Therefore, mean titres of sIgA antibodies specific for *S. mutans*, *S. sobrinus*, *S. mitis* and *S. sanguinis* were all higher at follow-up compared with baseline (Table 4-9). The differences were statistically significant for each of the antibody specificities with the exception of *S. sobrinus* specific sIgA (Figure 4-13). Together this data indicate that the expression of salivary proteins increased with increased age of children.

Of all the children who were followed up both children with caries had amongst the highest concentrations of lactoferrin and calprotectin at baseline. Additionally, the child with a dmft of five had the highest baseline concentrations of LL37 and the HNPs 1-3. The concentrations of salivary proteins increased over time in both children and remained the highest detected concentrations of lactoferrin, calprotectin and LL37 in the saliva of any of the children who were followed-up. The HNPs 1-3 decreased at follow-up in the child with a dmft of five but increased dramatically in the saliva from the child with a dmft of two (Figure 4-12). Antibody titres specific for oral streptococci increased longitudinally in the saliva of both children with caries. The child with a dmft of five had amongst the highest follow-up antibody titres of all the children who were followed-up (Figure 4-13).

Table 4-9: Longitudinal changes in the concentrations of salivary antimicrobial proteins in young children

Salivary protein	N	Mean	95% CIs of the mean		Median	Quartiles	
			lower	upper		Q1	Q3
Lactoferrin (ng/ml)							
time 1	21	1347.9	634.4	2061.4	895.3	523.2	1467.5
time 2	21	1321.6	653.9	1989.2	653.5	653.5	1675.1
Calprotectin (ng/ml)							
time 1	21	504.2	379.5	629.0	479.4	237.5	690.5
time 2	21	676.4	545.4	807.5	670.7	428.1	978.0
HNPs 1-3 (ng/ml)							
time 1	21	108.8	41.2	176.5	49.7	16.7	139.1
time 2	21	499.0	251.4	746.6	218.6	137.4	1172.7
LL37 (ng/ml)							
time 1	18	5.3	0.4	10.2	ND	2.1	4.9
time 2	18	7.5	ND	18.7	ND	ND	6.3
S. mutans specific sIgA (EU)							
time 1	17	246.2	149.2	343.1	217.0	134.5	281.0
time 2	17	381.8	207.6	556.0	295.0	188.0	385.5
S. sobrinus specific sIgA (EU)							
time 1	17	323.7	240.3	407.2	288.0	199.0	429.5
time 2	17	444.7	218.2	671.1	242.0	190.0	628.0
S. mitis specific sIgA (EU)							
time 1	17	265.1	129.3	401.0	166.0	117.0	254.0
time 2	17	603.6	500.5	706.7	653.0	472.0	753.0
S. sanguinis specific sIgA (EU)							
time 1	17	248.6	131.4	365.8	189.0	120.5	279.0
time 2	17	483.8	237.7	730.0	372.0	159.5	669.5

Comparisons of concentrations of salivary proteins measured from young children at baseline and at follow-up. In some instances the large 95% confidence intervals have resulted in negative values. ND: not detected.

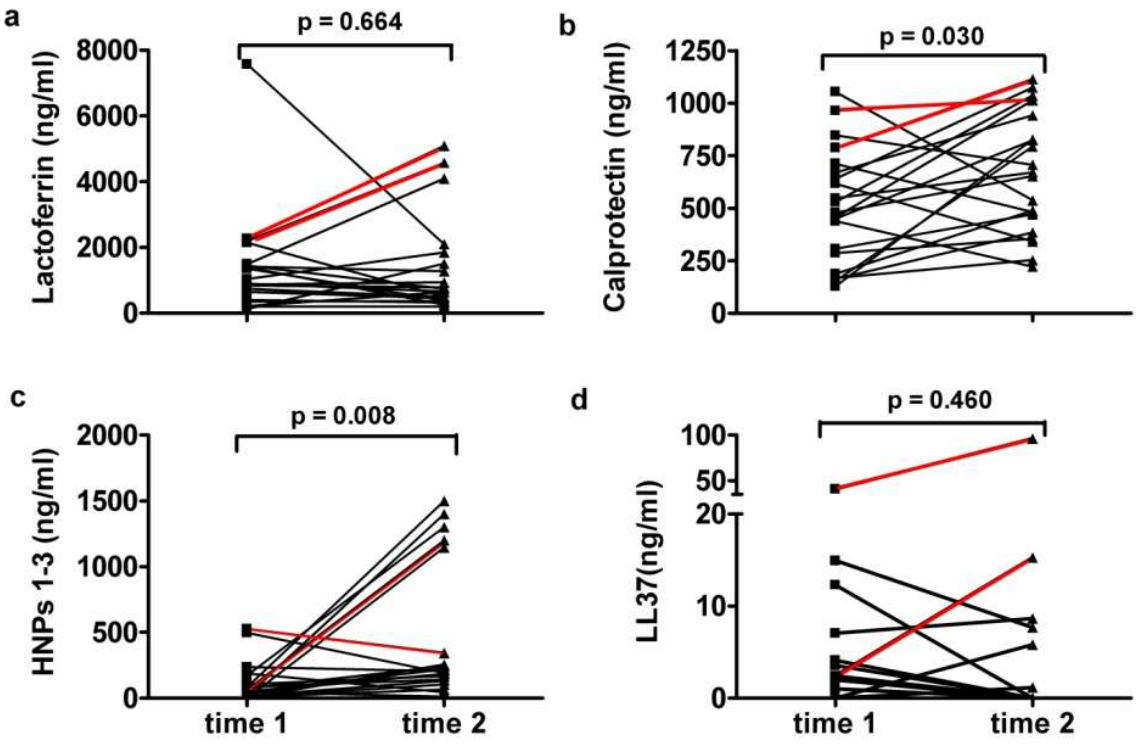


Figure 4-12: Longitudinal changes in the concentrations of salivary antimicrobial proteins in the saliva of young children

Scatter plots of concentrations (ng/ml) of a) lactoferrin (n = 21), b) calprotectin (n = 21), c) HNPs 1-3 (n = 21) and d) LL37 (n = 18, including three children with undetectable concentrations at both time points), measured from the saliva of young children at baseline and follow-up. Each data point represents a mean value for an individual study participant and connecting lines show the change over time for each participant. Red lines indicate children with detectable caries. Raw data are shown in each instance and differences were compared by Wilcoxon signed ranks test.

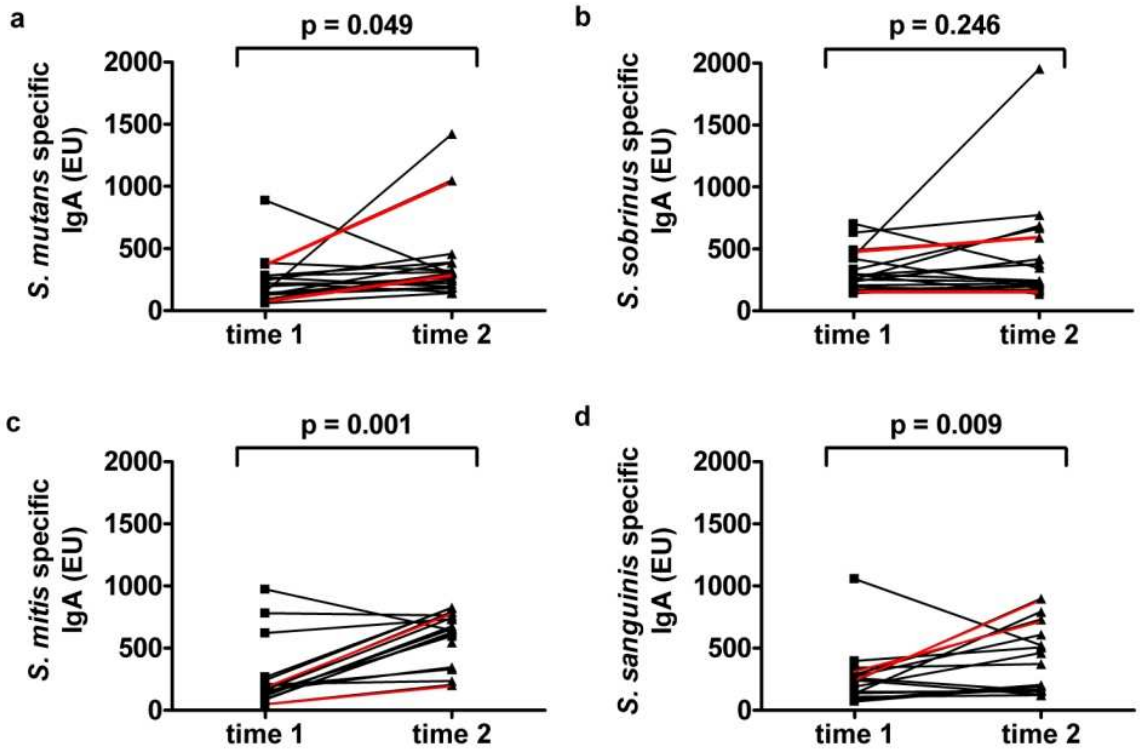


Figure 4-13: Longitudinal changes of sIgA antibody titres specific for oral streptococci in young children

Scatter plots of sIgA antibody titres (n = 17) specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* and d) *S. sanguinis* measured at baseline and follow-up. Each data point represents a mean value for an individual study participant and connecting lines show the change over time for each participant. Red lines indicate children with detectable caries. Raw data are plotted in each instance. Differences were compared using Wilcoxon signed ranks test.

4.2.5 How do changes in the cariogenic oral biofilm evolve with changes in the concentrations of salivary antimicrobial proteins.

It was hypothesised that changes in the oral biofilm may reflect changes in the developing salivary immune response, or indeed vice versa.

4.2.5.1 Changes in the oral biofilm coincident with changes in the salivary immune response

To investigate longitudinal changes of bacterial counts coincident with changes in salivary antimicrobial proteins the longitudinal fold change was calculated for each biological measure in each study participant (Table 4-10). In summary of data already reported the fold changes in bacterial counts demonstrated the most variability overall. However, for individual children the direction of fold change tended to be consistent between variables using the same method (i.e. culture or QPCR). There was also a general trend for fold increases in salivary proteins.

It was hypothesised that changes in the bacterial load of plaque or the numbers of cariogenic bacteria in plaque and saliva over time may correlate with changes in the expression of salivary antimicrobial proteins.

There was a mild to moderate positive correlation between the longitudinal fold changes in aerobic plaque flora with longitudinal fold changes in calprotectin (r^2 = 0.342 [Table 4-11 & Figure 4-14a]). Longitudinal fold increases in Mutans streptococci cultured from plaque were positively correlated with longitudinal fold increases in calprotectin and the HNPs 1-3 (r = 0.360 and 0.404, respectively [Table 4-11 & Figure 4-14b & c]). This finding was mirrored by fold increases in *S. mutans* (% Gram positive plaque flora), which was positively correlated with longitudinal fold changes in calprotectin and the HNPs 1-3 (r = 0.401 and 0.429, respectively [Table 4-11 & Figure 4-14d & e]).

A negative correlation was observed between the longitudinal fold increases in salivary *S. sobrinus* (CFU/ml) with longitudinal fold decreases in LL37 (r = -0.326 [Table 4-11]). However, the LL37 data set remained non-parametric following

log_{10} transformations (data not shown). Spearman rank correlations revealed no association for longitudinal fold changes of LL37 with salivary *S. sobrinus* (Rho = -0.045) or any of the remaining microbiological data sets (data not shown).

Longitudinal fold decreases in aerobic plaque flora, Mutans streptococci cultured from plaque, *S. mutans* (% Gram positive plaque flora) and salivary *S. sobrinus* (CFU/ml) were all negatively correlated with longitudinal fold increases in *S. mitis* specific sIgA antibody titres (Table 4-12 and Figure 4-15).

Longitudinal fold increases in *S. sobrinus* (% Gram positive plaque flora) were moderately positively correlated with longitudinal fold increases in *S. sobrinus* specific sIgA antibody titres (r = 0.612 [Table 4-12 and Figure 4-16]). Longitudinal fold increases in salivary *S. sobrinus* (CFU/ml) were not correlated with increases in *S. sobrinus* specific sIgA antibody titres.

Longitudinal fold increases in salivary *S. mutans* (CFU/ml) were moderately positively correlated with changes in *S. mutans*, *S. sobrinus* and *S. sanguinis* specific sIgA (r = 0.439, 0.488 and 0.586, respectively [Figure 4-16]).

Table 4-10: Longitudinal fold changes in bacterial counts and salivary proteins in young children

Code	AF	OS	MS	Mut saliva	Sob saliva	Mut % plaq	Sob % plaq	lacto	LL37	calp	HNPs	mut IgA	sob IgA	mit IgA	sang IgA	cort
025	0.3	0.7	<0.1	<0.1	<0.1	<0.1	11.0	2.2	6.3	1.4	19.1	3.3	1.4	4.3	3.8	5.5
031	30.7	1.6	<0.1	0.7	<0.1	4.9	2.6	0.9	1	1.8	1.6	1.9	2.7	5.8	2.4	4.4
037	2.3	1.2	670.0	17.2	1.2	5.8		1.1		0.8	6.7	9.3	4.5	3.1	11.7	9.7
039	128.0	11.8	$4x10^3$	6.1	0.3			0.8	1	2.6	73.0	0.8	1.2	1.1	5.9	0.6
041	0.2	0.1	<0.1	509.5	1.7	566.8	2.3	0.3	1.2	1.4	18.1					2.3
047	0.8	0.1	$7x10^5$	2.0	0.1	$6x10^3$	0.1	3.0	1	1.2	53.6	3.0	1.3	2.2	2.2	1.9
059	2.3	2.8	3.3	14.3	1.0	$2x10^4$	165.6	11.6		5.2	916.4					1.4
060	9.7	1.1	0.1	0.1	<0.1	<0.1	<0.1	0.9	$6x10^3$	1.2	14.9	1	0.4	1.7	0.5	0.6
064	14.2	12.8	<0.1	<0.1	0.3	0.3	0.2	2.8	<0.1	2.2	1.6					16.5
067	2.7	23.4	1	4.1	0.2	0.1	<0.1	0.7	<0.1	2.3	0.2					1.2
076	0.5	1	7.69	0.1	2.8	1.8	32.6	2.1	2.4	1.1	0.7	2.8	1.2	3.4	2.6	1.3
078	0.2	0.1	1	9.8	1.1	10.0	0.8	0.3	<0.1	2.0	0.9	0.7	0.9	4.5	1.2	0.1
079	0.2	1.1	1	1.7	0.1	0.1	<0.1	0.8	$1x10^3$	1.5	3.9	1.4	1.1	7.1	1.8	3.8
081	0.7	0.9	$2x10^3$	2.3	0.2	1.6	0.1	1.8		1.7	87.8	1.7	0.8	6.6	2.8	3.0
084	2.7	2.6	1	2.9	5.6	1.5	5.5	1.0	<0.1	1.5	7.6	2.4	1.2	5.4	1.1	2.3
086	1.4	3.2	<0.1	0.1	<0.1	<0.1	0.1	0.5	<0.1	0.6	6.8	0.7	1.1	4.2	1.0	2.9
092	0.3	4.4	<0.1	0.4	<0.1	<0.1	0.1	0.2	<0.1	0.5	0.3	1.3	0.5	5.6	2.3	4.4
093	0.4	0.8	1	<0.1	<0.1	7.7	3.7	1.1	<0.1	1.4	3.2	1.4	0.8	4.6	0.6	1.1
126	1.9	0.3	$8x10^4$	<0.1	450.0	352.7	2.3	0.7	0.5	6.4	4.4	0.3	0.5	0.7	0.5	2.0
128	0.4	0.4	1	0.4	$1x10^4$	269.2	680.0	0.2	<0.1	0.5	0.4	1.1	2.0	0.9	1.1	2.6
129	1.5	109.3	1	1.4	$6x10^3$	2.4	9.0	0.7	<0.1	0.7	7.5	1.4	1.8	1.2	1.3	1.1
133	1.5	106.6	1													
139						148.0	0.1									

Longitudinal fold changes of biological measures from plaque and saliva of study participants at baseline relative to follow-up. Pink boxes indicate a fold decrease, blue boxes indicate a fold increase, lilac boxes (1) are not detected at both times, yellow boxes (1) no change over time, black boxes indicate at least one data point missing and orange boxes indicate children with dmft ≥ 1. AF: aerobic flora, OS: oral

streptococci, MS: mutans streptococci, Mut and Sob saliva: salivary *S. mutans* and salivary *S. sobrinus*, Mut and Sob %: *S. mutans* and S. sobrinus % Gram positive plaque flora, lacto: lactoferrin, calp: calprotectin, HNPs: HNPs 1-3, Mut, Sob, Mit, Sang IgA: *S. mutans*, *S. sobrinus*, *S. mitis* and *S. sanguinis* salivary IgA, respectively.

Table 4-11: Association of longitudinal fold changes in bacterial counts with longitudinal fold changes in salivary antimicrobial proteins

	Antimicrobial protein			
Microbiological measurement	lactoferrin	calprotectin	HNPs 1-3	LL37
Diagnostic culture of plaque (CFU/ml)				
aerobic flora **N**	**21**	**21**	**21**	**18**
r	**0.232**	**0.342**	**0.207**	**0.106**
R^2	0.054	0.117	0.043	0.011
oral streptococci **N**	**21**	**21**	**21**	**18**
r	**0.101**	**-0.075**	**-0.069**	**-0.242**
R^2	0.010	0.006	0.005	0.059
mutans streptococci **N**	**21**	**21**	**21**	**18**
r	**0.260**	**0.360**	**0.404**	**0.270**
R^2	0.068	0.130	0.163	0.073
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive flora)				
S. mutans **N**	**20**	**20**	**20**	**17**
r	**0.270**	**0.401**	**0.429**	**0.006**
R^2	0.073	0.161	0.184	<0.001
S. sobrinus **N**	**19**	**19**	**19**	**17**
r	**0.150**	**0.041**	**0.106**	**-0.080**
R^2	0.023	<0.001	0.011	<0.001
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)				
S. mutans **N**	**21**	**21**	**21**	**18**
r	**-0.137**	**-0.132**	**0.268**	**0.077**
R^2	0.019	0.017	0.072	0.001
S. sobrinus **N**	**20**	**20**	**20**	**17**
r	**-0.311**	**-0.108**	**-0.135**	**-0.326**
R^2	0.097	0.012	0.018	0.106

Pearson correlation coefficient (r) of the relationship between longitudinal fold changes in log_{10} transformed bacterial counts with longitudinal changes in log_{10} transformed innate antimicrobial proteins. R^2 is also shown. Grey shading indicates low to moderate associations which are shown graphically in Figure 4-14.

Table 4-12: Associations of longitudinal fold changes in bacterial counts with longitudinal fold changes in sIgA antibodies

	Specific sIgA antibody			
Microbiological measurement	*S. mutans*	*S. sobrinus*	*S. mitis*	*S. sanguinis*
Diagnostic culture of plaque (CFU/ml)				
aerobic flora **N**	**17**	**17**	**17**	**17**
r	**-0.106**	**0.154**	**-0.320**	**0.178**
R^2	0.011	0.024	0.102	0.032
oral streptococci **N**	**17**	**17**	**17**	**17**
r	**0.011**	**0.165**	**-0.124**	**0.250**
R^2	>0.001	0.027	0.015	0.063
mutans streptococci **N**	**17**	**17**	**17**	**17**
r	**0.074**	**0.025**	**-0.428**	**0.171**
R^2	0.005	0.001	0.183	0.029
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive flora)				
S. mutans **N**	**16**	**16**	**16**	**16**
r	**-0.004**	**0.245**	**-0.478**	**-0.071**
R^2	<0.001	0.060	0.228	0.005
S. sobrinus **N**	**14**	**14**	**14**	**14**
r	**0.148**	**0.612**	**-0.275**	**0.054**
R^2	0.022	0.374	0.076	0.003
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)				
S. mutans **N**	**17**	**17**	**17**	**17**
r	**0.439**	**0.488**	**0.227**	**0.586**
R^2	0.191	0.238	0.052	0.344
S. sobrinus **N**	**16**	**16**	**16**	**16**
r	**-0.309**	**0.130**	**-0.526**	**-0.245**
R^2	0.095	0.017	0.277	0.060

Pearson correlation coefficient (r) of the relationship between longitudinal fold changes in log_{10} transformed bacterial counts with longitudinal fold changes in log_{10} transformed salivary IgA antibodies (*S. mitis* specific sIgA is raw data). R^2 is also shown. Grey shading indicates low to moderate associations which are shown graphically in (Figure 4-15 and Figure 4-16).

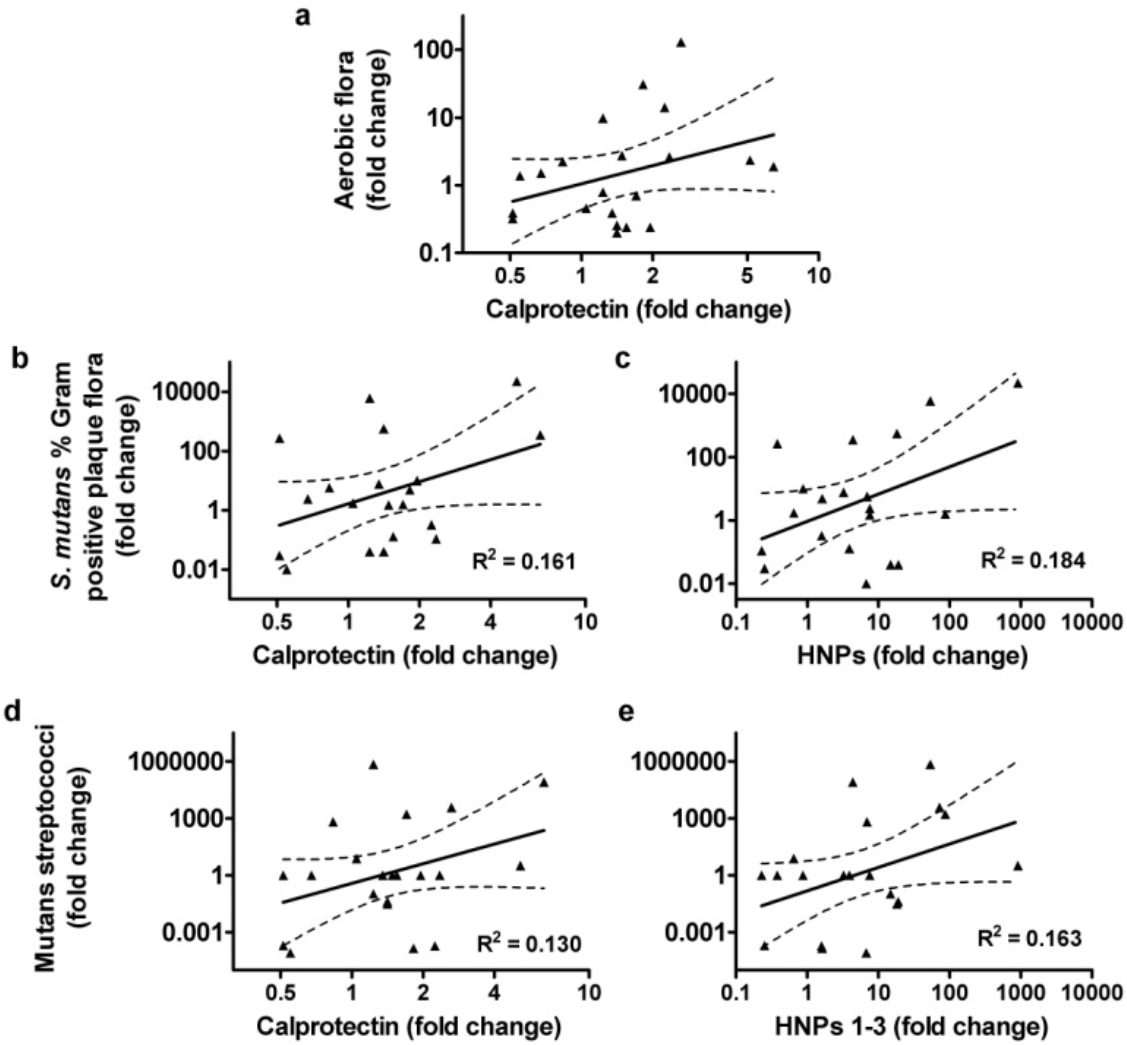


Figure 4-14: Associations of longitudinal fold changes in bacterial counts with longitudinal fold changes in salivary antimicrobial peptides

Scatter plots of longitudinal fold changes in log_{10} transformed a) aerobic plaque flora (b & c) *S. mutans* (%) of Gram positive plaque flora and (d & e) Mutans streptococci cultured from plaque with longitudinal fold changes in log_{10} transformed calprotectin (a, b & c) and the HNPs 1-3 (d & e). Each data point represents an individual study participant. The regression line (solid black line), 95% confidence intervals (dotted line) and R^2 are shown for each graph.

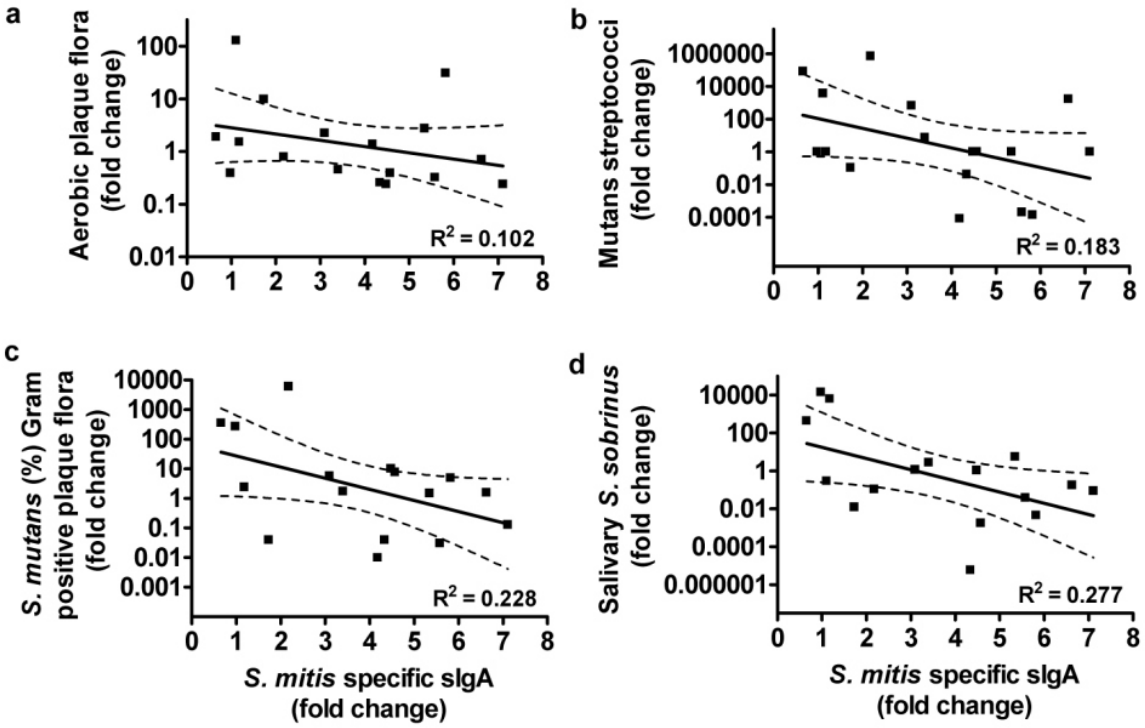


Figure 4-15: Associations of longitudinal fold changes in bacterial counts from plaque and saliva with longitudinal fold changes in *S. mitis* specific sIgA antibody titres

Scatter plots of associations of longitudinal fold changes in *S. mitis* specific sIgA antibody titres with longitudinal fold changes in $\log_{10}$ transformed a) aerobic plaque flora, b) plaque Mutans streptococci c) *S. mutans* (%) of Gram positive plaque flora and d) salivary *S. sobrinus*. Each data point represents an individual participant. The regression line (solid black line), 95% confidence intervals (dotted line) and R^2 are shown for each graph.

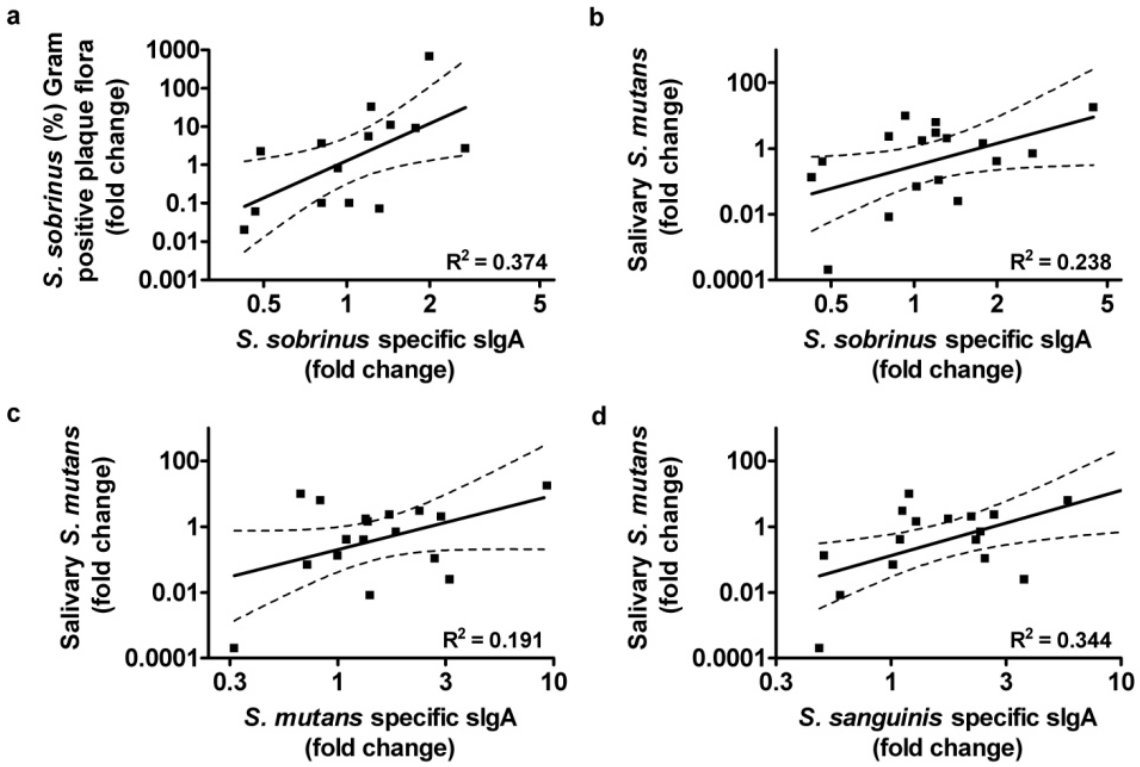


Figure 4-16: Associations of longitudinal fold changes in cariogenic bacteria from plaque and saliva with sIgA antibody titres specific for oral streptococci

Scatter plots of associations of longitudinal fold changes in log_{10} transformed a) *S. sobrinus* (%) of Gram positive plaque flora and (b, c &d) salivary *S. mutans* with longitudinal fold changes in log_{10} transformed sIgA antibody titres specific for (a & b) *S. sobrinus*, c) *S. mutans* and d) *S. sanguinis*. Each data point represents an individual participant. The regression line (solid black line), 95% confidence intervals (dotted line) and R^2 are shown for each graph.

4.2.6 Do changes in the concentrations of salivary antimicrobial proteins and the cariogenic oral biofilm relate to clinical disease scores in young children?

Of the 23 children recalled at follow-up, only two children had clinical evidence of caries, therefore negating any meaningful statistical analysis of the influence of the salivary immune response or oral biofilm on dental disease outcomes. For this reason a descriptive analysis of the longitudinal changes in the oral biofilm and salivary immune response in the children with detectable caries was included in the previous section.

4.2.7 Does SES influence the development of the cariogenic oral biofilm and the concentrations of salivary antimicrobial proteins in young children?

It was hypothesised that SES may influence the development of the oral biofilm or the evolution of the salivary immune response.

4.2.7.1 The impact of SES on bacterial counts in plaque and saliva

The longitudinal fold changes in bacterial numbers from both plaque and saliva were investigated according to baseline measures of SES. The influence of gender on oral biofilm development was first investigated. There were no statistically significant differences for longitudinal fold changes in bacterial counts from plaque or saliva according to the gender of children (data not shown).

Due to the low numbers of study participants there was insufficient evidence to indicate differences of the longitudinal fold changes in any bacterial measure with respect to the level of family income, parental education or SIMD (Table 4-13 & Table 4-14).

Table 4-13: Longitudinal fold changes in bacterial counts cultured from plaque with respect to SES of children at baseline

SES	Aerobic plaque flora			Oral streptococci			Mutans streptococci		
	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P
Income									
<£10,000	8	**9.7**	0.551	8	**1.0**	0.356	8	**4.6**	0.521
≥£10,000	11	**2.1**		11	**2.4**		11	**0.6**	
Education									
Secondary school	7	**0.3**	0.267	7	**1.5**	0.689	7	**0.5**	0.571
6th form or above	14	**2.2**		14	**2.2**		14	**2.8**	
SIMD									
Most deprived quintile	10	**1.9**	0.642	10	**1.3**	0.807	10	**1.8**	0.927
Quintiles 2-5	10	**1.3**		10	**1.6**		10	**1.4**	

Longitudinal fold changes in bacterial counts cultured from plaque and grouped according to baseline measures of SES were compared by independent samples t-tests. Geometric mean fold changes generated from back transformations of $\log_{10}$ transformed data are shown.

Table 4-14: Longitudinal fold changes of cariogenic bacteria quantified by TaqMan® QPCR from plaque and saliva with respect to SES of children at baseline

SES	*S. mutans* % Gram positive plaque flora			*S. sobrinus* % Gram positive plaque flora			salivary *S. mutans*			salivary *S. sobrinus*		
	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P
Income												
<£10,000	8	**7.3**	.539	7	**2.0**	.233	8	**0.7**	.962	8	**0.3**	.876
≥£10,000	11	**2.1**		10	**0.4**		11	**0.7**		10	**0.4**	
Education												
Secondary school	8	**2.3**	.717	6	**1.1**	.949	7	**0.6**	.677	6	**<0.1**	.103
6th form or above	12	**4.7**		12	**1.2**		13	**0.3**		13	**1.5**	
SIMD												
Most deprived quintile	10	**2.6**	.383	8	**0.6**	.587	10	**0.4**	.523	9	**0.2**	.277
Quintiles 2-5	9	**14.8**		9	**1.4**		9	**1.1**		9	**1.4**	

Longitudinal fold changes in bacterial counts quantified by TaqMan® QPCR from plaque and saliva and grouped according to baseline measures of SES were compared by independent samples t-tests. Geometric mean fold changes generated from back transformations of $\log_{10}$ transformed data are shown.

4.2.7.2 The impact of SES on the development of salivary immune responses

The effect of gender on the development of the salivary immune response was investigated. Statistically significant differences were found with respect to longitudinal increases in the concentrations of lactoferrin ($p = 0.014$), the HNPs 1-3 ($p = 0.006$) and *S. mutans* specific sIgA antibody titres ($p = 0.027$), all of which were found to be greater in females (Table 4-15).

Due to the low numbers of study participants there was insufficient evidence to indicate differences in the longitudinal fold changes of salivary antimicrobial proteins or sIgA antibody titres according to baseline measures of SES (Table 4-16 and Table 4-17).

Table 4-15: Longitudinal fold changes in salivary protein concentrations with respect to gender of study participants

Salivary antimicrobial proteins by gender of child	N	Mean fold change	P
lactoferrin			
female	9	1.7	0.014
male	12	0.6	
calprotectin			
female	9	1.4	0.884
male	12	1.5	
HNPs 1-3			
female	9	22.6	0.006
male	12	2.0	
LL37			
female	6	0.1	0.622
male	12	< 0.1	
S. mutans specific sIgA			
female	8	2.3	0.027
male	9	1.0	
S. sobrinus specific sIgA			
female	8	1.2	0.670
male	9	1.1	
S. mitis specific sIgA			
female	8	3.6	0.950
male	9	3.7	
S. sanguinis specific sIgA			
female	8	1.8	0.834
male	9	1.7	
cortisol			
female	9	2.0	0.971
male	12	2.0	

Longitudinal fold changes in salivary protein concentrations grouped according to gender of study participants were compared by independent samples t-tests. Geometric data generated from back transformations of $\log_{10}$ transformed data are shown (*S. mitis* specific sIgA is raw data).

Table 4-16: Longitudinal fold changes in concentrations of salivary antimicrobial proteins with respect to baseline measures of SES

SES	lactoferrin			calprotectin			HNPs 1-3			LL37		
	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P
Income												
<£10,000	8	**1.3**	.373	8	**1.9**	.231	8	**8.2**	.743	6	**1.9**	.103
≥£10,000	11	**0.8**		11	**1.3**		11	**5.9**		10	**<0.1**	
Education												
Secondary school	7	**1.4**	.303	7	**1.5**	.879	7	**8.1**	.541	5	**<0.1**	.975
6th form or above	13	**0.8**		13	**1.4**		13	**4.3**		12	**<0.1**	
SIMD												
Most deprived quintile	10	**1.1**	.312	10	**1.8**	.298	10	**8.1**	.389	7	**0.3**	.328
Quintiles 2-5	9	**0.7**		9	**1.3**		9	**3.3**		9	**<0.1**	

Longitudinal fold changes in the concentrations in salivary antimicrobial proteins grouped according to baseline measures of SES were compared by independent samples t-tests. Geometric mean fold changes generated from back transformations of $\log_{10}$ transformed data are shown.

Table 4-17: Longitudinal fold changes in sIgA antibody titres with respect to baseline measures of SES

SES	*S. mutans* specific sIgA			*S. sobrinus* specific sIgA			*S. mitis* specific sIgA			*S. sanguinis* specific sIgA		
	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P	N	mean fold change	P
Income												
<£10,000	5	**2.0**	.572	5	**1.1**	937	5	**2.6**	.138	5	**2.0**	.976
≥£10,000	10	**1.4**		10	**1.1**		10	**4.4**		10	**1.9**	
Education												
Secondary school	5	**2.1**	.247	5	**1.4**	.368	5	**4.7**	.216	5	**2.2**	.506
6th form or above	12	**1.3**		12	**1.0**		12	**3.3**		12	**1.6**	
SIMD												
Most deprived quintile	8	**1.4**	.858	8	**1.1**	.937	8	**1.5**	.416	8	**2.2**	.534
Quintiles 2-5	7	**1.5**		7	**1.1**		7	**2.0**		7	**1.7**	

Longitudinal fold changes in the titres of sIgA antibodies specific for oral streptococci grouped according to baseline measures of SES were compared by independent samples t-tests. Geometric mean fold changes generated from back transformations of $\log_{10}$ transformed data are shown (*S. mitis* specific sIgA are raw data).

4.2.8 Do changes in salivary cortisol concentrations influence the development of the cariogenic oral biofilm or the concentrations of salivary antimicrobial proteins in young children?

Previous cross-sectional analysis revealed no association of salivary cortisol concentrations with measures of socioeconomic status. A similar cross-sectional analysis was performed at the second time point and similarly no association of salivary cortisol concentrations with baseline measures of socioeconomic status were identified in three-year-old children. Nonetheless, cortisol has been shown to impact on the immune system and it was decided to investigate the longitudinal changes in salivary cortisol concentrations for correlations with longitudinal changes in the oral biofilm and salivary immune response.

Longitudinal salivary cortisol data was available for all children who were followed-up (n = 23). Salivary cortisol concentrations significantly increased longitudinally from 0.18 µg/dL at baseline to 0.27 µg/dL at follow-up (p = 0.009 [Figure 4-17]). There were no correlations between longitudinal fold changes in bacterial counts from plaque or saliva of young children with longitudinal fold changes in salivary cortisol concentrations (Table 4-18). Longitudinal fold changes in salivary antimicrobial proteins (lactoferrin, calprotectin, the HNPs 1-3 and LL37) were not correlated with longitudinal fold changes in salivary cortisol concentrations (Table 4-19). There were mild to moderate correlations for longitudinal changes in sIgA antibody titres with longitudinal changes in salivary cortisol concentrations, suggesting that as cortisol concentrations increased longitudinally so to did the titres for sIgA antibodies specific for oral bacteria (Table 4-19 and Figure 4-18).

Salivary cortisol concentrations increased longitudinally in both children with caries. In one child there was over a five-fold increase in the concentration of salivary cortisol at follow-up. The concentration of cortisol in this child was over double that of any other child at follow-up.

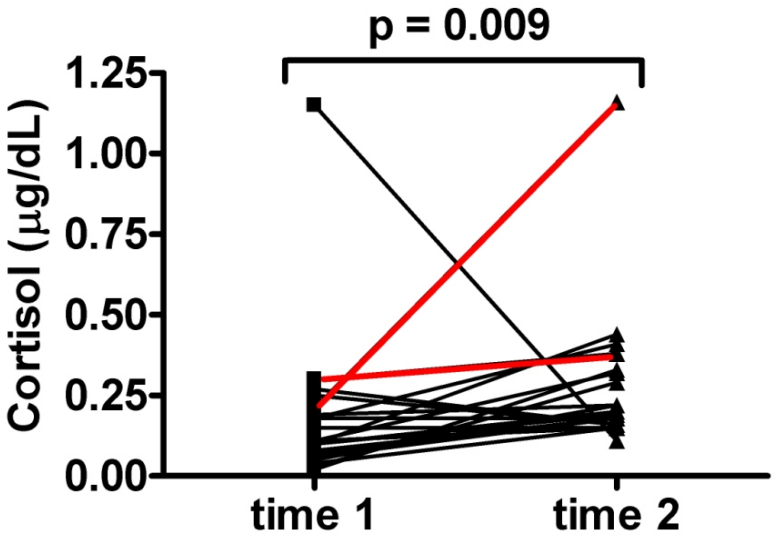


Figure 4-17: Longitudinal changes of salivary cortisol concentrations in young children

Scatter plot of salivary cortisol (µg/dL) measured at baseline and follow-up (n = 23). Each data point represents a mean value for an individual study participant and the connecting lines show the change over time for each participant. Red lines indicate children with detectable caries. Raw data were plotted. The difference was statistically significant by Wilcoxon signed ranks test.

Table 4-18: Associations of longitudinal fold changes in bacterial counts with longitudinal fold changes in salivary cortisol

Microbiological measurement	Cortisol
Diagnostic culture of plaque (CFU/ml)	
aerobic flora **N**	**21**
r, R^2	**0.053**, 0.003
oral streptococci **N**	**21**
r, R^2	**0.169**, 0.029
mutans streptococci **N**	**21**
r, R^2	**-0.248**, 0.062
Relative quantitation from plaque by TaqMan® QPCR (% Gram positive flora)	
S. mutans **N**	**20**
r, R^2	-**0.173**, 0.030
S. sobrinus **N**	**18**
r, R^2	**-0.009**, >0.001
Absolute quantitation from saliva by TaqMan® QPCR (CFU/ml)	
S. mutans **N**	**21**
r, R^2	**-0.261**, 0.068
S. sobrinus **N**	**20**
r, R^2	**-0.197**, 0.039

Pearson correlation coefficient (r) of the relationship between longitudinal fold changes in $\log_{10}$ transformed bacterial counts with longitudinal fold changes in $\log_{10}$ transformed salivary cortisol concentrations. R^2 is also shown.

Table 4-19: Associations of longitudinal fold changes in salivary antimicrobial proteins with longitudinal fold changes in salivary cortisol concentrations

Salivary antimicrobial proteins		Cortisol
lactoferrin	**N**	**21**
	r, R^2	**0.202**, 0.041
calprotectin	**N**	**21**
	r, R^2	**0.176**, 0.031
HNPs 1-3	**N**	**21**
	r, R^2	**0.032**, 0.001
LL37	**N**	**18**
	r, R^2	**0.007**, >0.001
S. mutans specific sIgA	**N**	**17**
	r, R^2	**0.521**, 0.271
S. sobrinus specific sIgA	**N**	**17**
	r, R^2	**0.370**, 0.137
S. mitis specific sIgA	**N**	**17**
	r, R^2	**0.283**, 0.080
S. sanguinis specific sIgA	**N**	**17**
	r, R^2	**0.416**, 0.173

Pearson correlation coefficient (r) of the relationship between longitudinal fold changes of log_{10} transformed salivary antimicrobial proteins with longitudinal fold changes of log_{10} transformed salivary cortisol concentrations. R^2 is also shown. Grey shading indicates mild associations and are shown graphically in Figure 4-18.

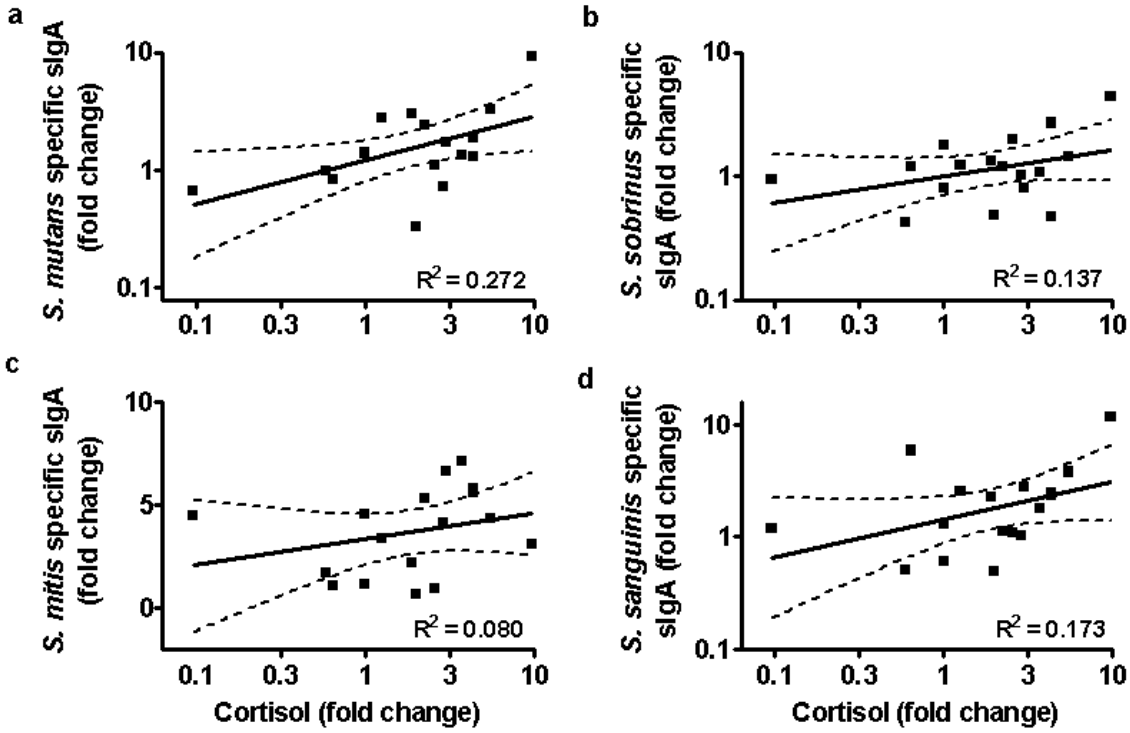


Figure 4-18: Associations of longitudinal fold changes of sIgA antibody titres with longitudinal fold changes of salivary cortisol

Scatter plots of associations of longitudinal fold changes of log_{10} transformed sIgA antibody titres specific for a) *S. mutans*, b) *S. sobrinus*, c) *S. mitis* (raw data) and d) *S. sanguinis* with longitudinal fold changes of log_{10} transformed salivary cortisol concentrations. Each data point represents an individual participant. The regression line (solid black line), 95% confidence intervals (dotted line) and R^2 are shown for each graph.

4.3 Discussion

In this study caries-associated biological risk factors were investigated as part of a pilot longitudinal clinical study. This work demonstrates that it is extremely challenging to follow-up children of this age and in this setting within the Childsmile programme. Nonetheless, repeated samples were obtained from a proportion of the original study population at specified intervals and longitudinal changes in biological variables were assessed.

The overwhelming limitation of this study was the small sample size. The effect of the small sample size was evident throughout the study with respect to the constraints of identifying statistically significant differences between groups of individuals. As a consequence, this study did not have the power to identify true population differences based on the statistical analysis. Thus, in many instances the only conclusion must be that there was insufficient evidence to identify any differences or changes over time.

The first and second aims of this study, to determine whether the children who returned for follow-up were representative of the study group at baseline and to provide a descriptive analysis of the microbiological, immunological, dietary and dental disease data of three-year-old children at follow-up were fulfilled. The children who returned for follow-up were found to be broadly representative of the study group at baseline and descriptions of the microbiological, immunological, dietary and dental disease data were presented.

The third aim of this study was to investigate the effect of NMES consumption on the detection of cariogenic bacteria in plaque and saliva. Frequent consumption of sugary foods is a well established risk factor associated with increased *S. mutans* colonisation and development of dental caries in young children (Habibian et al. 2001; Milgrom et al. 2000). In the present study no association was found with regard to the consumption of NMES (of which on average greater than 90% comprised sucrose) with the detection of cariogenic bacteria in three-year-old children. However, it is noteworthy that in 86% (n = 19/22 of children with available NMES consumption data) the percentage of daily calories represented by NMES exceeded the national guidelines of 10%. It is likely that

the data reported here were compromised by the small sample size. The FFQ used in this study is designed to estimate differences in the eating behaviours of populations of greater than 100 individuals and thus, there was insufficient power to detect real differences with regard to NMES consumption in this study. Furthermore, the low caries rate in the study population (only two children with measurable caries, for one of which there was no data for NMES consumption) negated even a descriptive analysis of the influence of NMES consumption on dental decay experience.

The fourth aim of this study was to investigate how the oral biofilm changes over time. Assessment of the bacterial load of plaque by microbial culture of the total aerobic flora revealed no change in the bacterial load of plaque over time, indicating that total numbers of aerobic and facultative anaerobic bacteria in the dental plaque of children remained constant from one to three-years of age. This was perhaps unexpected as during the sampling period the primary dentition continued to erupt in these children, providing new attachment sites and would have presumably contributed to increased bacterial colonisation. This is most likely explained by the sampling techniques employed in this study. The reduction in the proportion of total oral streptococci from 40% at baseline to 33% at follow-up likely reflects an increase in the diversity and maturation of the oral biofilm from one- to three-years of age. With the exception of Lactobacillus spp. anaerobic plaque bacteria, associated with mature oral biofilms, were not investigated within the present study. The numbers and diversity of anaerobic bacteria in the oral biofilm are known to increase with increased age (Kononen 2000; Sutter 1984). It is possible and likely that the numbers of anaerobic plaque bacteria increased during the sampling period, commensurate with previous reports in three-year-old children (Kononen et al. 1994). Indeed, consistent with this notion, Lactobacillus were undetectable by culture of plaque at baseline but were detected in three of 23 children at follow-up.

There were trends for increased colonisation by *S. mutans* with increased age of children. However, the data in support of this were compromised by the small follow-up rates. The complete eruption of the primary dentition by around three-years of age, specifically the molars provides new attachment sites for microbial adherence and presumably would contribute to increased *S. mutans* colonisation. The finding that the bacterial load of plaque did not change

longitudinally, together with the increase in *S. mutans* as a proportion of Gram positive plaque flora suggest that the proportion of *S. mutans* in dental plaque increased in the study group over time. This is in agreement with a previous study which reported longitudinal increases in *S. mutans* as a proportion of total oral flora in children aged three- to four-years (Parisotto et al. 2011).

Differences in the culture data compared to TaqMan® QPCR data were evident at both time points. Longitudinal fold changes in *S. mutans* cultured or detected from plaque were statistically correlated with each other ($r = 0.561$, $p = 0.01$), indicating that as detection of Mutans streptococci by culture increased over time so too did detection of *S. mutans* in plaque by TaqMan® QPCR. However, changes in salivary *S. mutans* did not correlate with changes in Mutans streptococci cultured from plaque or with *S. mutans* detected in plaque by TaqMan® QPCR. This study indicates that the tooth-associated dental plaque is a more clinically relevant and reliable method for the detection of *S. mutans*. Additionally, data reported here confirm TaqMan® QPCR to be a more sensitive method for enumeration of *S. mutans* in dental plaque.

Recent molecular studies describing the oral metagenome have indicated caries activity does not necessarily correlate with the presence of *S. mutans*. In one study 10% of children with rampant caries in the permanent dentition did not have detectable *S. mutans* by molecular techniques (Aas et al. 2008). A separate study utilising 454 pyrosequencing reported almost a complete absence of *S. mutans* in carious lesions, although other species such as Veillonella and Corynebacterium were found to be present (Belda-Ferre et al. 2011). Another recent study used Roche pyrosequencing to estimate the bacterial diversity of different carious lesions. In this study it was demonstrated that *S. mutans* was commonly associated with enamel caries but was undetected in dentine and deep dentine lesions (Simon-Soro et al. 2012a). Thus *S. mutans* appear to be most commonly associated with childhood caries which once established progresses into dentine and bacterial species other than *S. mutans* become important. Incidentally, in the present study the BASCD criteria used to estimate caries experience only detects caries into dentine and not white spot enamel lesions.

Future population-based studies of the oral metagenome and transcriptome associated with health and disease, particularly in young children are required to elucidate the role of other bacterial species, particularly the uncultivatable species in the development of dental caries. Childsmile's young target age group and multiple visit interventions could provide a platform from which to longitudinally investigate the development of the oral metagenome in children through to adolescence and all stages of disease. As oral health has been related to cancer and cardiovascular diseases (Aida et al. 2011), such a study could have far reaching implications beyond those specifically related to oral health.

A further aim of this study was to investigate changes in the oral biofilm coincident with the evolution of the salivary immune response. It was hypothesised that changes in the salivary immune response would influence the development of the oral biofilm. This is the first study to document changes in the concentrations of lactoferrin, LL37, calprotectin and the HNPs 1-3 in children of this age. The median concentrations of the HNPs 1-3 and LL37 were lower than reported in a study of children aged 11- to 15-years (Tao et al. 2005). Additionally, previous studies have reported increased sIgA levels with increased age in older children (Childers et al. 2003; Parisotto et al. 2011). Together this data support a role for the ongoing maturation and development of salivary immune responses during early childhood.

Previous cross-sectional analysis in one-year-old children indicated that the concentrations of antimicrobial proteins in saliva increased non-specifically in response to increased numbers of oral bacteria. However, longitudinal data presented here indicate some association of increased concentrations of the HNPs 1-3 with increased numbers of *S. mutans* in dental plaque that is independent of the numbers of total aerobic plaque flora. This finding is in agreement with a cross-sectional study in 13-year-old children which reported a significant correlation of the HNPs 1-3 with numbers of *S. mutans* in dental plaque (Phattarataratip et al. 2011).

Longitudinal increases in LL37 were not associated with longitudinal increases in *S. mutans* detected from plaque or saliva. However, detectable concentrations of LL37 were positively correlated with the proportion of *S. mutans* detected in plaque of three-year-old children and were significantly higher in three-year-old

children in whom Mutans streptococci were cultured from plaque. Low concentrations of the HNPs 1-3 (Tao et al. 2005) and LL37 (Davidopoulou et al. 2012) have previously been attributed to increased caries risk in young children. These antimicrobial proteins are genetically encoded and differences in their concentrations in saliva have been attributed to gene copy number and genetic polymorphisms (Dale et al. 2006). Thus, individuals with a reduced ability to produce adequate concentrations of antimicrobial proteins in the context of increased colonisation by *S. mutans* may become more susceptible to caries initiation. However, further studies are required to adequately determine whether low concentrations of antimicrobial proteins are a risk factor for caries in young children.

Sources of HNPs 1-3 and LL37 in the oral cavity include neutrophils, which enter the oral cavity via the gingival crevicular crevice and both peptides are released from submandibular glands, which are a major source of unstimulated saliva (Tao et al. 2005). This would suggest that the presence of these peptides in saliva is due to constitutive, non-specific immune mechanisms. However, longitudinal data reported here indicate some association with numbers of *S. mutans* in the dental plaque of young children. One concern with this finding is that the partial eruption of teeth is a source of inflammation with associated increases in neutrophil influx and concentrations of antimicrobial proteins in saliva. The presence of partially erupted teeth was not controlled for in this study and thus could confound the data reported here.

Neutrophils continuously enter the oral cavity via the gingival crevicular crevice and increased numbers of neutrophils are associated with increased severity of periodontal disease (Bhadbhade et al. 2012). However, there is no evidence that neutrophils are important for caries resistance since individuals with neutrophil deficiencies are not subject to higher prevalence of dental caries, except in rare circumstances (Antonio et al. 2010).

Other sources of HNPs 1-3 and LL37 include expression by salivary duct cells (Dale et al. 2006). Additionally, LL37 is expressed within the oral epithelium and its expression is inducible by bacterial stimulation. Expression of human beta-defensin 2 (hBD2) is also induced in response to bacterial stimulation (Dale and Fredericks 2005). The oral epithelium is capable of distinguishing between

commensal and pathogenic organisms via the use of differential signalling cascades in response to bacterial stimulation (Chung and Dale 2008). It is possible that LL37 expression by the oral epithelium may be regulated through a similar mechanism. This has yet to be investigated but could potentially provide a mechanism through which LL37 concentrations are associated with numbers of *S. mutans* in dental plaque.

The association for longitudinally increased sIgA antibody titres specific for *S. mutans* and *S. sobrinus* with increased numbers of *S. mutans* detected in saliva, suggests that specific sIgA antibodies increased in response to the presence of cariogenic bacteria in saliva. This association was not evident for *S. mutans* in dental plaque. One reason for this apparent dichotomy may be due in part to the mechanism of induction of the sIgA antibody response. Firstly, migration of antigen-sensitised B cells from the gut-associated lymphoid tissue to the salivary glands is an important route of antibody induction (Brandtzaeg 1996). Secondly, bacterial antigens stimulate the proliferation and differentiation of plasma cells locally in salivary glands (Marcotte and Lavoie 1998). In both instances oral bacteria present within dental plaque are not readily accessible for sIgA antibody induction by these routes, while bacteria present in saliva are. Furthermore, sIgA antibodies absorbed to bacteria makes them unavailable for measurement using the techniques employed here. Moreover, sIgA antibody responses are subject to significant circadian variation with higher levels at night compared with during the day (Dimitriou et al. 2002). Therefore, the time of day at which samples were collected, specifically the time since tooth-brushing, which disrupts the oral biofilm and releases bacterial cells into saliva, presumably making them available for sIgA induction and absorption, is likely important for the measurement of salivary proteins and should be considered for future clinical studies.

A recent study demonstrated that the breadth of the sIgA antibody response to different *S. mutans* epitopes within individuals is important for caries-resistance (Parisotto et al. 2011). Glucan-binding protein B (GbpB) is involved in accumulation and aggregation of *S. mutans* into the dental biofilm (Stipp et al. 2008) and it was reported that children with low levels of sIgA reactive with GbpB at baseline had a 7.5 higher chance of developing caries during the study period. Furthermore, antibody responses in children from the caries-free group

increased significantly to a greater number of *S. mutans* peptides compared with the caries active group. In the present study sIgA antibody titres were assessed using whole fixed bacterial cells. The use of purified antigens derived from cariogenic bacteria to assess sIgA responses are likely a more reliable indicator of caries susceptibility. Insufficient volume of saliva negated these investigations in the present study.

These data suggest that *S. mitis* specific sIgA antibodies may have some limited effect on the accumulation of *S. mutans* in dental plaque. This perhaps indicates the presence of antibodies directed against shared streptococcal antigens and may be one method that commensal species use to persist within the oral cavity. In support of the notion of cross-reactive antibodies was the finding that antibody titres within individuals were statistically, positively correlated with each other (data not shown). This could have been further investigated by prior absorption of saliva against each of the bacterial species to be tested but insufficient volume of saliva negated this analysis.

The inverse association of *S. mitis* specific antibodies with decreased *S. mutans* accumulation in dental plaque may also be explained indirectly through increased numbers of *S. mitis*. *S. mitis* is an early coloniser of the oral cavity and remains numerically significant throughout life (Hohwy et al. 2001). *S. mitis* successfully colonises numerous habitats in the oral cavity including the oral mucosa and dental plaque. Studies have demonstrated that the presence of *S. sanguinis* in dental plaque negatively correlates with the presence of *S. mutans*, thereby providing direct protection against *S. mutans* colonisation (Caufield et al. 2000). It therefore follows that increased *S. mitis* specific antibodies may result from greater accumulation of *S. mitis* in dental plaque and thus provides protection against *S. mutans* colonisation through competition. Due to the costs associated with running TaqMan® QPCR only the cariogenic species were quantified in the present study. Future studies of the oral metagenome and transcriptome could help to elucidate the role of non-cariogenic bacterial species and potentially identify bacterial species whose presence may protect or delay against caries initiation.

The sixth aim of this study was to investigate if the composition of the oral biofilm and the nature of the salivary immune response influence dental disease

outcomes in young children. This study was designed to measure clinical disease scores only at follow-up, as this is the time when tooth decay becomes more clinically relevant. Three-year-old national dental inspections are performed as part of the Childsmile evaluation and the same criteria was used for the present study. These inspections are not performed in dental surgeries. The BASCD criteria define the standards by which these inspections should be performed. However, the resources available in these settings are limited and thus a thoroughly detailed examination cannot be performed. White spot enamel lesions cannot be measured with consistency and so only dentine caries are recorded. Based on previous national dental inspection data for the prevalence of tooth-decay in Scottish three-year-olds and given the demographics of study participants, the caries rate for this study was anticipated to be between 25% to 40% (McMahon et al. 2010). Of the 23 children successfully recalled at follow-up, only two children had dental decay, representing less than 9% of the study group at follow-up. This negated any statistical analyses to investigate the effect of cariogenic bacteria or the salivary immune response on clinical disease scores.

A descriptive analysis of the changes in the composition of the oral biofilm and the salivary immune response in both children with caries was performed. While the merit of data from only two children is questionable some interesting trends were identified. Both of the children with measureable caries at age three-years had the highest baseline levels of *S. mutans* detected in saliva and plaque of all the children who returned for follow-up and amongst the highest of all the children who were recruited at baseline. This finding is consistent with previous reports that high numbers of *S. mutans* at an early age are a risk factor for caries development in the primary dentition (Alaluusua and Renkonen 1983). At follow-up the numbers of *S. mutans* detected in both plaque and saliva in both children decreased substantially, consistent with reports that *S. mutans* are less important in lesion progression (Simon-Soro et al. 2012a). Furthermore, both children had amongst the highest baseline concentrations of innate antimicrobial proteins of all children who were followed-up, which remained amongst the highest concentrations detected at follow-up.

Of additional interest was the finding that both children with measurable caries were followed-up via domiciliary visits. This suggests perhaps that the parents of children with caries opted out of bringing their child along to follow-up

appointments in a clinical health centre setting. Thus, causing a bias in the caries rates reported here and could account for the lower than expected caries rates in this cohort. However, whether parents actively opted out of the study or simply just did not respond or show up for follow-up appointments is not known. A recent study which investigated the use of positive consent on participation rates of caries studies in Wales concluded that parents of children with caries experience were more likely to opt out their child from dental surveys if positive consent was used than parents with caries-free children. Positive consent was obtained from parents of all children at baseline who participated in the present study.

A major limitation of the present longitudinal study was the poor follow-up rates, which were only 37% of the original study group at baseline. The use of domiciliary visits, although resource intensive, were productive for successful follow-up participation of those who failed to attend health centre appointments. Moreover, there were measurable differences in the levels of *S. mutans* detected in the plaque and saliva of children according to the recruitment method used. One reason for this may be due to tooth-brushing habits. Parents who brought their child along to their allocated appointment in the health centre and therefore actively engaged with the study may have been more likely to ensure the child brushed their teeth on that morning compared with the 'hard to reach' parents who participated with minimal effort on their part.

The final aim of this longitudinal study was to investigate if socioeconomic inequalities or life stresses associated with changes in the salivary immune response or oral biofilm development. Children from families with a relatively poor income at baseline demonstrated the greatest increases in *S. mutans* in plaque over the study period. Conversely, the greatest increases in plaque *S. mutans* were found to be in children living in relatively less deprived areas or from parents with a higher level of education. This finding seems to indicate an underlying disparity within the data for assessing socioeconomic status in this study. Indeed, living in a deprived area was statistically associated with parents who obtained a secondary school education ($p = 0.005$, by Fisher's exact) but not with an annual income of £10, 000 or less ($p = 0.748$, by Fisher's exact). The cut off point for the dichotomisation of annual income received was arbitrarily

chosen in an attempt to compare the most deprived families with those who were relatively less deprived. Figures from the Scottish Government suggest the annual income for a family living in Glasgow is £22, 000. Thus, a cut off point closer to this figure may have more successfully reflected the socioeconomic position of families as indicated by the other measures. Moreover, the finding that children from a relatively more affluent background (as indicated by a higher level of parental education and living in a less deprived area) were associated with greater increases in the proportion of *S. mutans* in dental plaque reflects previous cross-sectional data which demonstrated that these children also had higher numbers of *S. mutans* cultured and detected in plaque and saliva at baseline. Despite these disparities the overwhelming limitation with regard to assessing the effect of socioeconomic status and life stresses on biological risk factors of caries in the present study is most likely due to the small sample size and insufficient power to detect statistically significant differences.

There was no evidence to suggest that salivary cortisol concentrations were influenced by the socioeconomic status of children. As previously discussed the findings reported here are likely limited by potential biases associated with the measurement of salivary cortisol at a single time point and the small sample size. Future studies should ideally look to measure salivary cortisol at numerous times throughout the day in order to determine a basal cortisol level for each individual.

Salivary cortisol concentrations increased significantly over the study period. This is consistent with a previous study (Watamura et al. 2004) and indicates that circadian regulation of the hypothalamic-pituitary axis continues to mature during early childhood. There was no evidence that salivary cortisol concentrations influenced the development of the oral biofilm or the concentrations of salivary innate antimicrobial proteins. However, increases in salivary cortisol were associated with increases in sIgA antibody titres specific for oral bacteria. This was surprising given previous studies have documented lower levels of antigen specific sIgA antibodies following vaccination under conditions of chronic stress (Cohen et al. 2001). However, the longitudinal findings reported here reflect previous cross-sectional observations of higher sIgA antibody titres in children with higher concentrations of salivary cortisol. Whether increases in specific sIgA antibody titres truly reflect regulation by

cortisol remains unclear. Both salivary cortisol and sIgA antibodies are subject to circadian regulation, although their patterns of expression are opposing and thus unlikely to result in the observed longitudinal associations. It is likely these associated longitudinal increases in reflect the normal development and maturation of immune and stress responses in young children.

4.4 Conclusion

Work reported here demonstrates that it is extremely challenging to follow-up children of this age and in this setting. However, by the methods employed here, variables hypothesised to influence dental caries were obtained and successfully measured in young children. These studies were performed during the early development of the Childsmile programme, which has now expanded to become part of a national dental service for all children in Scotland. Thus, provided lessons are learned from the recruitment methods used here, the potential to perform large-scale clinical studies within the Childsmile programme has increased. In particular, dental practice participation will likely be crucial to recruit substantial numbers of very young children from within the Childsmile programme. Additionally, given the demographics, domiciliary visits proved successful for the recruitment of 'hard to reach' children. Alternatively, the collection of samples from three-year-old children undergoing national dental inspections within nurseries could provide a useful opportunity for sample collection and would allow access to a large cross-section of children from across the socioeconomic spectrum. Thus, Childsmile's young target age group and multiple visit interventions could provide a valuable platform from which to perform large-scale longitudinal clinical studies with enormous potential related to both systemic and oral diseases.

The frequency of detection and relative proportion of *S. mutans* in dental plaque increased over time. Coincidentally, the salivary immune response continued to mature and develop as indicated by increases in both innate and adaptive immune mediators. Data reported here suggest that increased concentrations of the HNPs 1-3 and LL37 may be partly influenced by increases in the proportion of *S. mutans* in dental plaque and low concentrations of these antimicrobial proteins in saliva may be a risk factor for caries development. These findings

warrant further investigation in a larger study, particularly in relation to dental disease scores. The role of sIgA antibodies in limiting the accumulation of cariogenic bacteria in dental plaque remains unclear. Assessment of sIgA antibodies to whole bacterial cells likely includes the detection of and is confounded by the presence of cross-reactive antibodies of low-specificity. Studies investigating sIgA antibody responses to specific *S. mutans* epitopes involved with their adherence and persistence within the oral biofilm may have more merit. Moreover, future studies should ideally collect samples at specified times and the time since tooth-brushing should be known. The dental data reported here were compromised by a high drop out rate and obvious recall bias. However, the limited data available support previous reports that caries development is associated with high levels of *S. mutans* in dental plaque at one-year of age. Given recent evidence from oral metagenomic studies indicating the importance of *S. mutans* in the initiation of white spot enamel lesions it may be more important for future studies to accurately measure caries initiation, since by the time caries enters into dentine the aetiology of the disease changes to become less mineral and more tissue dependent. Thus, factors such as the concentrations of antimicrobial proteins may have limited protective effect once the disease is established. Future studies of the oral metagenome and transcriptome in young children are required to identify the important processes involved with disease initiation. There was insufficient evidence to indicate whether salivary cortisol concentrations were associated with socioeconomic status or oral biofilm development. Future studies should ideally aim to assess basal cortisol levels from children across the socioeconomic spectrum.

Chapter 5: The adaptive immune response to *Streptococcus mutans*

5.1 Introduction

The adaptive immune response can offer protection against colonisation by *S. mutans* and ultimately against the development of dental caries. Transient protection against *S. mutans* is offered by topically administered anti-*S. mutans* monoclonal antibodies (Ma et al. 1987). Active immunisation stimulates protective IgA production in response to streptococcal protein antigens and confers protection in animal models (Culshaw et al. 2007; Taubman and Nash 2006) Moreover, greater peripheral blood mononuclear cell proliferation and higher levels of sIgA were observed in caries resistant compared to susceptible individuals, suggesting protective immunity may be generated (Hocini et al. 1993; Parkash et al. 1993). These studies remain somewhat controversial and the mechanisms driving this apparent protective immunity are unknown. The potential exists for individuals to raise an antibody response against *S. mut*ans. However, to date, this has proved challenging to effectively manipulate. Rationale design of therapeutic strategies may be aided by further characterisation of the early interactions between the host immune system and *S. mutans* that are prerequisite to protective antibody generation.

Dendritic cells (DCs) are critical in the initiation of pathogen-specific adaptive immune responses through T cell activation (Banchereau and Steinman 1998). Immature DCs residing within peripheral tissues, are highly endocytic and express low levels of major histocompatability complex class-II (MHCII) and co-stimulatory molecules. Upon antigen encounter, DCs up-regulate MHCII and co-stimulatory molecules and migrate to secondary lymphoid tissue, a process known as DC maturation. Once arriving in the lymph node mature DCs present antigen in the context of MHCII to T helper cells. Antigen-specific T helper responses culminate in effector responses and subsequent B cell activation and specific antibody production (Figure 5-1). Thus, DCs play an important role bridging innate and adaptive immune responses and represent a critical step in the induction of protective immunity. Crucially, following DC activation decisions regarding the nature of the immune response are made rapidly (Itano et al. 2003). Co-stimulatory signals and the cytokine environment elicited by activated DCs at the time of T cell polarisation have a powerful influence on the nature of T cell proliferation and effector functions (O'Garra 1998).

In the current study a novel system was employed to dissect the early immune response in the context of *S. mutans* infection (Ravindran et al. 2007; Rush et al. 2009). The peptide designated Eα was expressed together with green fluorescent protein (GFP) and recombinant EαGFP was created. Uptake of exogenous EαGFP and subsequent antigen processing and presentation by DCs results in the expression of Eα-MHCII peptide complexes on the surface of activated DCs. The monoclonal antibody recognises Eα-MHCII peptide complexes and can thus be used to detect and quantify antigen presentation in the context of *S. mutans* infection (Figure 5-2). Furthermore, transgenic TCR TEa T cells also recognise Eα-MHCII peptide complexes and can be used to detect T cell responses to presented antigen (Figure 5-2).

In an attempt to detect native bacterial antigen presentation by DCs, attempts were made to insert the EαGFP peptide construct into the Streptococcal plasmid pAYBG854S (Yoshida and Kuramitsu 2002) and to transfect this plasmid vector into S. *mutans*. However, after repeated attempts the expression of EαGFP could not be stably maintained in *S. mutans*. Thus, exogenous EαGFP in conjuction with *S. mutans* was used in the current studies. Using this system the key initial stages of the adaptive immune response following *S. mutans* exposure were characterised.

These studies aimed to characterise the *In vitro* and *In vivo* phenotype of DCs in response to *S. mutans*.

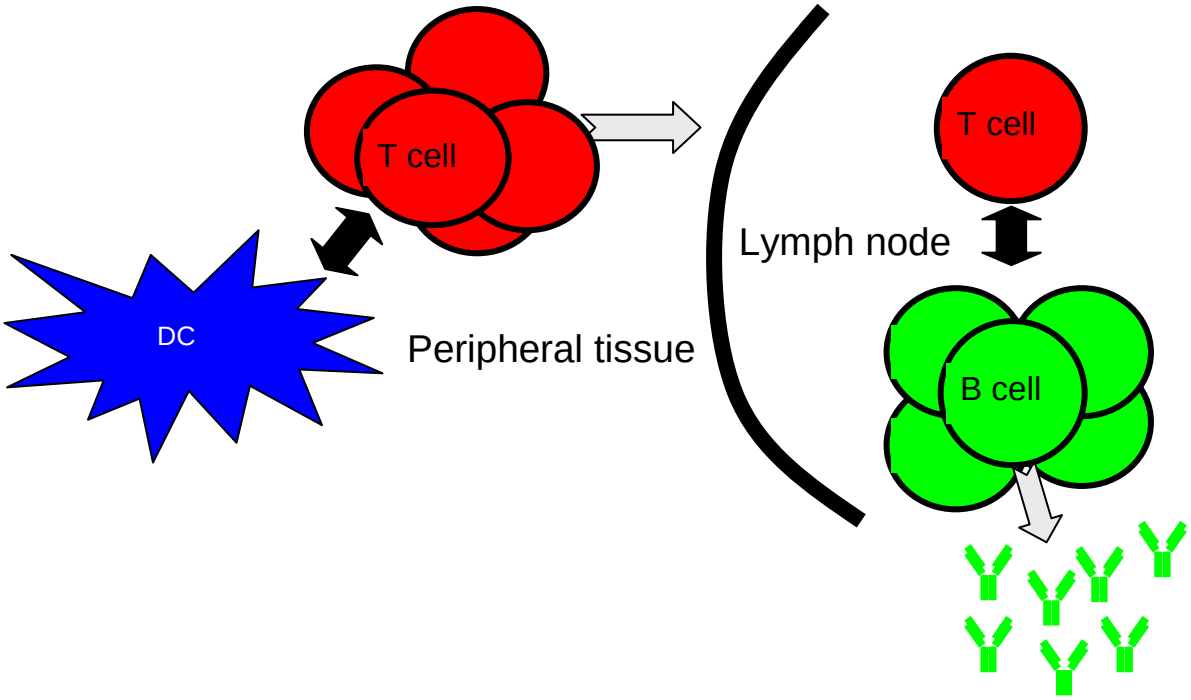


Figure 5-1: Schematic overview of the adaptive immune response

Dendritic cells (DCs) are the major antigen presenting cells. They capture antigen in peripheral tissues which promotes their maturation via induction of MHCII and co-stimulatory molecules and migration to secondary lymphoid tissues where they present antigen to naïve CD4 T cells. Upon recognition of their cognate antigen CD4 T cells proliferate and migrate to effector sites. Some activated T cells migrate to the B cell compartment where they interact with and provide help to B cells, promoting affinity maturation and antibody production.

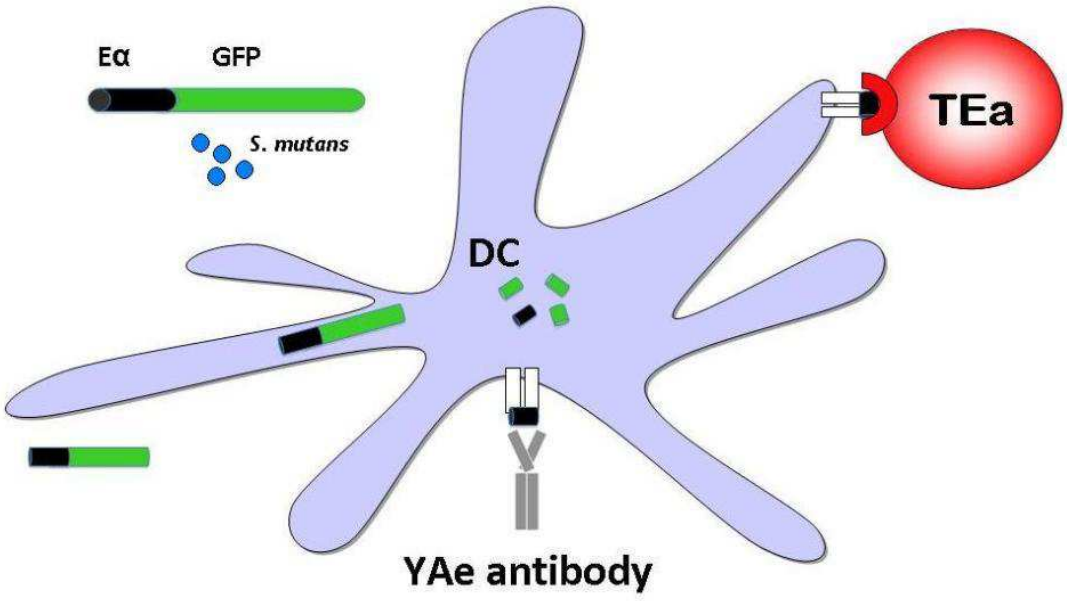


Figure 5-2: Schematic of the EaGFP system

A peptide designated 'Eα' was expressed with green fluorescence protein (GFP) and recombinant EαGFP was created. Eα peptide binds I-A^b MHC class II. The MHC class II-Eα peptide complex is recognised by the monoclonal antibody YAe, allowing for detection and quantification of antigen presentation. The T cell response to DC antigen presentation was investigated using C57BL/6 mice expressing the T cell receptor transgenic TEa T cell, which also recognises the MHC class II-Eα peptide complex. Thus, addition of exogenous EαGFP to *S. mutans* and DC co-cultures allows for detection of antigen presentation and subsequent incubation of peptide pulsed DCs with TEa T cells allows for quantitation of T cell proliferation in the context of *S. mutans* infection.

5.2 Results

5.2.1 In vitro dendritic cell activation and maturation following exposure to S. mutans

The initiation of an adaptive immune response to *S. mutans* was first investigated by assessing the ability of bone-marrow-derived DCs (BMDCs) to phagocytose *S. mutans*. After 4 h co-culture, chains of green fluorescent *S. mutans* were visible within the BMDC (Figure 5-3a). Following 24 h exposure a significant accumulation of *S. mutans* were visible within the BMDC (Figure 5-3b). The images shown were kindly provided by Dr John Butcher.

To elucidate the effects of phagocytosis of *S. mutans* on DC activation, BMDCs were co-cultured with live or heat-killed (HK) *S. mutans*, stained with antibodies specific for cell surface receptors and analysed by flow cytometry. It was observed that a ratio of 1 DC to 100 HK *S. mutans* stimulated optimal activation. Excessive bacterial proliferation in the culture medium starved the DC of nutrients, resulting in a statistically significant reduction in DC viability. It was observed that a reduced ratio of 1 DC to 10 live *S. mutans* allowed the bacteria to proliferate and ensured the survival of the DC (Figure 5-4). Thus, heat-killed *S. mutans* was co-cultured with DCs at a ratio of 100:1 and live *S. mutans* at a ratio of 10:1 for all subsequent investigations.

Cell surface expression of co-stimulatory molecules: CD40, CD80, CD86 and MHC class II by CD11c positive cells in response to *S. mutans* was assessed by the mean fluorescent intensity (MFI). There was a general trend to up-regulation of co-stimulatory molecules. The intensity of CD40 staining increased after 4 h and by 24 h the intensity increased 10-fold in response to both live and heat-killed *S. mutans*. At 4 and 24 h there was a three-fold increase in CD80 staining and increased CD86 staining. MHC class II staining was only modestly increased following 24 h exposure to both heat-killed and live *S. mutans* (Figure 5-5a). Representative histograms demonstrating minimal background staining and increased MFI of co-stimulatory molecules are shown (Figure 5-5b).

The percentage of CD11c positive cells expressing co-stimulatory molecules was also assessed to determine whether the proportion of cells expressing co-stimulatory molecules was altered in response to co-culture with *S. mutans* (Figure 5-5c). The percentage of cells expressing CD40, CD80 and CD86 increased following exposure to *S. mutans*, although this did not reach statistical significance. Commensurate with the constitutively high expression of MHC class II on BMDCs, the percentage of CD11c positive cells expressing MHC class II remained unchanged in response to *S. mutans* exposure.

Taken together this data demonstrate that BMDCs become activated and undergo maturation as indicated by the up-regulation of co-stimulatory molecules in response to the phagocytosis of *S. mutans In vitro*.

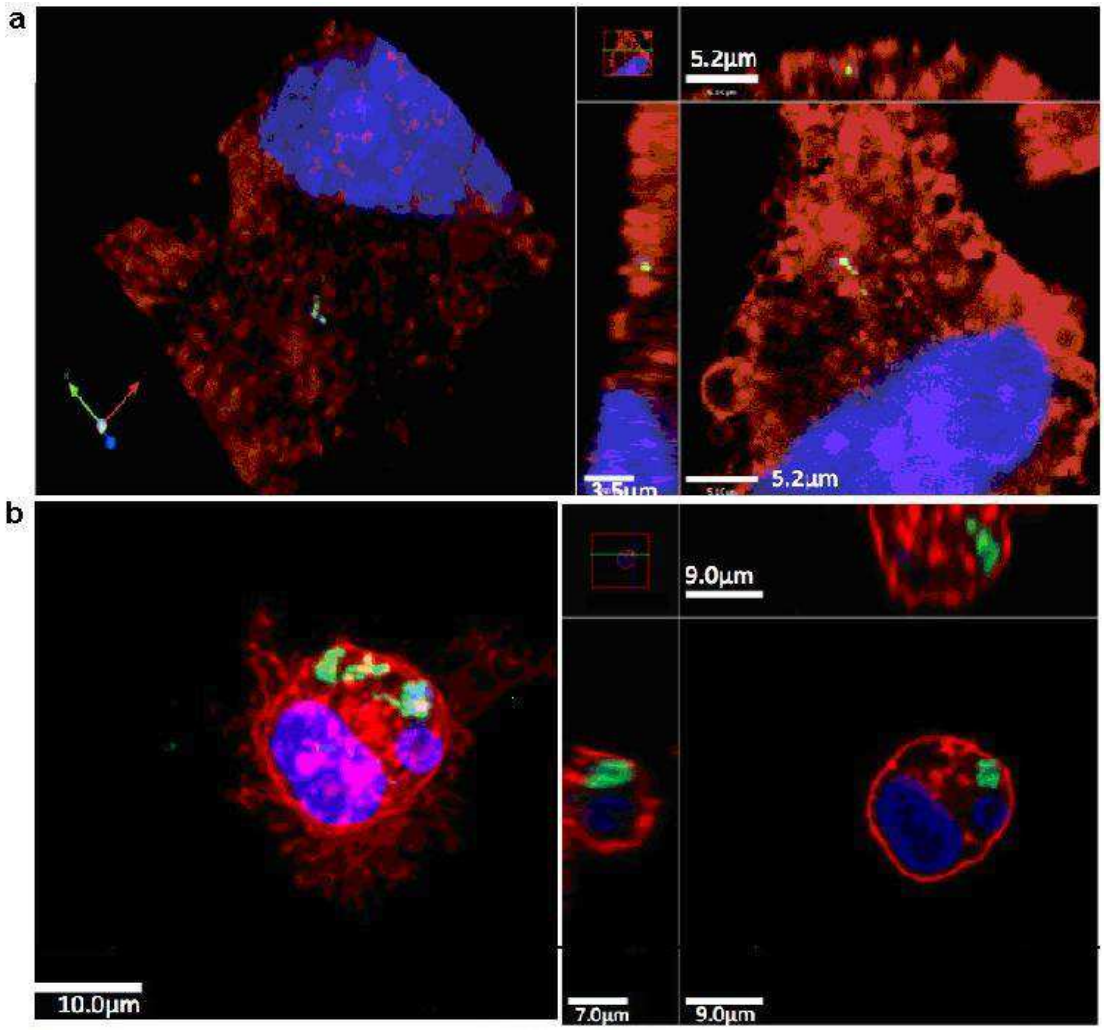


Figure 5-3: Phagocytosis of *S. mutans*-activated dendritic cells

Bone marrow derived DCs (BMDCs) were generated from C57BL/6 mice and co-cultured with green fluorescent *S. mutans* (pDM15). DC membranes were stained with Alexa-fluor 647 (red), and nuclei stained with DAPI (blue). *S. mutans* are visible within BMDCs after (a) 4 h and (b) 24 h of co-incubation. Inset panels demonstrate views of x, y and z axis, confirming the intracellular location of *S. mutans* (630x magnification, 7x digital zoom). Images courtesy of Dr John Butcher.

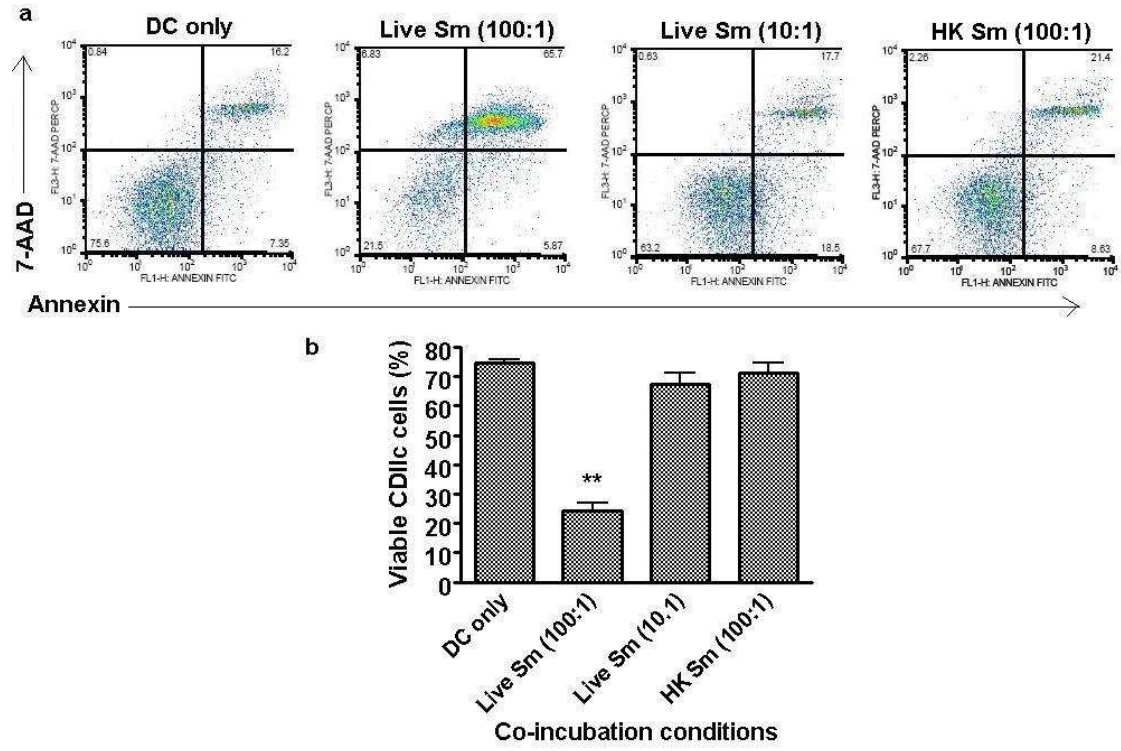


Figure 5-4: DC viability following *S. mutans* co-culture

Seven day old BMDCs were co-cultured with media only control, live *S. mutans* UA159 (Sm) at a ratio of 100 or 10 bacterial cells per DC or heat-killed (HK) *S. mutans* at a ratio of 100 bacterial cells per DC for 18 h. Cells were harvested and stained with anti-CD11c and analysed for incorporation of Annexin V (FITC) and 7-AAD by flow cytometry. (a) Representative flow cytometric dot plots showing the proportion of CD11c cells positive for annexin V and 7-AAD. (b) Bar chart showing the percentage of viable (7AAD negative, annexin V negative and CD11c positive) cells. Data are mean and SEM of a single experiment performed in duplicate and analysed by ANOVA (** $p < 0.01$ compared with DC only media control).

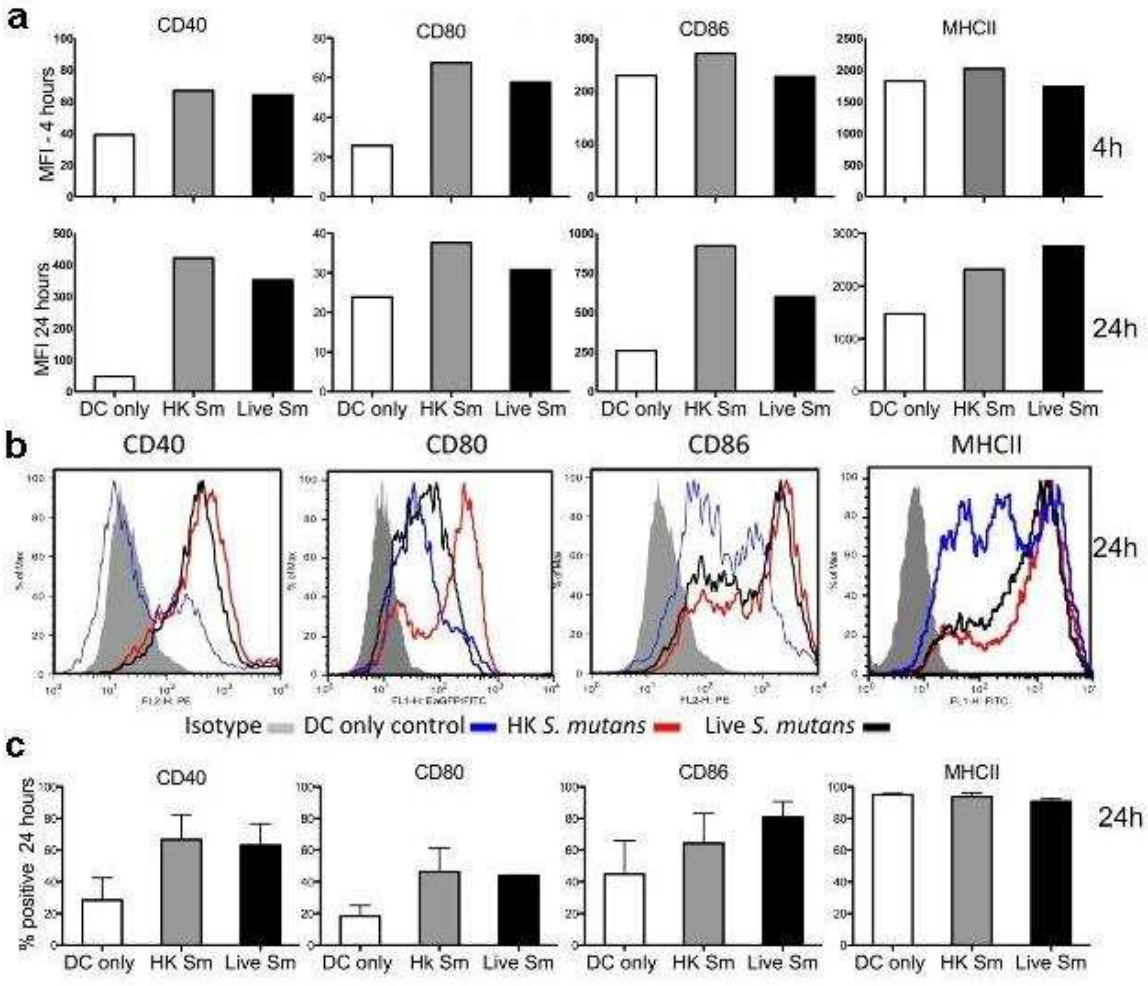


Figure 5-5: Up-regulation of co-stimulatory molecules by DCs in response to *S. mutans*

BMDCs were co-cultured with either live or heat-killed *S. mutans* UA159 for 4 and 24 h, then stained with CD11c and CD40, CD80, CD86 or MHC II specific antibodies and analysed by flow cytometry. (a) Mean fluorescent intensity (MFI) of BMDCs after 4 or 24 h of co-culture. Data are representative of three independent experiments. (b) Representative histograms of cell surface staining of CD11c positive cells, as in (a) demonstrating minimal background staining. (c) Percentage of BMDCs stained positive for co-stimulatory molecules after 24 h of co-culture. Data are mean and SEM of three independent experiments.

5.2.2 In vitro antigen presentation by dendritic cells following exposure to S. mutans

Antigen presentation by BMDCs co-cultured with live or heat-killed *S. mutans* was investigated using the EαGFP system (Figure 5-2). The addition of exogenous EαGFP to the co-culture system allowed for the detection and quantification of antigen presentation in the context of *S. mutans* infection using the monoclonal antibody, Y-Ae, which recognises MHCII-Eα peptide complexes. There was an increase in the cell surface expression of MHCII-Eα complexes as indicated by an increase in the MFI of Y-Ae staining when BMDCs were cultured with exogenous EαGFP. This was further increased in response to simultaneous stimulation with heat-killed or live *S. mutans* (Figure 5-6b). There was a statistically significant increase in the percentage of CD11c positive cells expressing MHCII-Eα complexes when BMDCs were cultured with exogenous EαGFP alone (27%) compared with the media only control (6%). The percentage of CD11c positive cells expressing cell surface MHCII-Eα complexes was further increased by the addition of heat-killed *S. mutans* (39%) or live *S. mutans* (31%). In each case the increased percentage of CD11c positive cells expressing cell surface MHCII-Eα complexes was statistically significant compared with the appropriate negative control (i.e. without EαGFP). The addition of heat-killed *S. mutans* together with exogenous EαGFP modestly increased the percentage of cells presenting MHCII-Eα complexes, which reached only borderline statistical significance ($p = 0.051$, by Student's *t* test) compared with EαGFP alone. Addition of live *S. mutans* together with exogenous EαGFP also increased the percentage of CD11c positive cells expressing MHCII-Eα complexes but this did not reach statistical significance compared with EαGFP alone (Figure 5-6c). Representative flow cytometric plots demonstrate the gated populations for each condition and the isotype controls confirm minimal background staining (Figure 5-6a). Thus, these data demonstrate that in addition to the ability to become activated, BMDCs are capable of processing and presenting antigen in the context of *S. mutans* infection *In vitro*.

As a specificity control, antigen presentation by BMDCs co-cultured with live or heat-killed *E. coli* was investigated using the EαGFP system. Cell surface expression of MHCII-Eα peptide complexes was increased by BMDCs co-cultured

with live *E. coli* and exogenous EαGFP simultaneously compared with heat-killed *E. coli* and EαGFP or exogenous EαGFP alone (Figure 5-7b). In agreement with previous data, a statistically significant increase in the percentage of CD11c positive cells presenting MHCII-Eα peptide complexes was observed when BMDCs were co-cultured with exogenous EαGFP alone (40%) compared with the media control (5% [Figure 5-7c]). The percentage of CD11c positive cells presenting MHCII-Eα peptide complexes was statistically significantly increased when BMDCs were co-cultured simultaneously with live *E. coli* and exogenous EαGFP (38%) compared with live *E.coli* only (2%). The percentage CD11c positive cells presenting MHCII-Eα peptide complexes increased when BMDCs were co-cultured simultaneously with heat-killed *E.coli* and EαGFP (43%) compared with heat-killed *E.coli* alone (13%), although the difference did not reach statistical significance. Representative flow cytometric plots demonstrate the gated populations for each condition and the isotype controls confirming minimal background staining are shown (Figure 5-7a). Together these data confirm the EαGFP system is a useful model to investigate antigen presentation in the context of bacterial infection. Moreover, antigen presentation was not compromised by the presence of either *S. mutans* or *E. coli*.

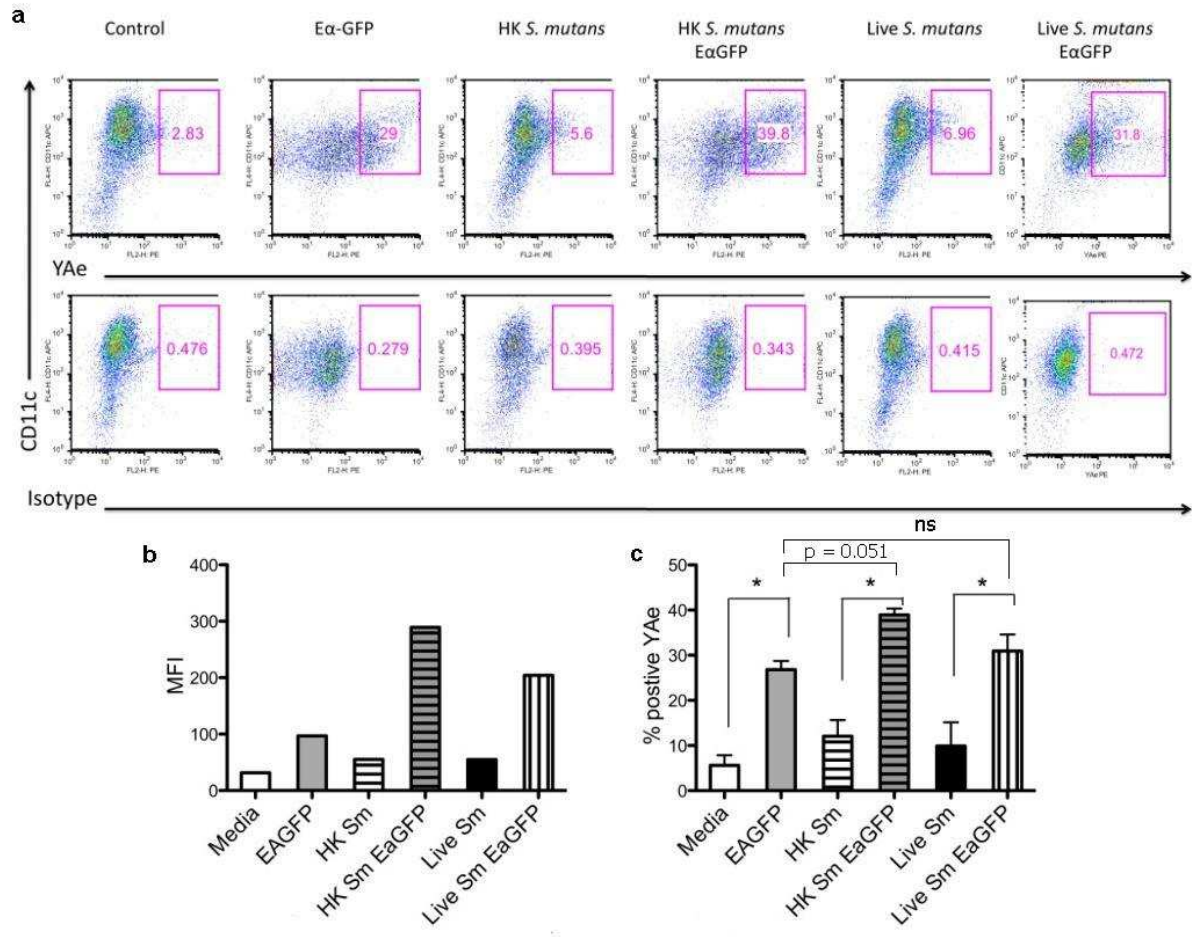


Figure 5-6: Antigen presentation by BMDCs cultured with live *S. mutans*

BMDCs were cultured with exogenous EαGFP alone, heat-killed or live *S. mutans* UA159 with and without exogenous EαGFP for 18 h. Cells were then harvested and stained with CD11c and Y-Ae specific antibodies. (a) Representative flow cytometric scatter plots showing the proportion of cells positive for CD11c and Y-Ae. Isotype controls demonstrating minimal background staining are also shown. (b) MFI of Y-Ae staining representative of three independent experiments. (c) Percentage of Y-Ae positive CD11c positive cells; data are mean and SEM from three independent experiments. (* $p < 0.05$, $p = 0.051$ and ns: not significant by Student's *t* test compared with indicated control).

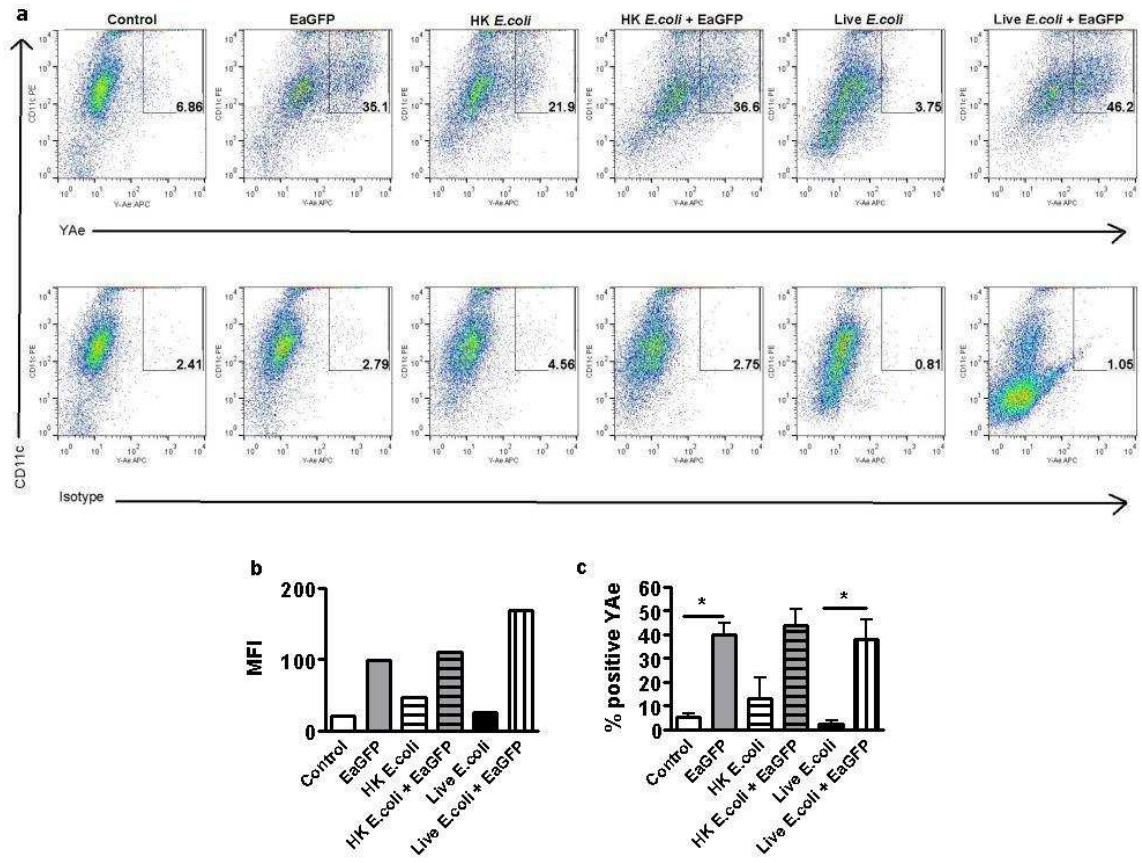


Figure 5-7: Antigen presentation by BMDCs cultured with live *E. coli*

BMDCs were cultured with exogenous EαGFP alone, heat-killed or live *E. coli* DH5A with and without exogenous EαGFP for 18 h. Cells were then harvested and stained with CD11c and Y-Ae specific antibodies. (a) Representative flow cytometric scatter plots showing the proportion of cells positive for CD11c and Y-Ae. Isotype controls demonstrating minimal background staining are also shown. (b) MFI of Y-Ae staining representative of two independent experiments. (c) Percentage of Y-Ae positive CD11c positive cells; data are mean and SEM from two independent experiments. (* $p < 0.05$ by Student's *t* test compared with indicated control).

5.2.3 In vitro cytokine secretion by dendritic cells following exposure to S. mutans

Culture supernatants from BMDCs exposed to live or heat-killed *S. mutans* were assessed for the presence of cytokines IL-10, IL-12 and the chemokine CCL20. BMDCs co-cultured with live or heat-killed *S. mutans* expressed greater concentrations of IL-10, IL-12 and the chemokine CCL20 compared with the media only control (Figure 5-8). The difference reached statistical significance only for CCL20 ($p < 0.05$). The concentration of IL-10 was higher (900 vs 355 pg/ml), while IL-12 was lower (19.6 vs 35.9 ng/ml) in the supernatants of BMDCs co-cultured with live *S. mutans* compared with heat-killed *S. mutans*, although these differences did not reach statistical significance. There was however, a statistically significant reduction in the concentration of CCL20 in the supernatant from BMDCs co-cultured with live *S. mutans* compared with heat-killed *S. mutans* (52.6 vs 127.4 pg/ml, $p < 0.05$).

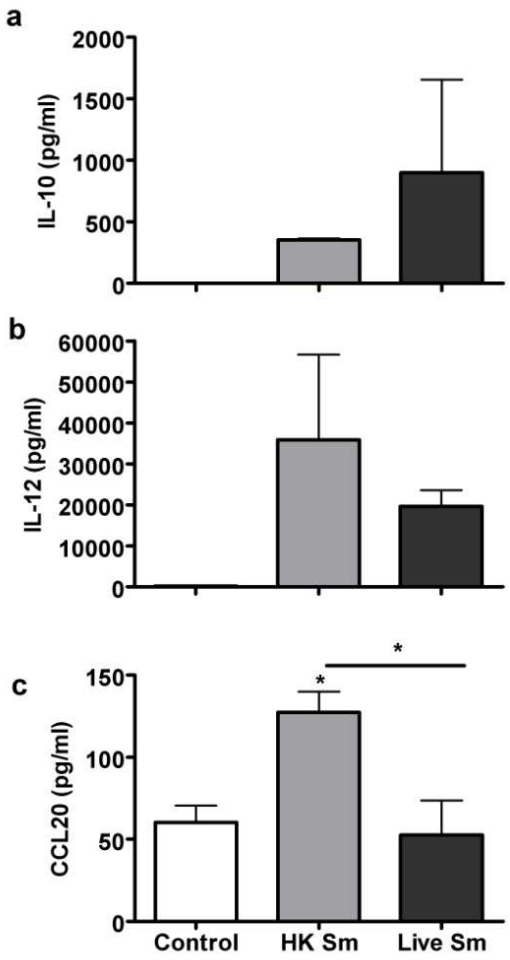


Figure 5-8: Cytokine secretion by BMDCs in response to live *S. mutans*

BMDCs were cultured with media only, heat-killed and live *S. mutans* UA159 for 18 h. Culture supernatants were collected and investigated for concentrations (pg/ml) of secreted (a) IL-10, (b) IL-12 and (c) CCL20 by Luminex^{TM}. Data are mean and SEM from two individual experiments. (* $p < 0.05$ by Tukey comparison vs control).

5.2.4 In vitro T cell proliferation by dendritic cells following exposure to S. mutans

In order to elucidate the functional impact of these observations, T cell receptor transgenic TEa T cells specific for MHCII-Eα peptide complexes were co-cultured with BMDCs previously pulsed with antigen as previously described. Subsequent T cell proliferation was measured by incorporation and detection of EdU (5-ethynyl-2'-deoxyuridine) into the DNA of proliferating cells. Representative flow cytometric scatter plots of gated populations of proliferating CD4 positive cells are shown (Figure 5-9a). There was statistically significant ($p < 0.001$ by Student's *t* test) antigen-specific T cell proliferation in response to BMDCs exposed to exogenous EαGFP or heat-killed *S. mutans* together with exogenous EαGFP. Interestingly, antigen-specific T cell proliferation did not occur in response to live *S. mutans* with exogenous EαGFP (Figure 5-9).

To confirm that T cell proliferation in response to live *S. mutans* had not occurred prior to the incorporation of EdU, the experiment was repeated at an earlier time point. At this time, TEa mice were unavailable. T cell receptor transgenic OT-II C57BL/6 mice contain CD4+ T cells that express a TCR specific for chicken $OVA_{323-339}$ peptide bound to the MHCII molecule $I\text{-}A^b$ (Barnden et al. 1998). Thus, to further investigate T cell proliferation in the context of *S. mutans* infection, BMDCs (derived from C57BL/6 mice) were co-cultured with OVA peptide under the same activation conditions as previously described, and subsequent T cell proliferation was quantified by EdU incorporation. Representative flow cytometric contour plots are shown (Figure 5-10a). There was statistically significant antigen-specific T cell proliferation in response to BMDCs pulsed with heat-killed *S. mutans* together with OVA peptide (39%) compared with heat-killed *S. mutans* only (2%, [$p < 0.001$, Figure 5-10b]). The percentage of antigen-specific proliferating T cells at this earlier time point was greater than following 96 h co-culture with antigen pulsed BMDCs. However, even at this earlier time point there was no antigen-specific T cell proliferation in response to live *S. mutans* with exogenous OVA peptide.

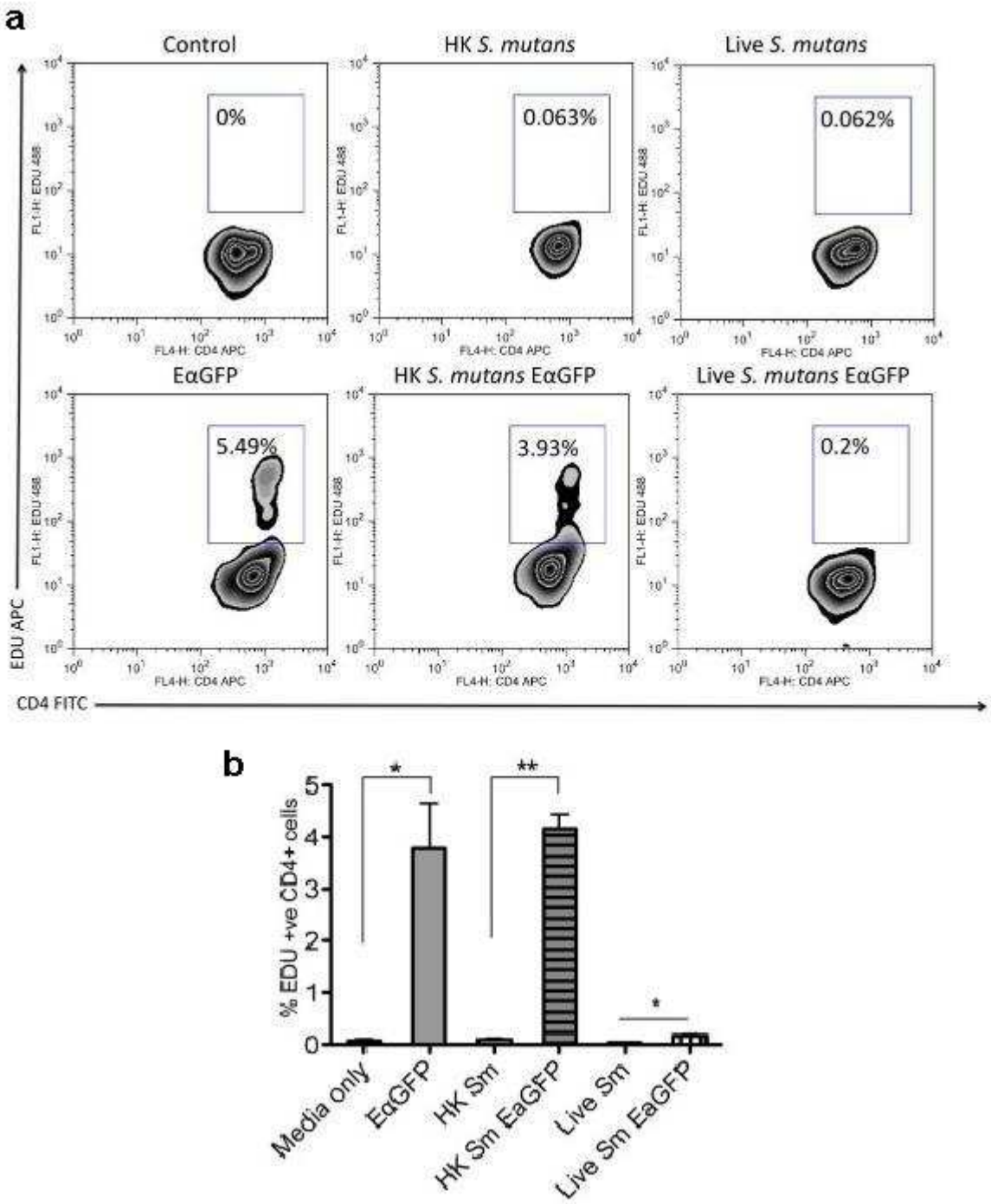


Figure 5-9: *In vitro* T cell proliferation following exposure to live *S. mutans*

BMDCs derived from C57BL/6 mice were co-cultured with exogenous EαGFP alone, heat-killed or live *S. mutans* with and without exogenous EαGFP for 18 h. BMDCs were harvested, washed and co-cultured with TEa T cells *in vitro*. T cell proliferation was analysed by flow cytometry after 96 h, staining for CD4 and EdU incorporation. (a) Representative flow cytometric contour plots demonstrating EdU incorporation into proliferating CD4 positive cells. (b) Percentage of proliferating CD4 positive cells following challenge. Data shown are mean and SEM (* $p < 0.05$, ** $p < 0.005$ by Student's t test compared with indicated control).

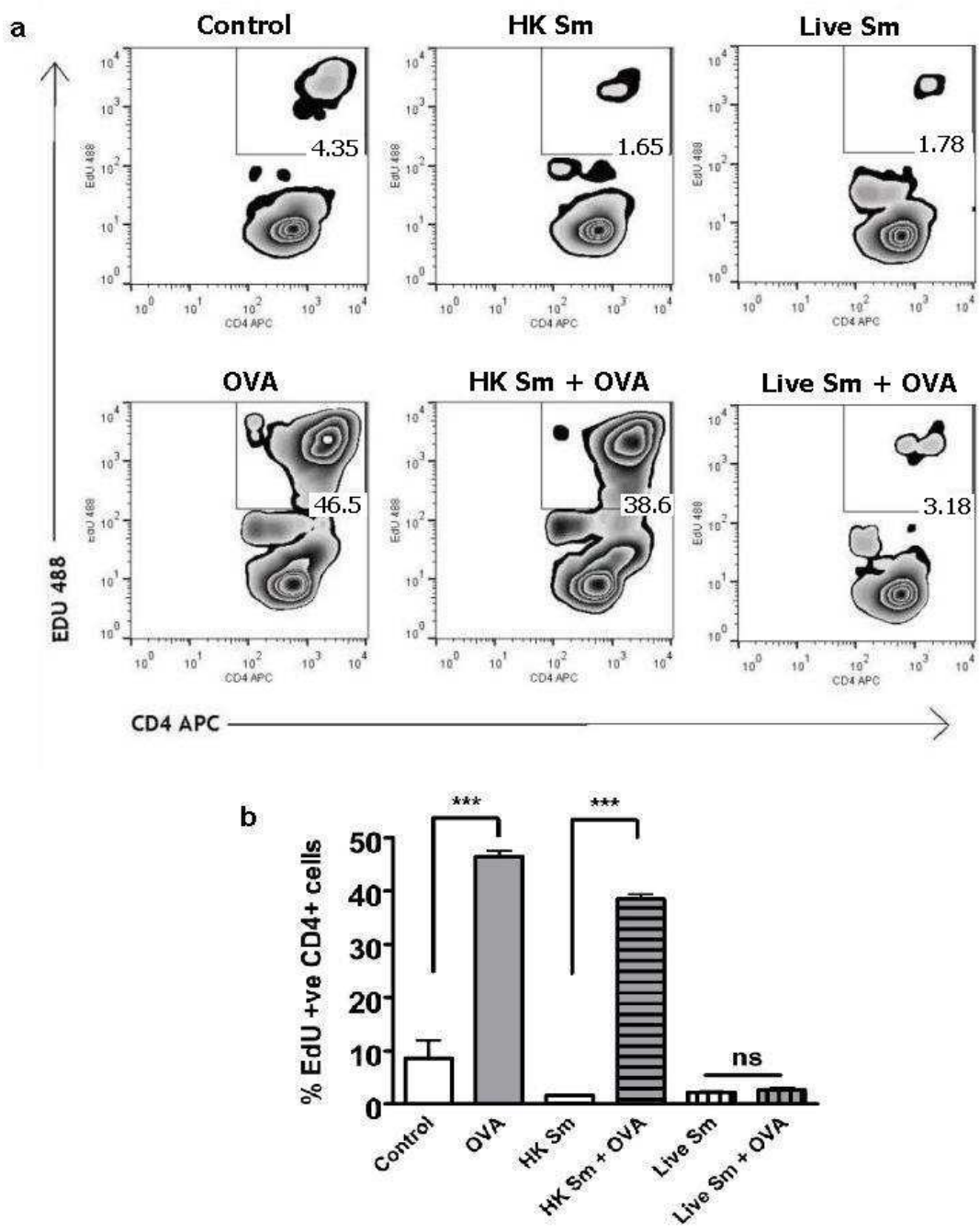


Figure 5-10: *In vitro* T cell proliferation following exposure to live *S. mutans* at an earlier time point

BMDCs derived from C57BL/6 mice were co-cultured with exogenous OVA peptide alone, heat-killed or live *S. mutans* with and without exogenous OVA peptide for 18 h. BMDCs were harvested, washed and co-cultured with TEa T cells *in vitro*. T cell proliferation was analysed after 72 h by flow cytometric staining for CD4 and EdU incorporation. (a) Representative flow cytometric contour plots demonstrating EdU incorporation into proliferating CD4 positive cells. (b) Percentage of proliferating CD4 positive cells following challenge. Data shown are mean and SEM of a single experiment performed in triplicate (*** $p < 0.001$ by Student's t test compared with indicated control).

5.2.5 In vitro cytokine secretion by DC/T cell co-culture following S. mutans exposure

Culture supernatants from BMDCs and T cell co-cultures were assessed for cytokines released in response to exposure to live or heat-killed *S. mutans* (Figure 5-11). The concentrations of IL-2, IL-10, IL-12, IFN-γ and IL-17 were significantly increased in the culture supernatants in response to exposure to heat-killed *S. mutans* compared with the media only controls. The concentrations of IL-2, IL-12, IFN-γ and IL-17 were significantly decreased in the culture supernatants in response to live *S. mutans* compared with heat-killed *S. mutans* exposure. The concentration of IL-10 was significantly increased in the culture supernatants in response to live *S. mutans* compared with the media only control (1648 pg/ml vs not detected, $p < 0.001$) and was also found to be significantly higher when compared with exposure to heat-killed *S. mutans* (380 pg/ml, $p< 0.001$ [Figure 5-11b]). There was no difference in the concentrations of the chemokine CCL20 in the culture supernatants following exposure to heat-killed or live *S. mutans*. These data indicate that cytokine profiles elicited by BMDC/T cell co-cultures in response to heat-killed or live *S. mutans* differ significantly. The cytokine milieu at the time of T cell polarisation is important for the determination of T cell effector function and thus, likely influenced the abrogated T cell response following live *S. mutans* exposure described earlier.

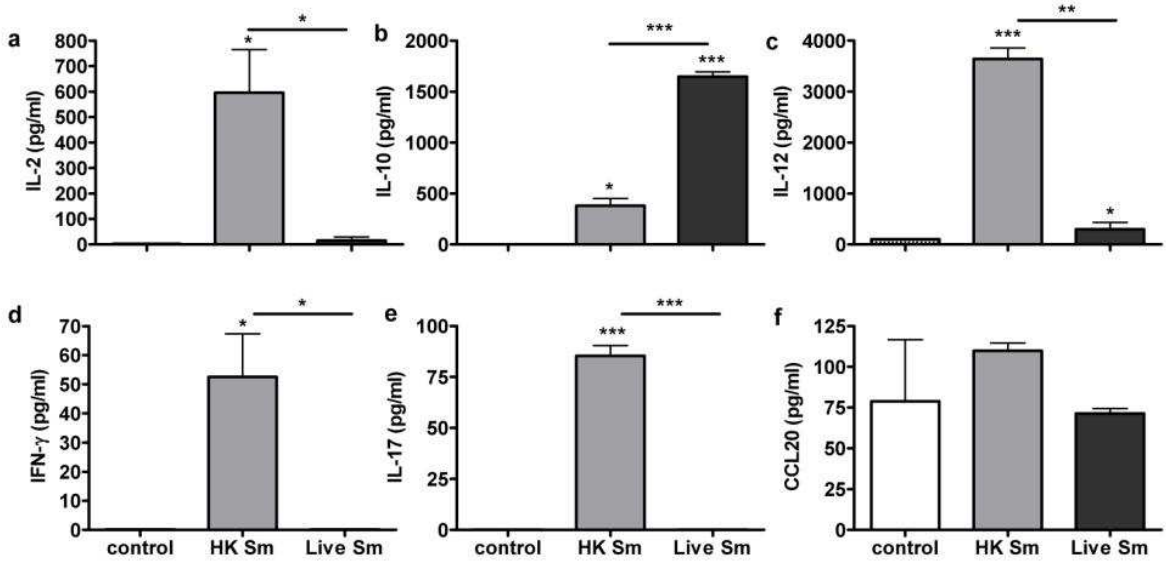


Figure 5-11: Cytokine secretion by DC/T cell co-cultures in response to live *S. mutans* exposure

BMDC/T cell co-cultures were exposed to media only, heat-killed or live *S. mutans* UA159 for 72 h. Culture supernatants were collected and investigated for concentrations (pg/ml) of secreted (a) IL-2, (b) IL-10 and (c) IL-12 (d) IFN-γ, (e) IL-17 and (f) CCL20 by Luminex^{TM}. Data are mean and SEM from two individual experiments each measured in triplicate. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Tukey comparison vs media only control or indicated group).

5.2.6 In vivo dendritic cell recruitment in response to S. mutans

The effect of oral exposure to *S. mutans* on dendritic cell recruitment *in vivo* was investigated. Cells from the draining lymph nodes (DLNs) of the oral cavity were collected 6, 24 and 48 h following intra-oral exposure to *S. mutans* or carrier only control. R848, an imidazoquinoline compound that activates DCs via TLR7 (Chaperot et al. 2006) was administered orally as a positive control. Total numbers of cells in the DLN increased only modestly in animals 6 and 24 h following oral exposure to *S. mutans*, compared with the carrier-only control (Figure 5-12a and b, respectively). By 48 h following oral exposure total cell numbers decreased and were equivalent to numbers in the carrier control group (Figure 5-12c). There were no differences in the numbers or percentage of CD11c positive cells recruited to the DLN of animals 6, 24 or 48 h following oral challenge with *S. mutans* (Figure 5-12d-i). In contrast, total cell numbers in the DLN of R848-treated animals (1.9×10^6 cells) markedly increased 24 h post-treatment compared with the carrier-control (6.8×10^5 cells, $p < 0.05$ [Figure 5-12b). This was partly mediated by a statistically significant increase in the number of CD11c positive cells recruited to the DLN (1.2×10^5 cells) compared with the carrier control (4.4×10^4 cells, $p < 0.05$ [Figure 5-12e). However, the percentage of CD11c positive cells 24 h following R848 was no different compared with the carrier control (Figure 5-12h) suggesting the proportion of CD11c negative cells also increased. By 48 h, R848-treated animals showed a statistically significant decrease in the percentage of CD11c positive cells in the DLN compared with the carrier control ($p < 0.05$ [Figure 5-12i]).

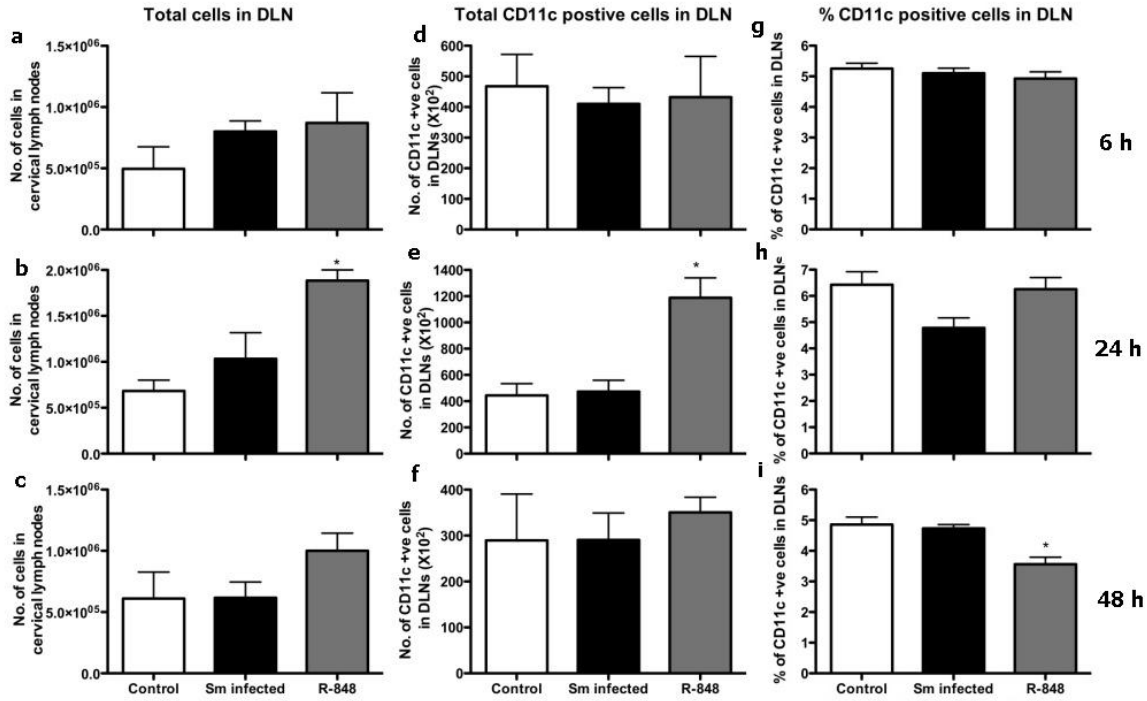


Figure 5-12: Dendritic cell recruitment to the DLN in response to oral exposure to *S. mutans*

C57Bl/6 mice were orally exposed to *S. mutans* UA159. Cervical lymph nodes were harvested 6, 24, and 48 h following oral exposure to carrier-only control, *S. mutans* UA159 or R848. Single cell suspensions were prepared and strained for CD11c and analysed by flow cytometry. (a-c) Total number of cells in cervical lymph nodes. (d-f) Total number of CD11c+ cells in the DLN. (g-i) Percentage of CD11c positive cells in the DLN. Data shown are mean ± SEM of data from two independent experiments (24 h), or a single experiment (6 h and 48 h). In each experiment, n = 3 mice per group. * $p < 0.05$ vs. control by Turkey comparison.

5.2.7 In vivo dendritic cell activation in response to S. mutans

CD11c positive cells from the DLNs of animals orally challenged with *S. mutans* showed no difference in the expression of co-stimulatory molecules (CD40, CD80 and CD86) or MHCII compared with carrier-only control animals at any time point (Figure 5-13a). Additionally, the proportion of CD11c positive cells expressing co-stimulatory molecules also remained unchanged in *S. mutans* exposed animals at all time points, with the exception of the proportion of CD11c positive cells expressing CD40 which was significantly higher 24 h following oral exposure compared with control animals ($p < 0.05$, Figure 5-13b). Cell surface expression of co-stimulatory molecules by CD11c positive cells from the DLNs of R848 challenged animals was statistically significantly increased at 6 and 24 h compared with control animals (Figure 5-13a). Furthermore, the proportion of CD11c cells stained positive for CD80 at 6 h and CD40 and CD86 24 h following oral exposure to R848 were statistically significantly higher compared with control animals (Figure 5-13b). Representative histograms of CD11c positive cells expressing co-stimulatory and MHCII molecules with minimal background staining at 24 h following oral exposure are shown.

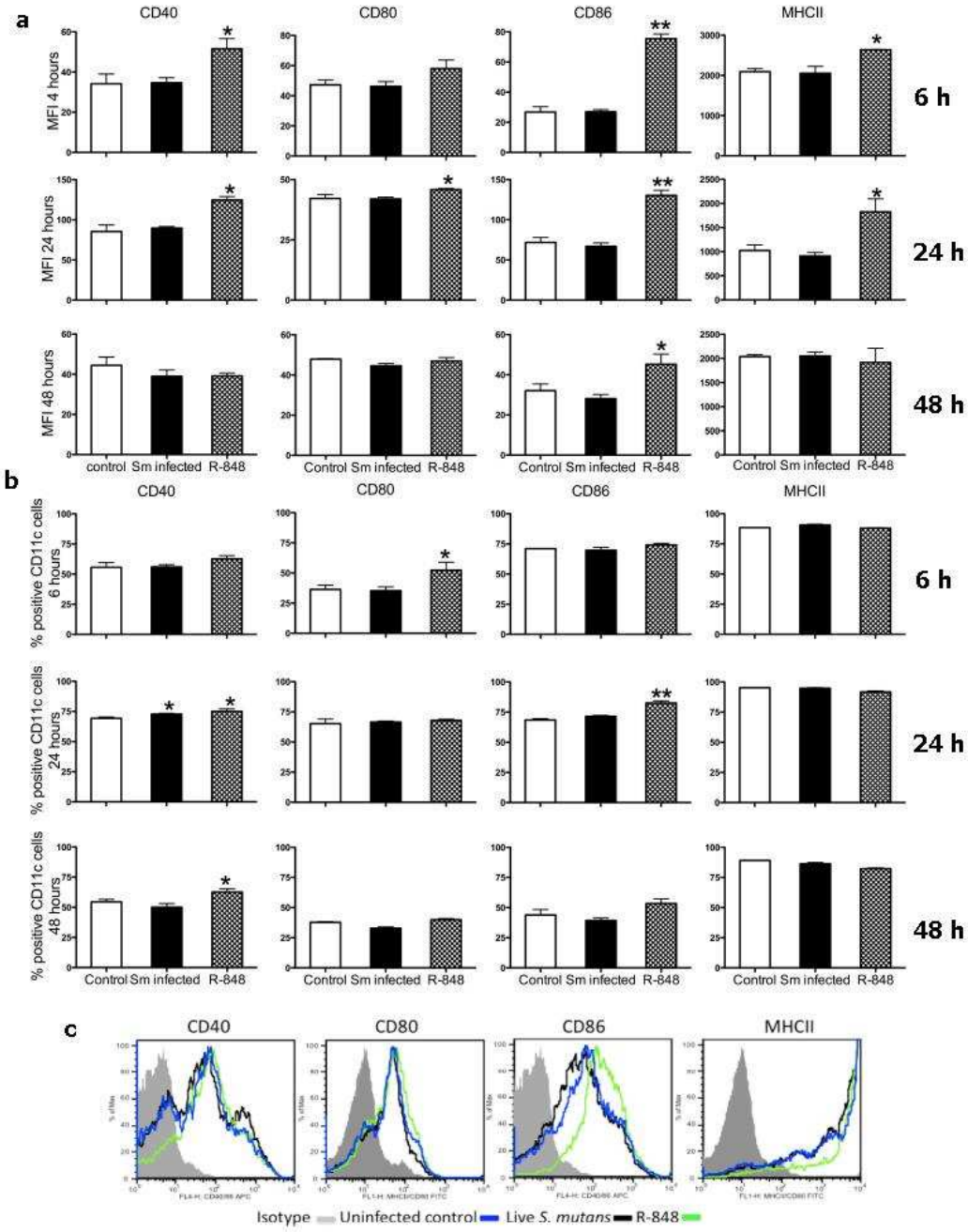


Figure 5-13: *In vivo* dendritic cells fail to become activated in response to *S. mutans*

C57Bl/6 mice were orally exposed to *S. mutans*. Cervical lymph nodes were harvested 6, 24 and 48 h following oral exposure to *S. mutans* UA159, PBS or R848. Single-cell suspensions were prepared and CD11c+ cells were assessed for expression of CD40, CD80, CD86 and MHCII by flow cytometric analysis. (a) Mean fluorescent intensity (MFI) of cells expressing CD40, CD80, CD86 or MHCII 6, 24 or 48 h following challenge. (b) Percentage of CD11c+ cells stained positive for CD40, CD80, CD86 or MHCII. Data are mean ± SEM of data from two independent experiments (24 h), or a single experiment (6 and 48 h). In each experiment n = 3 mice per group. * $p < 0.05$, ** $p < 0.005$ vs. control by Tukey comparison. (c) Representative histograms of CD11c+ cell expression of CD40, CD80, CD86 and MHCII at 24 h post-exposure to *S. mutans* or R848.

5.3 Discussion

DC uptake, processing and presentation of bacterial antigens to T-cells represent ill-defined yet key stages in the development of adaptive immunity to oral pathogens. Work described here used a novel system to dissect the early immune responses following oral exposure to *S. mutans*. Up-regulation of MHCII and co-stimulatory molecules by BMDCs *In vitro* demonstrated their ability to become activated in the context of *S. mutans* infection. Furthermore, BMDCs effectively processed and presented administered antigen in the presence of live and heat-killed *S. mutans*. When cultured with heat-killed *S. mutans* and antigen, BMDCs drove antigen-specific T cell proliferation. However, BMDCs exposed to live *S. mutans* did not. Consistent with this finding, CD11c positive cells from the DLN of mice orally challenged with live *S. mutans* failed to become activated as indicated by a failure to up-regulate co-stimulatory molecules and MHCII. The cytokine profiles elicited by BMDCs and T cells following exposure to live *S. mutans* may provide clues to explain the abrogated T cell proliferation in response to live *S. mutans*.

Interleukin 2 (IL-2) is secreted by most T cells immediately following antigenic stimulation and induces the proliferation of antigen-specific T cells (Gaffen and Liu 2004). Moreover, DCs also secrete IL-2 in the initial stages following bacterial stimulation to promote antigen-specific T cell stimulation (Granucci et al. 2001). The failure of DC/T cell co-cultures to produce IL-2 following *in vitro* exposure to live *S. mutans* could account for the failure of BMDCs to induce antigen-specific T cell proliferation. Furthermore, a significant reduction in the ability of BMDCs to secrete IL-12 in response to live *S. mutans* indicates that despite the detection of significant up-regulation of co-stimulatory molecules and detectable antigen presentation, BMDCs were not functionally mature. The effector cytokine profile elicited in response to heat-killed *S. mutans* (high concentrations of IL-12, IFN-γ and IL-17 together with low concentrations of IL-10) suggests a robust immune response along the Th1 and Th17 lineages were generated. The failure of live *S. mutans* to promote IL-12 secretion by activated BMDCs could explain the failure of BMDCs to induce antigen-specific T cell proliferation, and could thus account for the subsequent reduction in IL-2, IFN-γ and IL-17 production by T cells. The enhanced production of IL-10 by BMDC/T

cell co-cultures exposed to live *S. mutans* indicates that the cells actively responded to the presence of live *S. mutans* albeit with a distinctly un-inflammatory cytokine profile compared with exposure to heat-killed *S. mutans*. Interleukin-10 is associated with Th2-type responses; it would have been of interest to investigate other Th2 cytokines, such as IL-4, IL-6 and IL-13. However, it seems unlikely that live *S. mutans* induced a Th2-type response as there was a complete failure of T cells to proliferate along any lineage. The lack of T cell proliferation suggests that live *S. mutans* are capable of modulating or suppressing T cell priming by DCs.

Antigen targeting to DCs in the absence of inflammation is known to induce tolerogenic T cells (Finkelman et al. 1996). A semi-mature tolerogenic DC subset expressing high levels of MHCII and co-stimulatory molecules, low level expression of pro-inflammatory cytokines and IL-12 together with enhanced production of IL-10 have been identified and are consistent with the *In vitro* DC phenotype described here (Lutz and Schuler 2002). Moreover, tolerogenic DCs can promote antigen-specific T cell unresponsiveness by converting naïve T cells into Treg cells in the absence of T cell proliferation (Rutella et al. 2006). The T cells in BMDC/T cell co-cultures were not investigated phenotypically beyond the production of cytokines in the co-culture media. Future studies may look to characterise the T cells from such co-culture experiments to determine whether they are CD4+ CD25+ FoxP3+ Treg cells that produce TGF-ß (Chen 2006).

The mechanisms driving this anti-inflammatory response to *S. mutans* are not known. Previous studies of oral bacteria have demonstrated the ability of bacterial pathogens to suppress protective immunity by inhibiting the maturation and migration of DCs from the site of infection to the draining lymph nodes. In one study uptake of the oral pathogen *Porphyromonas gingivalis* by DCs lead to suboptimal DC maturation that was dependent on the expression of major fimbriae (Fim A), a bacterial adhesin associated with the ability of *P. gingivalis* to induce periodontal disease (Jotwani and Cutler 2004). In two separate studies it was demonstrated that lipopolysaccharide (LPS) from *P. gingivalis* stimulates DCs to secrete IL-10 but not IL-12 both *in vitro* (Jotwani et al. 2003) and *in vivo* (Pulendran et al. 2001). Thus, down-regulating antigen presentation and subsequent T cell responses.

A number of studies have identified the ability of the immune system to distinguish between pathogenic and commensal bacteria and thus these data reported here raise the question as to whether *S. mutans* can be considered a member of the commensal oral flora? Commensal bacteria have developed mechanisms to suppress the immune response. *Fusobacterium nucleatum* stimulates the release of the pro-inflammatory cytokines IL-6 and IL-8 by epithelial cells, but *S. gordonii* fails to trigger an immune response (Hasegawa et al. 2007). It has been demonstrated that the bacterial enzyme enolase expressed on the cell surface of *S. sobrinus* stimulated the release of IL-10. It was also shown that pre-treatment of mice with recombinant enolase from *S. sobrinus* failed to stimulate a primary immune response against T-cell dependent antigens. Interestingly, *S. sobrinus* enolase shares 90% homology with enolase from *S. mutans* (Veiga-Malta et al. 2004).

It seems unlikely that the lack of co-stimulation caused the abrogated T cell response as the molecules investigated were all up-regulated in response to both heat-killed and live *S. mutans*. The induction of semi-mature DCs giving rise to T cell unresponsiveness appears more likely. Further in-depth study is required to unravel the exact mechanisms involved *in vivo*.

Adoptive transfer of either Eα or OVA-peptide transgenic TCR T cells can be used to investigate antigen-specific T cell proliferation in the context of *S. mutans* infection *In vivo*. Thus, in the context of either model, oral challenge with *S. mutans* in conjunction with Th2/Th1 biasing adjuvants, such as the potent mucosal adjuvant cholera toxin may further elucidate the context to which *S. mutans* may modulate effector T-cell responses *In vivo*. Furthermore, the influence of such responses on the B-cell and antibody responses would be of particular interest given the suggestion that a poor specific antibody response may correlate with increased colonisation with oral pathogens (Hocini et al. 1993; Parkash et al. 1993).

Interestingly, despite the high bacterial exposure following oral challenge with *S. mutans*, there were no detectable changes in the expression of co-stimulatory molecules by CD11c positive cells. The appearance of activated DCs in the DLN of R848-treated animals within 6 to 24 h of bacterial exposure indicates that DC activation and migration from the oral mucosa to the DLN can be observed.

Moreover, DC migration to the DLN in response to subcutaneous bacterial challenge has been shown to occur within 24 h of exposure (Ravindran et al. 2007). The apparent absence of DC activation and migration to DLNs *In vivo* may be related to the finding that *In vitro* BMDCs failed to produce CCL20 in response to *S. mutans* exposure. CCL20 is highly chemoattractive for lymphocytes and immature DCs and is rapidly secreted in response to inflammatory stimuli (Schutyser et al. 2003). Thus, the failure to produce CCL20 in response to *S. mutans* exposure would result in reduced lymphocyte and monocyte influx to the site of infection and subsequent DC migration to the DLN. Alternatively, low numbers of DCs migrating to DLN are difficult to differentiate from resident cells. It would be of interest to investigate DCs resident within the oral mucosa to determine if the local activation followed by migration occurs (Cutler and Jotwani 2004). Such studies would likely require enrichment of DCs from mucosal tissues or DLNs in order to accurately define their phenotype in response to pathogen exposure.

It is possible that the DC subsets involved in *S. mutans* uptake and antigen presentation *In vivo* are not CD11c positive and were thus overlooked in these studies. However, previous reports have indicated that the majority of DCs in the mouse are CD11c positive (Banchereau and Steinman 1998). One study investigating the migration of CD11c positive DC subsets in murine oral mucosal tissues in response to inflammatory stimuli clearly identified the presence of three different subsets of CD11c positive DC residing in oral mucosal tissues, all of which were found to migrate to the DLNs under inflammatory conditions (Nudel et al. 2011). Thus, it seems unlikely that CD11c negative cells present in the oral cavity would be solely responsible for *S. mutans* specific antigen presentation, although this cannot be ruled out.

Previous reports have demonstrated rapid antigen presentation in the DLNs after infection and immunisation (Itano et al. 2003). Indeed, in the present study activated DCs appeared rapidly in the DLNs of R848-treated animals. However, the timing of antigen presentation to orally exposed antigens remains unclear. In the aforementioned study investigating the migration of CD11c positive DC subsets in murine oral mucosal tissues, antigen presentation could be detected days and weeks following challenge under local inflammatory conditions (Nudel

et al. 2011). Thus, the route of challenge and chronicity of infection are important considerations.

Investigating the initiation of an immune response to *S. mutans* is complex. The acquisition of *S. mutans* to the oral biofilm of young children is not met with the inflammation characteristic of plaque-induced gingivitis or other pathogenic oral pathogens and data reported here support a lack of inflammatory stimuli following oral challenge of mice with *S. mutans*. And yet, studies have demonstrated that colonisation with *S. mutans* is correlated with the appearance of salivary IgA antibodies specific for *S. mutans*, suggesting that a secretory immune response can be generated (Gahnberg et al. 1985; Smith et al. 1998; Smith and Taubman 1992). Whether or not protective immunity is generated remains controversial, although these data clearly indicate that at least under certain circumstances antigen presentation of *S. mutans* antigens must occur in order to subsequently generate secretory immunity. A repeated challenge, chronic infection model may aid identification of activated DCs in the DLN several days or weeks following infection.

Another possibility is that antigen presentation of oral bacterial antigens occurs in the mesenteric lymph nodes following indigestion of oral bacteria, leading to the induction of plasma B cells which migrate from the gut to the salivary glands and produce *S. mutans* specific antibodies locally in the oral cavity (Brandtzaeg and Johansen 2005).

Understanding the initiation of adaptive immunity in the oral cavity will provide insights and potential interventions for oral infectious diseases and potentially further our understanding of the impact of oral disease on systemic conditions. The observations reported here detail intriguing events, and these models could be used to further our understanding of oral adaptive immune responses.

Chapter 6: *S. mutans In vitro* biofilm model

6.1 Introduction

Previous data suggest that natural infection with *S. mutans* is not met with an inflammatory response that is characteristic of pathogenic microorganisms. Therefore, studies investigating the potential for passive vaccination strategies may have great therapeutic potential for preventing dental caries, particularly among young children.

In the presence of dietary sucrose numbers of Mutans streptococci in plaque increase and they have the ability to cause caries (Rolla 1989). A substantial body of literature supports the importance of the contribution of Gtfs and Gbps to caries development, and there is evidence that immunisation with Gtfs and Gbps can reduce caries development upon subsequent challenge with Mutans streptococci in animal models. Moreover, immunisation gives rise to antibody that can interfere with the production of glucans by Gtfs (Taubman et al. 1995, 2001). Passive immunisation with monoclonal antibodies raised against streptococcal antigen (SA) I/II reduced re-accumulation of Mutans streptococci in humans (Ma et al. 1987). Additionally, passive immunisation with monoclonal antibodies raised against *S. mutans* Gtf inhibited re-colonisation by *S. mutans* and significantly decreased caries development in rats (Hamada et al. 1991). Thus, oral administration of Gtf specific antibody, timed to coincide with colonisation by Mutans streptococci, could block their integration into the developing oral biofilm. However, passive administration of partially humanised monoclonal antibodies caries the risk of aberrant immune responses and thus are not always considered the most appropriate vaccine candidates for use in humans (Khazaeli et al. 1994). The production of monoclonal antibodies is time consuming and laborious, involving repeated animal immunisations and hybridoma generation. The advancement of technologies has allowed for the identification of single chain variable fragments (ScFv). ScFvs represent a fusion of the variable regions of heavy and light chains of immunoglobulin molecules, linked by a small peptide linker. Thus, the antigen binding domain of antibodies are expressed as a single peptide. This technology aids rapid and easy identification of antibody fragments in a form that is suitable for genetic manipulation and can readily be applied to large-scale production for therapeutic uses.

Previously, an ScFv fusion protein with activity towards *S. mutans* Gtf was found to significantly reduce the development of dental caries in a rat model (Kruger et al. 2006). However, the ScFv Gtf specific fragment was identified in llama and thus may not be considered the most appropriate vaccine candidate for use in humans (Szynol et al. 2004).

Human phage display libraries were recently constructed from B cells harvested from 57 donors. This library was screened against purified *S. sobrinus* Gtf-coated surfaces to select human ScFv specific for Gtf. Specific ScFv were then linked in frame with Fc of human IgG1 to produce antibody fragments known as "minibodies" (Figure 6-1). These minibodies have the advantage in that they are fully humanised and thus eliminate the risk of generating aberrant immune responses. Moreover, the small size allows for faster delivery and better penetration into tissues, which may be particularly relevant for access to the oral biofilm (Abiko 2000). These Gtf specific minibodies were further screened for Gtf binding activity by ELISA. Minibodies with the ability to inhibit Gtf mediated glucan synthesis from sucrose could provide a potential passive caries vaccine (Sui et al. 2008). A minibody with the ability to bind to Gtf was identified and denoted 'SS2'. Preliminary data revealed SS2 could significantly inhibit the ability of *S. mutans* Gtf mediated glucan synthesis from sucrose and *S. mutans* biofilm formation was significantly reduced in an *in vitro* sucrose-dependent biofilm model ($p < 0.03$) (Sui et al. 2009). Thus, the *in vitro* sucrose-dependent biofilm model was optimised and validated in order to determine the inhibitory capacity of SS2 on Mutans streptococcal biofilm formation.

Despite, the apparent low immunogenicity of *S. mutans* infection, the potential does exist to mount a natural immune response to *S. mutans*. Secretory immunity can be generated, namely through IgA and IgG, which comprise the functional arm of the adaptive immune system in the oral cavity (Taubman and Nash 2006). Antimicrobial peptides comprise an important innate response to bacteria present at mucosal surfaces. Antimicrobial peptides are released from numerous sources within the oral cavity in response to bacterial colonisation and are thought to have an important role in the maintenance of oral health (Dale and Fredericks 2005).

In vitro studies of antimicrobial peptides have attempted to investigate the antimicrobial properties of these peptides and others on the growth of *S. mutans* (Ouhara et al. 2005; Phattarataratip et al. 2011). However, the majority of these studies have investigated the antimicrobial effects only on planktonic cultures of *S. mutans*. Furthermore, these studies have used concentrations of antimicrobial peptides that are far higher than those found in the saliva of young children (Table 4-4). Several clinical studies have identified that low concentrations of antimicrobial peptides in the saliva of young children, such as LL37 (Davidopoulou et al. 2012) and the human neutrophil peptides (HNPs 1-3) (Tao et al. 2005) may be associated with increased risk of dental caries. Previous data reported in this thesis suggested a correlation between the recovery of *S. mutans* in dental plaque and increased concentrations of LL37 in the saliva of young children (Figure 4-8). Therefore, it was of interest to investigate the antimicrobial activity of LL37 on biofilm formation by *S. mutans*.

An intriguing study previously demonstrated increased planktonic growth responses of *S. mutans* in response to the presence of the stress hormones adrenaline and noradrenaline (Roberts et al. 2002). This report, together with the suggestion that increased concentrations of cortisol in the saliva of one-year-old children were associated with increased numbers of *S. mutans* in saliva and dental plaque (section 3.2.5), warranted further investigation. Thus, the *In vitro* sucrose-dependent *S. mutans* biofilm model was employed to further elucidate this response.

The aim of this chapter was to optimise and validate the use of an *in vitro* sucrose-dependent biofilm model. Once optimised this model was used to investigate the ability of the minibody SS2 to inhibit Mutans streptococcal biofilm formation, to investigate *S. mutans* growth responses to cortisol and investigate the minimum inhibitory concentration of the antimicrobial peptide LL37 required to inhibit biofilm formation by *S. mutans*.

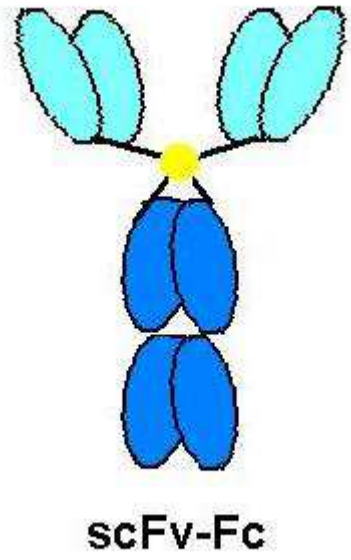


Figure 6-1: Diagrammatic representation of recombinant minibody

Single-chain variable fragments (depicted in light blue are linked in frame with Fc portion (heavy chain constant domains [CH2 and CH3 domains]) of human IgG1 (depicted in dark blue).

6.2 Results

6.2.1 Sucrose-dependent S. mutans biofilm formation

Morphological changes in *S. mutans* biofilms grown with or without sucrose were investigated by visualising by scanning electron microscopy. *S. mutans* grown in the presence of sucrose on Thermonox™ cover slips demonstrated clearly visible production of extensive extracellular matrix, which was absent from *S. mutans* biofilms grown in the absence of sucrose (Figure 6-2a and b). Similarly, biofilm mass, quantified by crystal violet incorporation, was significantly greater in the presence of sucrose ($p < 0.005$, Figure 6-2c).

An *In vitro* sucrose-dependent biofilm model was developed, optimised and validated. The conditions investigated included sucrose concentration, number of bacteria used as the initial inoculum and the pH of starting media. Modifying sucrose concentration demonstrated that biofilm formation did not increase with sucrose above 0.25%, suggesting this was sufficient for growth (Figure 6-3). Altering the starting inoculum demonstrated that *S. sobrinus* biofilm formation increased with each increased initial inoculum (Figure 6-3).

Changing the pH of the growth medium demonstrated that sucrose-dependent biofilm formation was significantly greater when grown in a medium adjusted to a starting pH 6.5 compared with pH 6.8 (Figure 6-4, $p < 0.005$) or pH 7 ($p > 0.001$). These data suggest that *S. sobrinus* biofilm formation is optimal at pH 6.5.

From the optimisation experiments it was decided to use an initial inoculum of 1.5×10^7 CFU/ml with sucrose at 0.25% in a media with a pH adjusted to 6.5 for all subsequent experiments.

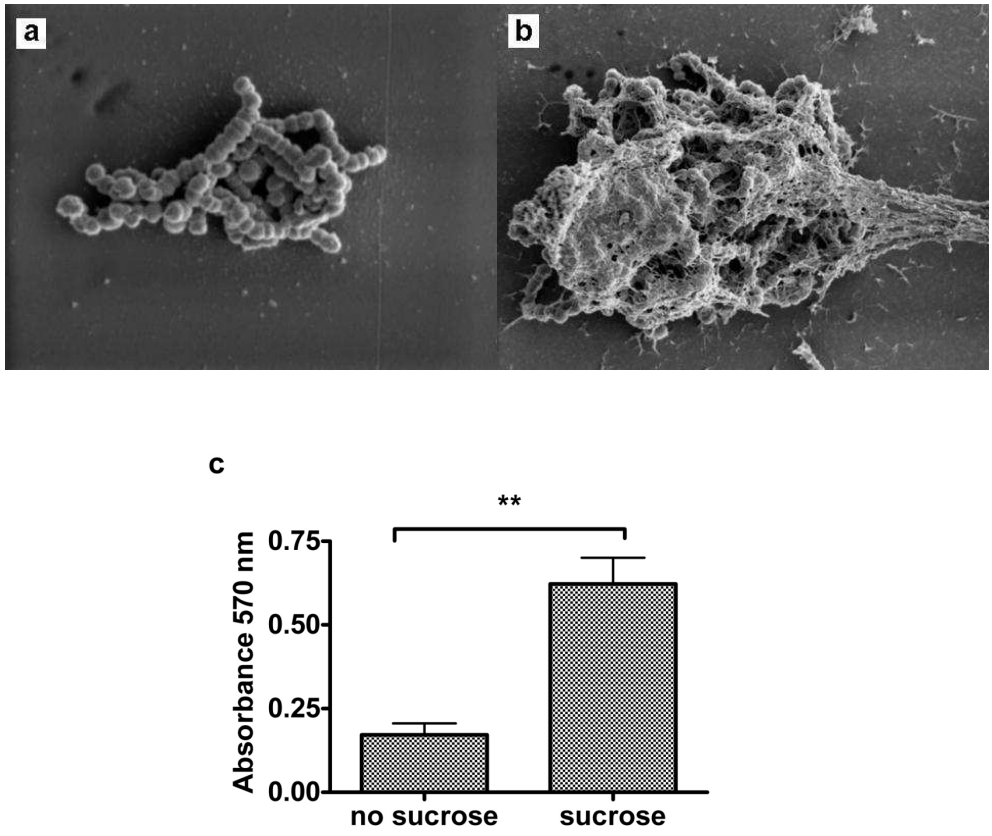


Figure 6-2: Sucrose dependent *S. mutans* biofilm formation

Biofilm formation by *S. mutans* ATCC 10449 in the presence or absence of sucrose was assessed. Scanning electron microscopy images of *S. mutans* grown on Thermanox® cover slips (a) without sucrose (x 7000 mag) and (b) with 1% sucrose (x 4500 mag), at 37˚C, in 5% CO_2 for 24 h. (c) Biofilms grown on peg lids at a starting inoculation of 1.5 x 10^7 CFU/well *S. mutans* with or without 0.25% sucrose at 37˚C, 5% CO_2 for 4 h were quantified by crystal violet incorporation. Data are mean and SEM of absorbance (OD 570 nm), measured in triplicate. ** $p < 0.005$ by independent samples t-test.

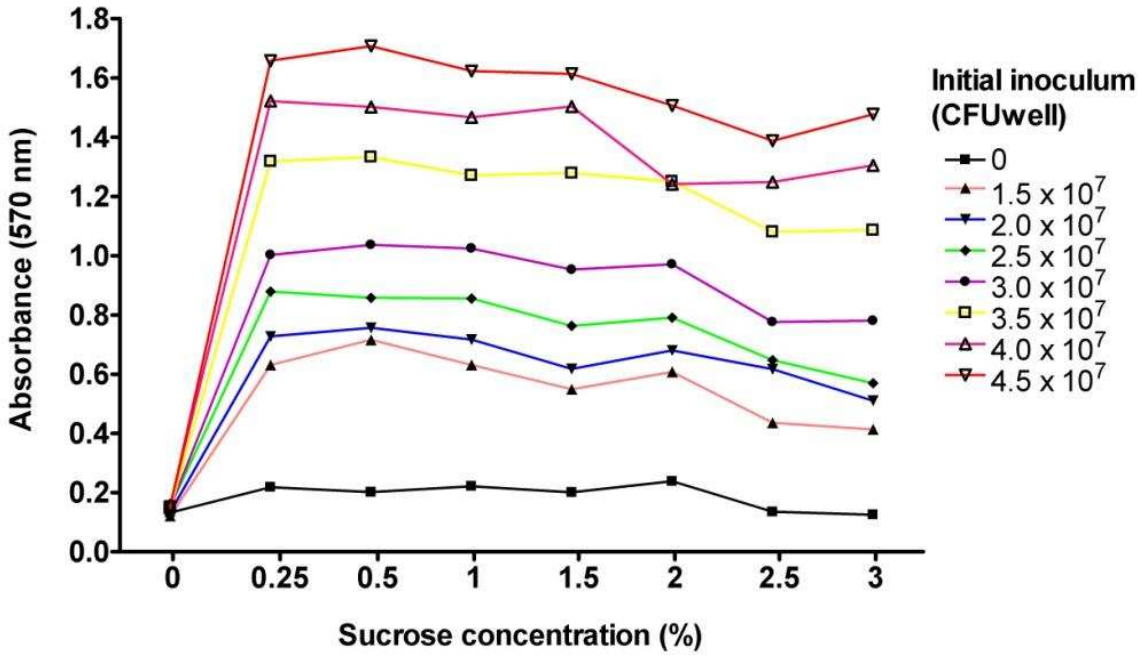


Figure 6-3: Increased *S. sobrinus* biofilm formation with increased initial inoculation

S. sobrinus biofilm formation using different starting inoculums (CFU/well) and with different sucrose concentrations (%) were assessed. Biofilms were grown on peg lids using starting inoculations of *S. sobrinus* 6715, ranging from 0 – 4.5 x 10^7 CFU/well, with concentrations of sucrose, ranging from 0 – 3.5%, at 37˚C, in 5% CO_2 for 4 h. Biofilm formation was quantified by crystal violet incorporation. Data are mean absorbance (OD 570 nm) of two independent experiments measured in triplicate.

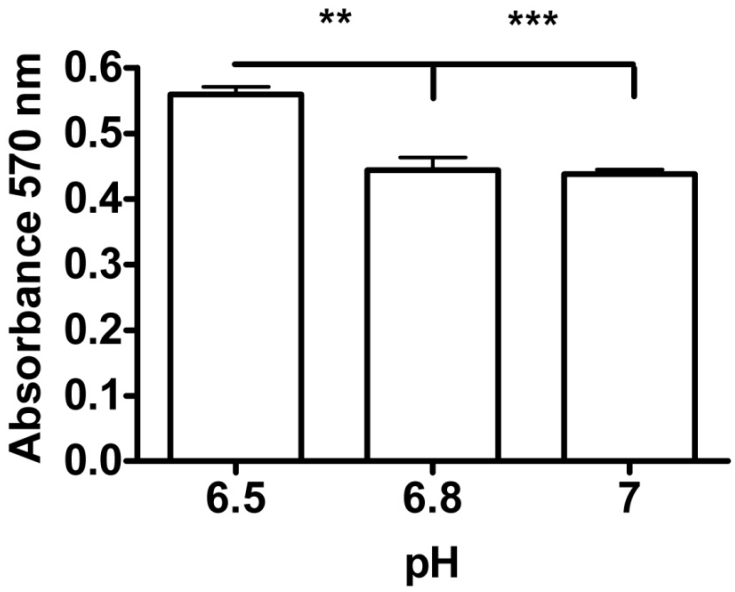


Figure 6-4: Increased *S. sobrinus* biofilm formation with increasingly acidic starting pH

S. sobrinus biofilm formation at different starting pH of culture media. Biofilms inoculated with 1.5 x 10^7 *S. sobrinus* 6715 (CFU/well) grown on peg lids in 0.25% sucrose, at 37˚C, in 5% CO_2 for 4 h in media of different starting pH were quantified by crystal violet incorporation. Data are mean and SEM of absorbance (OD 570 nm), measured in triplicate (*** $p < 0.001$, ** $p < 0.01$ by ANOVA).

6.2.2 Inhibition of Mutans streptococcal biofilm formation

To investigate inhibition of biofilm formation, conditions for the *In vitro* biofilm assay were optimised and validated. As a positive control rat IgG anti-*S. sobrinus* Gtf was prepared by active immunisation of Sprague Dawley rats with *S. sobrinus* Gtf (outlined in section 2.6.5) (Taubman et al. 1995). *S. sobrinus* was used for all optimisation and validation experiment. Once optimised the conditions were then used to investigate *S. mutans* biofilm inhibition using rat IgG anti-*S. mutans* Gtf as a positive control. Due to the ratio of water-soluble versus water-insoluble Gtfs produced by *S. sobrinus* and *S. mutans* it was more economical and time efficient to extract the primarily water-soluble Gtfs from *S. sobrinus*. Serum IgG from sham-immunised rats was used as control antibody (Taubman et al. 1995).

Inhibition of biofilm formation was expressed as a percentage relative to no antibody controls.

6.2.2.1 Optimisation of biofilm inhibition assay

Inhibition of *S. sobrinus* biofilm formation by rat IgG anti-*S. sobrinus* Gtf was assessed with two different concentrations of antibody and different starting inoculations of *S. sobrinus* (Figure 6-5). Using a high concentration (4.2 µg/ml) of antibody, inhibition of *S. sobrinus* biofilm formation ranged from 92% – 62% and was highly statistically significant at each starting inoculation assessed ($p < 0.001$, Figure 6-5a). A lower antibody concentration (2.1 µg/ml) demonstrated 50% inhibition of biofilm formation with a starting bacterial inoculum of $1.5x10^7$ CFU/well. At higher starting inoculums, the antibody demonstrated reduced efficacy but still caused statistically significant inhibition of biofilm formation (Figure 6-5b). Based on this data it was decided to use a starting inoculation of 1.5×10^7 CFU/well and rat IgG anti-*S. sobrinus* Gtf at a concentration of 2.1 µg/ml for all subsequent experiments as 50% inhibition of biofilm formation by rat IgG anti-*S. sobrinus* Gtf was considered optimal for use a positive control.

Previous data suggested 0.25% sucrose was suitable for growth (Figure 6-3). However, it was unclear from this data if inhibition of biofilm formation could be affected by the percentage of sucrose used. Therefore, using a starting

inoculation of 1.5 x 10^7 CFU/well, the effect of different sucrose concentrations on antibody-mediated inhibition of biofilm formation was investigated (Figure 6-6). Inhibition of biofilm formation demonstrated a dose response relationship, with greater inhibition at increased sucrose concentrations. Inhibition of biofilm formation was significant at all sucrose concentrations assessed ($p < 0.005$ compared to sham IgG control antibody). Based on these data it was decided to proceed with sucrose at 0.25% for all subsequent experiments as sufficient inhibition of biofilm formation in conjunction with adequate biofilm formation was observed.

Biofilm growth was optimal at a starting pH of 6.5 (Figure 6-4). The effect of starting pH on inhibition of biofilm formation by rat IgG anti-*S. sobrinus* Gtf was investigated. Inhibition of biofilm formation was statistically significant at all starting pH assessed although was markedly reduced at pH 6.5 compared to pH 6.8 or pH 7 (Figure 6-7). This data suggest that while biofilm formation is optimal at pH 6.5, inhibition of biofilm formation by rat IgG anti-*S. sobrinus* Gtf is optimal at a starting pH closer to neutral. Following 4 h sucrose-dependent biofilm formation the pH of the growth medium can fall to approximately pH 5.5 (data not shown). Such a drop in pH may modify the activity of the antibody. From these data it was decided to perform subsequent biofilm experiments in a growth medium adjusted to pH 6.8.

The *In vitro* biofilm model was optimised using *S. sobrinus*. However, *S. mutans* is the most frequently isolated of the Mutans streptococci and is the primary agent associated with dental caries, particularly among young children (Kristoffersson et al. 1985). Therefore, it was important to determine the optimum conditions for assessing inhibition of *S. mutans* sucrose-dependent biofilm formation. The conditions optimised for *S. sobrinus* were used and inhibition of biofilm formation was assessed at different concentrations of rat IgG anti-*S. mutans* Gtf (Figure 6-8). Inhibition of *S. mutans* biofilm formation was dose-dependent over the range of antibody concentrations assessed.

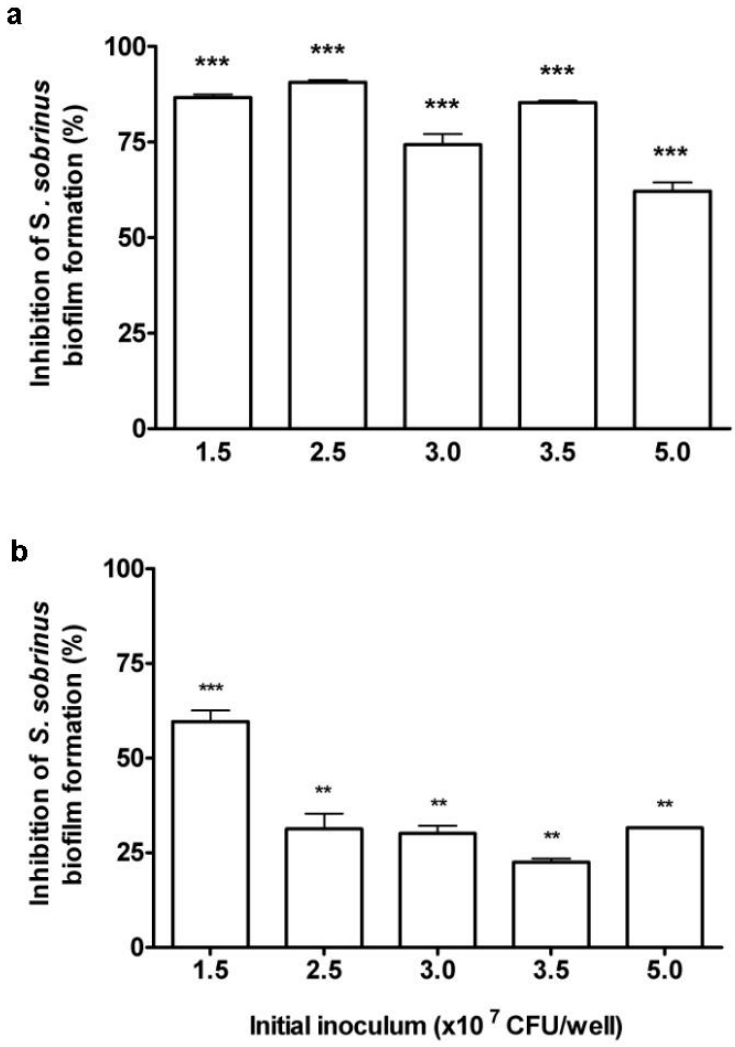


Figure 6-5: Inhibition of *S. sobrinus* biofilm formation by rat IgG anti-*S. sobrinus* Gtf

Inhibition of *S. sobrinus* biofilm formation, at different starting inoculations of *S. sobrinus* 6715 (CFU/well) by rat IgG anti-*S. sobrinus* Gtf were assessed. Biofilms grown on peg lids with 0.25% sucrose at 37˚C in 5% CO_2 for 4 h were quantified by crystal violet incorporation. Data are mean and SEM of percentage inhibition of *S. sobrinus* biofilm formation by rat IgG anti-*S. sobrinus* Gtf at a concentration of (a) 4.2 µg/ml and (b) 2.1 µg/ml, measured in triplicate and relative to no antibody controls with and without sucrose. *** $p < 0.001$, ** $p < 0.005$ by independent samples t-tests compared to sham IgG control antibody.

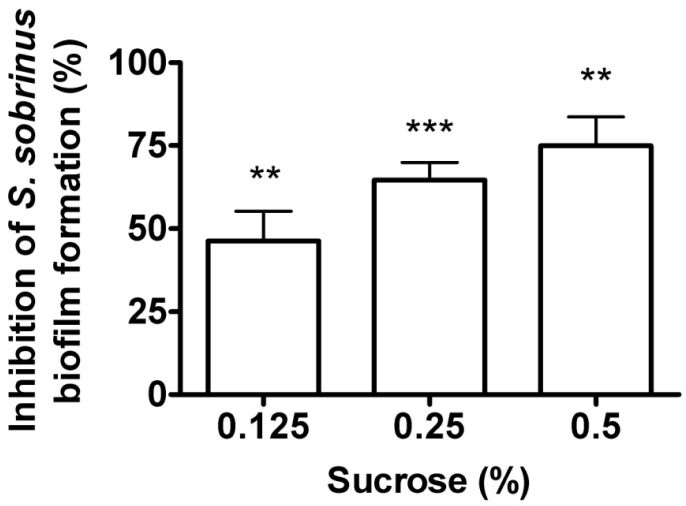


Figure 6-6: Inhibition of *S. sobrinus* biofilm formation by rat IgG anti-Gtf at different sucrose concentrations

Inhibition of *S. sobrinus* biofilm formation by rat IgG anti-*S. sobrinus* Gtf was assessed at different sucrose concentrations. Biofilms were grown on peg lids and inoculated with *S. sobrinus* 6715 at 1.5 x 10^7 CFU/well, at sucrose concentrations ranging from 0.125 – 0.5%, with 2.1 µg/ml rat IgG anti-*S. sobrinus* Gtf, and grown at 37˚C, in 5% CO_2 for 4 h. Biofilm formation was quantified by crystal violet incorporation. Data are mean and SEM of percentage inhibition of *S. sobrinus* biofilm formation, measured in triplicate and relative to no antibody controls with and without sucrose. *** $p < 0.001$, ** $p < 0.005$ by independent samples t-tests compared to sham IgG control antibody.

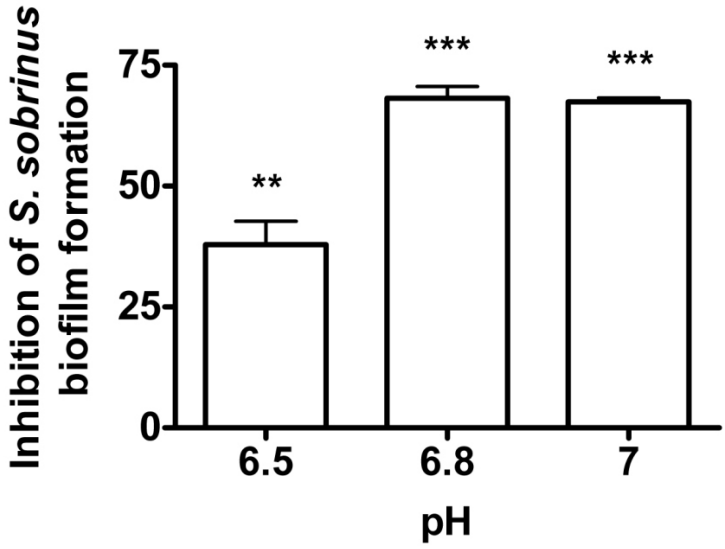


Figure 6-7: Inhibition of *S. sobrinus* biofilm formation is optimal at neutral pH

Inhibition of *S. sobrinus* biofilm formation by rat IgG anti-*S. sobrinus* GTF was assessed under different pH environments. Biofilms inoculated with 1.5 x 10^7 *S. sobrinus* 6715 (CFU/well) with 2.1 µg/ml rat IgG anti-*S. sobrinus* Gtf grown on peg lids in 0.25% sucrose, at 37˚C, in 5% CO_2 for 4 h in media of different starting pH were quantified by crystal violet incorporation. Data are mean and SEM of percentage inhibition of *S. sobrinus* biofilm formation by rat IgG anti-S. *sobrinus* Gtf at different pH, measured in triplicate and relative to no antibody controls with and without sucrose. *** $p < 0.001$, ** $p < 0.01$ by independent samples t-tests compared to sham IgG control antibody.

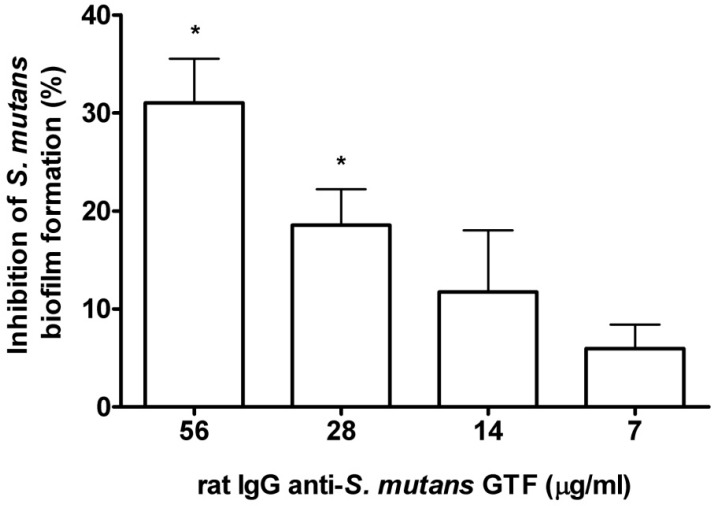


Figure 6-8: Inhibition of *S. mutans* biofilm formation by rat IgG anti-*S. mutans* Gtf

Inhibition of *S. mutans* biofilm formation at different concentrations of rat IgG anti-*S. mutans* Gtf was assessed. Biofilms inoculated with 1.5 x 10^7 *S. mutans* 28BE3 CFU/well and grown on peg lids at 37˚C, in 5% CO_2 with a starting pH of 6.8 for 4 h were quantified by crystal violet incorporation. Data are mean and SEM of percentage inhibition of *S. mutans* biofilm formation by rat IgG anti-*S. mutans* Gtf, measured in triplicate and relative to no antibody controls with and without sucrose. * $p < 0.05$, by independent samples t-tests compared to sham IgG control antibody (data not shown) assessed under respective conditions.

6.2.2.2 Inhibition of Mutans streptococcal biofilm formation by minibody SS2

The assay conditions optimised above, a starting inoculation of 1.5 x 10^7 CFU/well, with 0.25% sucrose in a starting media at pH 6.8, were used to investigate the ability of the minibody SS2 to inhibit Mutans streptococci sucrose-dependent biofilm formation.

Inhibition of *S. sobrinus* biofilm formation was dose-dependent over the range of minibody concentrations assessed, decreasing with each two-fold dilution of the minibody (Figure 6-9a). Inhibition of biofilm formation by SS2 was highly statistically significant over the range 16.6 - 132.5 µg/ml ($p < 0.001$) and remained significant at the lowest minibody concentration assessed 8.2 µg/ml ($p < 0.05$).

S. mutans sucrose-dependent biofilm formation was inhibited by SS2 in a dose-dependent manner over the range 7.8 µg/ml – 62.5 µg/ml (Figure 6-9b). Increasing the minibody concentration above 62.5 µg/ml did not further inhibit of *S. mutans* biofilm formation. Inhibition of *S. mutans* biofilm formation by SS2 was not significant at minibody concentrations below 31.3 µg/ml, almost four times the minibody concentration that was required for statistically significant inhibition of *S. sobrinus* biofilm formation.

These data indicate that the minibody SS2 has the ability to inhibit sucrose-dependent biofilm formation by Mutans streptococci.

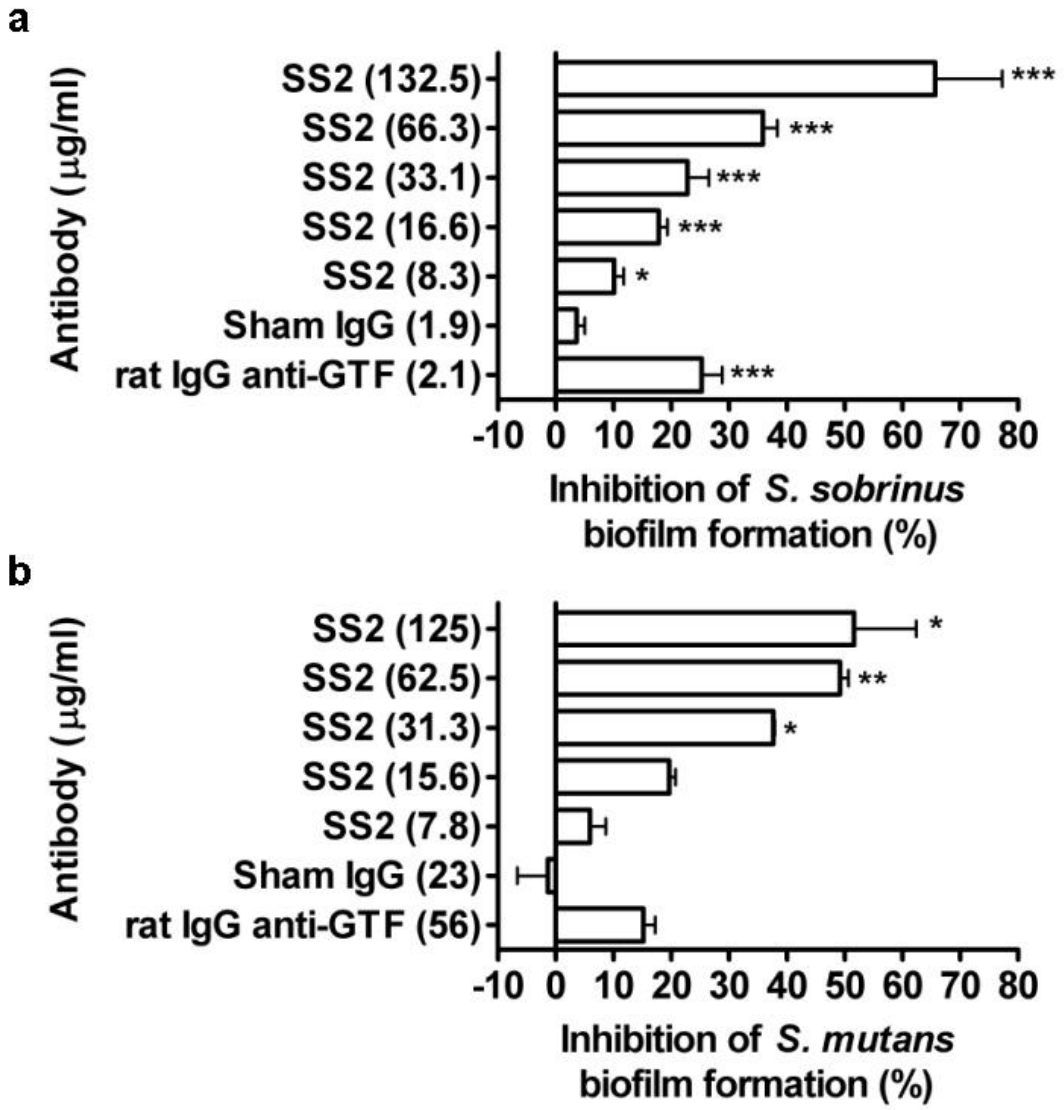


Figure 6-9: Inhibition of Mutans streptococcal biofilm formation by Sc Fv SS2

Inhibition of Mutans streptococcal biofilm formation by the minibody Sc Fv SS2 was assessed. Biofilms inoculated with 1.5 x 10^7 CFU/well were grown on peg lids in 0.25% sucrose at 37˚C, in 5% CO_2 at pH 6.8 for 4 h, were quantified by crystal violet incorporation. Data are mean and SEM of percentage inhibition of biofilm formation of (a) *S. sobrinus* 6715 and (b) *S. mutans* 28BE3, relative to no antibody controls with and without sucrose. Each data set is representative of two independent experiments, measured in triplicate. *** $p < 0.001$, ** $p < 0.005$ and * $p < 0.05$ by independent samples t-tests compared with sham IgG control antibody.

6.2.3 Mutans streptococci growth responses to cortisol

Data from a cross-sectional study into caries-associated risk factors in children aged one-year indicated that increased concentrations of salivary cortisol were associated with increased numbers of *S. mutans* recovered from both plaque and saliva of one-year-old children. These data were intriguing, and together with the suggestion that *S. mutans* is capable of modulating its growth response in the presence of the catecholamines adrenaline and noradrenaline (Radford et al. 2000), warranted further investigation to determine if *S. mutans* is also capable of modulating growth responses to cortisol.

6.2.3.1 Planktonic growth response to cortisol

The planktonic growth kinetics of *S. mutans* in the presence or absence of cortisol, with and without sucrose were assessed over time by measuring the absorbance of cultures every hour over a 24 hour period. Cultures were grown in half strength BHI to provide a minimal media that ensured growth responses were related to cortisol and sucrose supplements and not due to the nutrients present within the original growth media. *S. mutans* cultures supplemented with cortisol only (10, 50 and 100 nmol) entered logarithmic growth quicker than cultures containing both cortisol and sucrose or sucrose only (Figure 6-10a). Commensurate with these findings, after 24 hours of culture, the greatest numbers of *S. mutans* came from cultures supplemented with cortisol only. The CFU/ml were statistically significantly higher in cultures supplemented with either 100 or 50 nmol cortisol ($p < 0.001$ and $p < 0.05$, respectively) compared with the no supplement control (Figure 6-10b). There were no differences in the CFU/ml of *S. mutans* cultures supplemented with sucrose only compared with those supplemented with both sucrose and cortisol. *S. mutans* cultures supplemented with sucrose had significantly lower counts compared with the no supplement control, irrespective of the presence of cortisol. It was hypothesised that *S. mutans* cultures grown in the presence of sucrose switched from a planktonic free-living phenotype to a biofilm forming phenotype, characterised by the expression of Gtfs and Gbps. Thus, the growth responses in the presence of sucrose differed significantly from the growth responses in the presence of cortisol. To elucidate these growth responses, *S. mutans* biofilms grown in the

presence and absence of sucrose and with varying concentrations of cortisol were further investigated using the sucrose-dependent biofilm model.

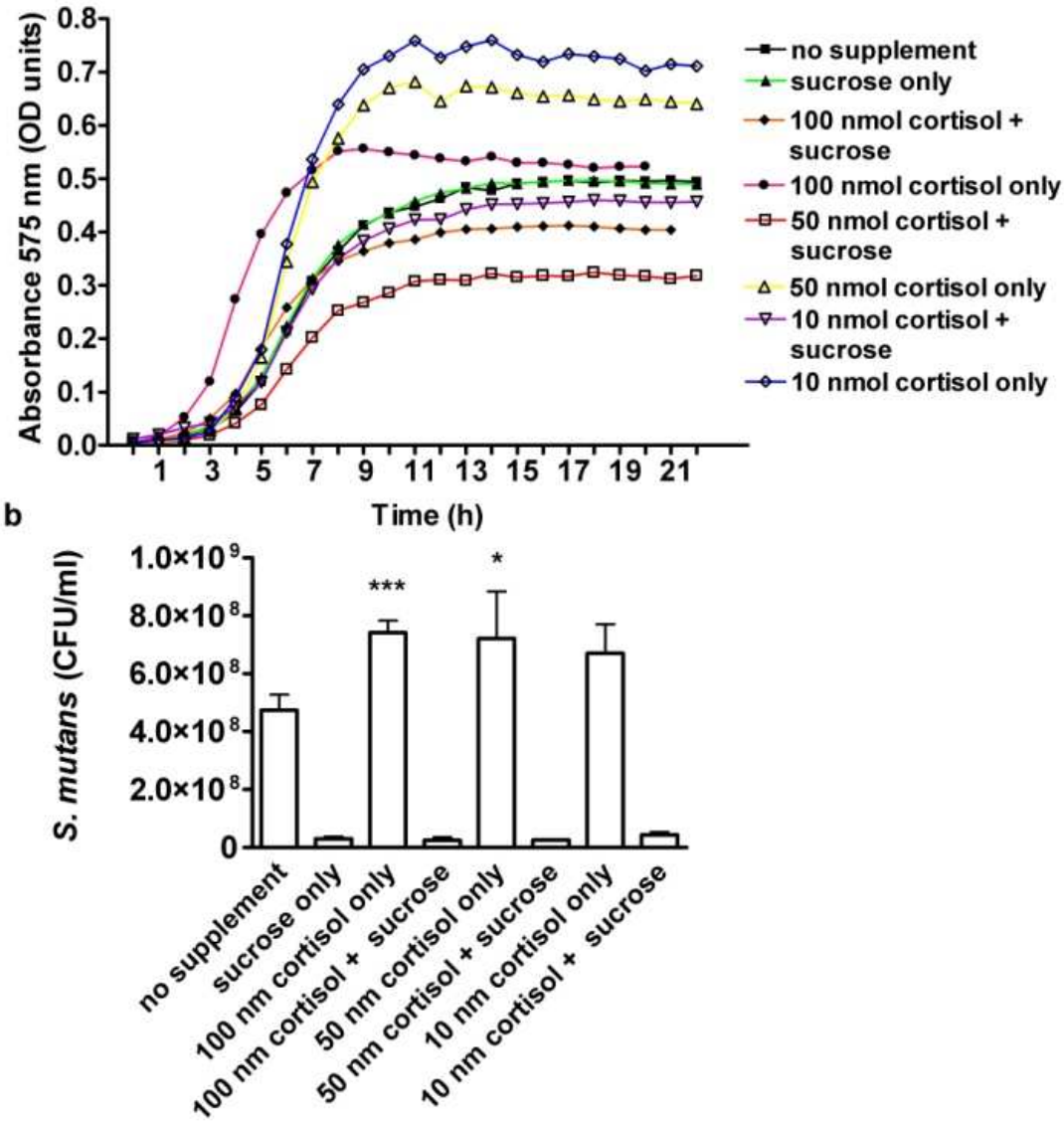


Figure 6-10: Planktonic growth responses of *S. mutans* to cortisol

Cultures of *S. mutans* grown in 1/2 strength BHI in the presence or absence of cortisol at varying concentrations, with or without 0.25% sucrose were assessed. Cultures were incubated at 37˚C in 5% CO_2 for 24 h. (a) Growth kinetics of *S. mutans* cultures over time. Data are mean of triplicates of absorbance at 570 nm (OD units) of *S. mutans* culture supplemented with 10, 50 and 100 nmol of cortisol. (b) Planktonic *S. mutans* CFU/ml after 24 h incubation. Results are from two independent experiments, each measured in triplicate. *** $p < 0.001$, * $p < 0.05$, by independent samples t-tests, compared with no supplement control.

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6.2.3.2 *S. mutans* biofilm growth response to cortisol

The biomass of *S. mutans* biofilms grown on pegs lids in the presence or absence of sucrose with different concentrations of cortisol were assessed by quantification of crystal violet incorporation after 4 and 24 h growth. After 4 and 24 h, the biomass of *S. mutans* biofilms grown with cortisol (10, 50 and 100 nmol) did not differ significantly from *S. mutans* control biofilms grown in media alone (Figure 6-11a and b). Furthermore, *S. mutans* biofilms grown in the presence of cortisol and 0.25% sucrose did not differ significantly from the sucrose only controls. *S. mutans* biofilms grown with sucrose had significantly greater biomass compared with biofilms grown in the absence of sucrose ($p < 0.001$, by Tukey comparison). The biomass of *S. mutans* biofilms grown in the absence of sucrose (i.e. no supplement or with cortisol only) did not increase from 4 to 24 h. In contrast, the biomass of *S. mutans* biofilms grown with sucrose (with or without cortisol) continued to increase from 4 to 24 h ($p < 0.001$, by Tukey comparison). Together these data confirm that sucrose is important for *S. mutans* biofilm formation and suggests cortisol does not influence *S. mutans* biofilm formation. However, given the enhanced planktonic growth of *S. mutans* in the presence of cortisol, and given that *S. mutans* biofilm formation in the presence of sucrose is characterised by the expression of large amounts of extracellular polymeric glucans (Figure 6-2b), it was hypothesised that the CFU/peg may be greater in *S. mutans* biofilms supplemented with sucrose and cortisol compared with the sucrose only control and that differences in cell number may have been masked by the expression of extracellular glucans using the crystal violet assay.

The CFU/peg of 24 h *S. mutans* biofilms grown under the conditions outlined previously were quantified by different techniques. Initially, culture techniques were used to quantify the number of *S. mutans* CFU/peg. There were no significant differences in the number of *S. mutans* CFU/peg from biofilms grown with cortisol compared with the no supplement control (Figure 6-12a). *S. mutans* CFU/peg were greater when biofilms were grown with sucrose but the difference was not statistically significant compared with the no supplement control. Furthermore, *S. mutans* CFU/peg were found to be marginally lower in biofilms grown with both cortisol (100 and 50 nmol) and sucrose compared with sucrose alone, although the differences were not statistically significant. The culture

data were highly variable and this was likely related to the phenomenon that *S. mutans* biofilms grown with sucrose are extremely adherent to the biofilm matrix (polyethylene peg). To remove the biofilm from the peg and allow quantification of *S. mutans*, biofilms were subjected to sonication. It was previously observed that sonication of planktonic *S. mutans* cultures did not kill bacterial cells but disrupted the bacterial chains and gave rise to higher CFU/ml compared with cultures that were not subjected to sonication (data not shown). Thus, although considered unlikely, it was possible that sonication of *S. mutans* biofilms variably killed the bacterial cells and thus gave rise to the inherent variation in the CFU/peg data. Moreover, there was potential variation introduced from an inability to remove all of the biofilm from the pegs. To further investigate the CFU/peg, *S. mutans* biofilms grown under these same conditions were quantified by estimating amount of DNA instead of CFU/ml, using a CyQuant® assay. Although still reliant on sonication to remove the biofilm from the pegs, this method was selected to overcome the potential variation in viability following sonication.

The CyQuant® assay allows for the quantification of relative cell numbers by use of a dye that emits a strong fluorescence signal when bound to cellular DNA and thus quantifies the total number of cells. In agreement with the culture data, there were no statistically significant differences in the CFU/peg of *S. mutans* biofilms grown with cortisol (100 and 50 nmol) compared with the no supplement control (Figure 6-12b). The CFU/peg was greater for *S. mutans* biofilms grown with sucrose, although the differences were not statistically significant. Furthermore, there were no statistically significant differences in the CFU/peg from *S. mutans* biofilms grown with both cortisol and sucrose compared with sucrose only.

In an attempt to ensure complete removal of *S. mutans* cells from biofilms formed on peg lids, enzymatic digestion in conjunction with sonication was used, rendering the sample suitable for TaqMan® QPCR. Pegs were incubated with a mixture of lysozyme and mutanolysin, followed by sonication. DNA was extracted and purified from the supernatants and *S. mutans* CFU/peg was quantified by TaqMan® QPCR using *S. mutans* specific primers and probe (cross-refence methods). In agreement with previous observations using culture and CyQuant, there were no statistically significant differences in the CFU/peg from

S. mutans biofilms grown in the presence of cortisol (10, 50 and 100 nmol), compared with the no supplement control (Figure 6-12c). *S. mutans* CFU/peg were greater in biofilms grown with sucrose but the differences were not statistically significant. There was an increase in the CFU/peg for *S. mutans* grown with 100 nmol cortisol and 0.25% sucrose compared with sucrose only but the difference was not statistically significant. Furthermore, there were no differences in *S. mutans* CFU/peg in biofilms grown with 50 or 10 nmol cortisol together with 0.25% sucrose compared with *S. mutans* biofilms grown with sucrose only.

The use of the CyQuant® assay and TaqMan QPCR to quantify *S. mutans* CFU/peg by detecting DNA were found to be more sensitive with respect to the number of CFU/peg detected, with TaqMan QPCR being the most sensitive compared with the culture data. Both CyQuant® and TaqMan QPCR detect the total number of cells and thus cannot discriminate between live and dead bacteria in the biofilm. To determine whether the metabolic activity of *S. mutans* biofilms grown with cortisol with or without sucrose differed, a REDOX indicator was included in the growth media. Cortisol had no effect on the metabolic activity of *S. mutans* biofilms. *S. mutans* biofilms grown with sucrose both with or without cortisol were statistically significantly more metabolically active as indicated by a greater reduction of alamarBlue® compared with *S. mutans* biofilms grown in the absence of sucrose ($p < 0.001$, by Tukey comparison). However, *S. mutans* biofilms grown with both cortisol and sucrose were significantly less metabolically active compared with sucrose only (Figure 6-13).

Together this data indicate that cortisol modulates the growth response of planktonic cultures of *S. mutans*, although has little or no influence on *S. mutans* biofilms.

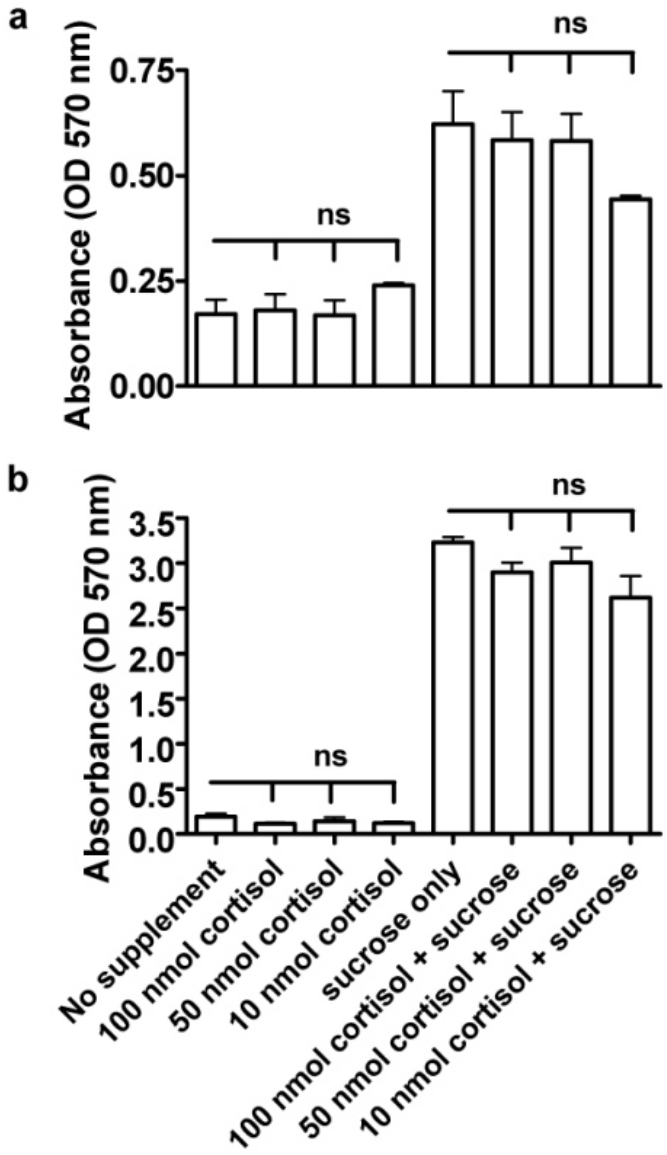

Figure 6-11: *S. mutans* biofilm biomass in the presence of cortisol and sucrose

S. mutans biofilm formation with cortisol at varying concentrations, with or without 0.25% sucrose was assessed. Biofilms grown on peg lids in 37˚C, 5% CO_2 for were quantified using crystal violet incorporation. Data are mean and SEM of absorbance (OD 570 nm) of (a) 4 h and (b) 24 h biofilms, measured in triplicate on three independent occasions. ns = not significant by Tukey comparison, compared with indicated control.

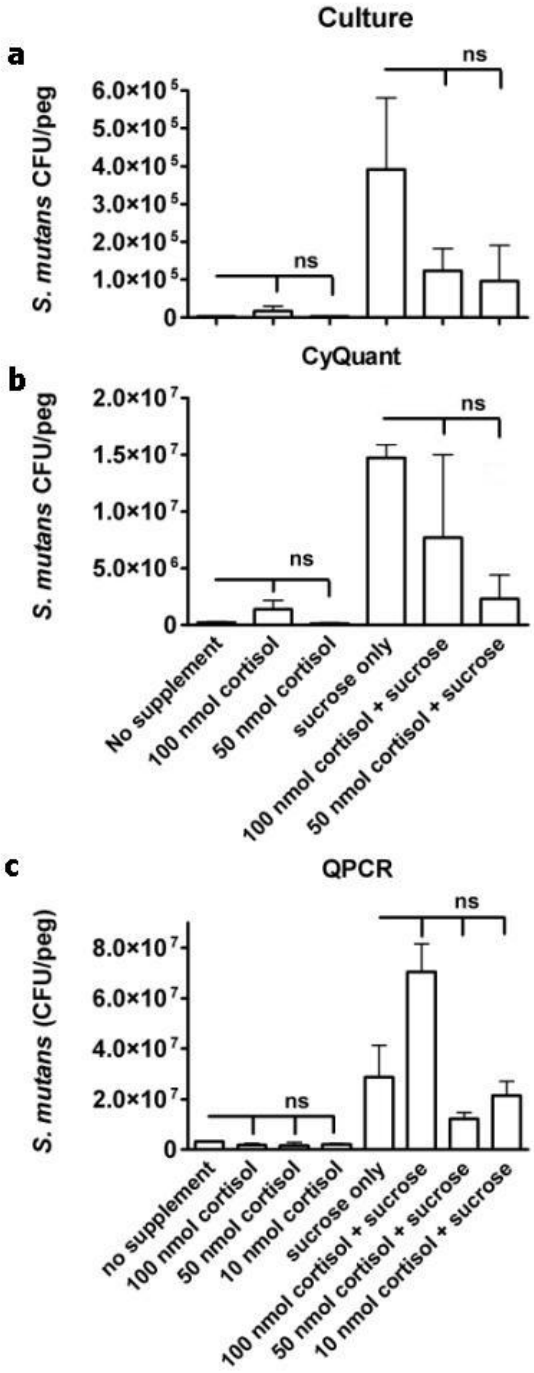

Figure 6-12: Quantification of *S. mutans* cell numbers in biofilms grown with sucrose and cortisol

S. mutans biofilms were grown on peg lids in the presence or absence of cortisol at 100, 50 and 10 nmol, with or without 0.25% sucrose for 24 h and quantified by (a) culture, data are mean and SEM of CFU/peg from two independent experiments, measured in triplicate (b) CyQuant® assay, data are mean and SEM from two independent experiments, measured in triplicate and (c) TaqMan® QPCR, data are mean and SEM of CFU/peg, measured in triplicate. ns = not significant by Tukey comparison with indicated controls.

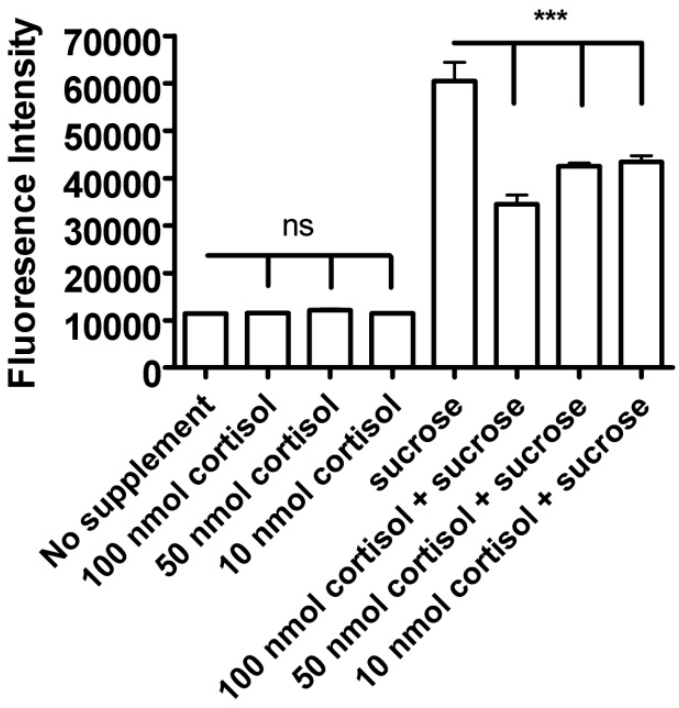

Figure 6-13: Metabolic activity of *S. mutans* biofilms with sucrose and cortisol

S. mutans biofilms grown on peg lids with cortisol, with or without 0.25% sucrose for 24 h were assessed for metabolic activity by alamarBlue® reduction. Data are mean and SEM of triplicate fluorescent intensity data, where increased fluorescence intensity indicates higher reduction of alamarBlue®. ns= not significant, *** $p < 0.001$ by Tukey comparison with indicated controls.

6.2.4 Inhibition of S. mutans biofilm formation by LL37

The ability of the antimicrobial peptide LL37 to inhibit the formation of *S. mutans* biofilms was assessed using the sucrose-dependent *In vitro* biofilm model. Using a starting biofilm inoculation of 1.5 x 10^7 CFU/well, *S. mutans* biofilm formation with LL37 was reduced at all concentrations assessed compared with the no LL37 control. The percent inhibition was statistically significant at LL37 concentrations of 32 and 64 µg/ml ($p < 0.05$ and $p < 0.001$, respectively [Figure 6-14a]). The inhibitory effect of LL37 appeared to be dose-dependent. At a starting inoculation of 3 x 10^7 CFU/well, *S. mutans* biofilm formation was not inhibited at concentrations of LL37 below 32 µg/ml and the percent biofilm inhibition was statistically significant only at a concentration of 64 µg/ml ($p < 0.001$, Figure 6-14b).

This data suggest that LL37 is capable of inhibiting *S. mutans* sucrose-dependent biofilm formation.

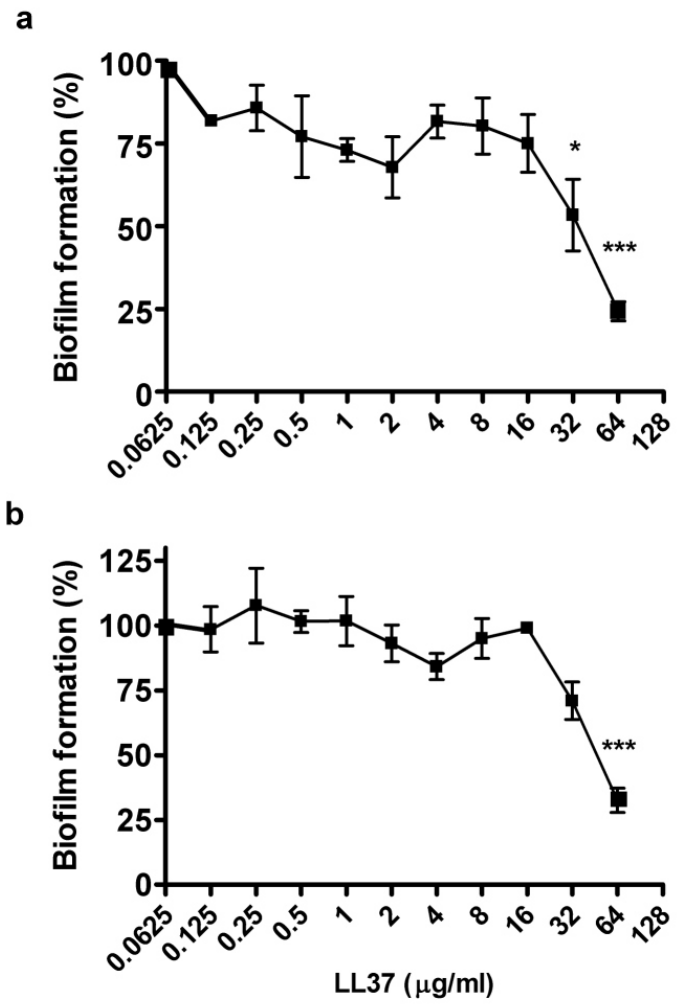

Figure 6-14: *S. mutans* biofilm inhibition by LL37

Inhibition of *S. mutans* biofilm formation using concentrations of LL37 (a kind gift from Dr Donald Davidson of the University of Edinburgh) at two-fold dilutions ranging from 64 – 0.125 µg/ml was assessed. Biofilms were grown on peg lids with 0.25% sucrose at 37˚C for 24 h with a starting inoculum of (a) 1.5 x 10^7 CFU/well and (b) 3.0 x 10^7 CFU/well. Biofilm formation was quantified by crystal violet incorporation. Data are mean and SEM of percent biofilm formation relative to no LL37 control. *** $p < 0.001$, * $p < 0.05$, by Tukey comparison relative to no LL37 control.

6.3 Discussion

Work presented here described an optimised sucrose-dependent *S. mutans* biofilm model that can be used to investigate novel molecules for their antimicrobial and potentially therapeutic properties. The use of a sucrose-dependent biofilm model, in which the bacterial cells actively form biofilm on inverted pegs is arguably more representative of *In vivo* biofilm formation than the commonly used microtitre plate assays that allow cells to settle in the bottom of the well with gravity assisted bacterial accumulation aiding biofilm formation (Ahn et al. 2012; Wang et al. 2012). Furthermore, Gtf-mediated adherence and accumulation in the presence of sucrose represents the major mechanism contributing to the ability of the Mutans streptococci to initiate dental caries (Banas and Vickerman 2003). Nonetheless, this single species biofilm model does not represent the complex microbial community found in dental plaque.

Using this model, it was identified that the minibody denoted SS2 had the ability to significantly inhibit Mutans streptococcal biofilm formation, presumably due to its ability to bind Gtf and thus block Gtf mediated glucan production from sucrose. Interestingly, the single chain variable fragments used to develop SS2 were identified by their ability to bind Gtf isolated from *S. sobrinus*. However, unsurprisingly given that there is greater than 50% homology between the *S. mutans* and *S. sobrinus* Gtfs (Russell et al. 1988), SS2 was able to inhibit the formation of both *S. mutans* and *S. sobrinus* biofilms, albeit using higher doses to achieve inhibition of *S. mutans* biofilm formation. Moreover, *S. mutans* produces greater quantities of insoluble-glucans than *S. sobrinus*, which may render the biofilm harder to penetrate. Given that *S. mutans* are more numerically significant in the dental biofilm than *S. sobrinus,* and *S. mutans* is the primary aetiological agent associated with dental caries initiation in young children, the development of minibodies with activity specifically targeted against *S. mutans* Gtf would likely yield better results.

Development of human monoclonal antibodies with the ability to inhibit Gtf activity may have potential for passive antibody application to children. Topical application of antibody would likely have limited effect on bacteria already

established within the oral biofilm. However, oral administration of antibody timed to coincide with or precede the acquisition of *S. mutans* may block their integration into the developing oral biofilm. Earlier acquisition of *S. mutans* is associated with the increased prevalence of dental caries (Alaluusua and Renkonen 1983). Thus, delaying or blocking the entry of *S. mutans* into the developing oral biofilm would potentially reduce the burden of dental caries in young children. The therapeutic potential of the minibody SS2 is currently being investigated *In vivo* using a rodent model of dental caries. If SS2 protects against the development of dental caries in this model the next stage would be to enter SS2 into human clinical trials as a passive vaccine candidate for dental caries.

Having established a biofilm model, this was employed to investigate the effect of cortisol on *S. mutans* growth responses. A number of pathogenic organisms possess the ability to recognise and respond to host hormones (Lyte 1993). The release of stress hormones such as adrenaline, noradrenaline and cortisol can be detected in saliva and could act as environmental cues to alter the growth of oral bacteria. A previous study documented significantly increased planktonic growth responses by *S. mutans* in response to noradrenaline and adrenaline (Roberts et al. 2002). Data reported here demonstrate that the planktonic growth of *S. mutans* in the presence of cortisol was significantly increased. In contrast, cortisol did not have any discernable influence on *S. mutans* biofilm formation, both in terms of biomass and the recoverable cell numbers. Reasons for this may be related to the determination of CFU/ml of *S. mutans* cultures and biofilms grown in the presence of sucrose. Expression of Gtfs and Gbps together with the synthesis of extracellular polymeric glucans in the presence of sucrose results in highly aggregated clusters of cells. Thus, cultures supplemented with sucrose are 'stickier' and so it is less likely that one cell is equivalent to one CFU even after sonication to disrupt cell aggregates. It is likely therefore, that the determinations of the CFU/ml of planktonic cultures grown in the presence of sucrose were highly underestimated and subsequently the effects of cortisol on planktonic growth responses were overestimated. This effect also influenced the quantitative determination of recoverable numbers from *S. mutans* biofilms grown in the presence of sucrose by culture, which gave rise to variable results. The use of CyQuant® and TaqMan® QPCR assays confirmed no detectable differences in the number of *S. mutans* cells forming

biofilms in the presence of cortisol. The prevalence of dental caries follows a socioeconomic status related gradient of distribution (Sisson 2007). However, these disparities cannot be fully explained by differences in access to health care, genetic disposition or health damaging behaviours. In recent decades evidence has emerged that the physiological stress response may provide a common link between low socioeconomic status and chronic disease, including dental caries (Quinonez et al. 2001; Reisine and Litt 1993). Thus, oral bacteria with the ability to respond to host hormones and modulate their growth responses accordingly could disrupt the homeostasis of the oral biofilm and influence disease. While the formation of *S. mutans* biofilms remained unchanged in response to cortisol, it cannot be determined from these investigations whether the gene expression profiles were altered. It would be of interest to perform functional gene expression analysis on *S. mutans* biofilms in the presence of cortisol. The use of TaqMan® QPCR to quantify the number of *S. mutans* CFU/peg demonstrated that it is possible to extract DNA from these biofilms which could be used for functional gene expression analysis. These studies would provide a more comprehensive understanding of host-microbiological interactions in the oral cavity and could provide clues as to why individuals from deprived backgrounds are subject to a higher prevalence of oral diseases.

The sucrose-dependent *S. mutans* biofilm model was also used to determine the minimum concentration (MIC) of LL37 that was required to inhibit *S. mutans* biofilm formation. Unsurprisingly, the minimum inhibitory dose of LL37 required to inhibit biofilm formation was dose-dependent according to the number of *S. mutans* used in the initial inoculum. The concentrations of LL37 required to significantly inhibit *S. mutans* sucrose-dependent biofilm formation were found to be far higher than the mean concentrations reported in this thesis in children aged one-year (2.53 ng/ml) and three-years (6.3 ng/ml) and by others in children aged 13-years (15.8 ng/ml) (Phattarataratip et al. 2011) and 11- 13-years (3.07 µg/ml) (Tao et al. 2005). Thus, data reported here suggest that LL37 has little or no influence on the inhibition of *S. mutans* biofilm formation at concentrations found in the saliva of young children. However, notably the MIC reported here to inhibit *S. mutans* sucrose-dependent biofilm formation is considerably lower than MIC concentrations of LL37 previously reported to

inhibit planktonic cultures of *S. mutans* and other oral streptococci (Ji et al. 2007; Ouhara et al. 2005).

In conclusion, the *In vitro* sucrose-dependent *S. mutans* biofilm model described here is a useful model for investigating the inhibitory potential of a given molecule of interest. And thus represents an important first stage in the analysis of molecules with potentially therapeutic potential prior to application in *In vivo* and clinical studies.

Chapter 7: General Discussion

The primary aim of this thesis was to investigate the biological risk factors associated with dental caries experience in children aged one- to three-years as part of a pilot clinical study of Childsmile participants. These studies documented for the first time the longitudinal increases in the concentrations of lactoferrin, calprotectin, LL37 and the HNPs 1-3 in the saliva of children of this age. There was some suggestion that the concentrations of these salivary antimicrobial proteins were positively correlated with numbers of *S. mutans* in dental plaque. Whether the concentrations of salivary antimicrobial proteins influenced the susceptibility of dental caries experience could not be determined in these studies. The low rates of caries prevalence most likely reflected the low follow-up rates. However, Childsmile intervention has been associated with a reduction in caries prevalence among young children (McMahon et al. 2011), and it cannot be ruled out that this influenced the low caries rates in three-year-old participants reported here.

An important aim of these studies was to investigate the potential biological mechanisms underlying socioeconomic disparities in the dental health of young children. No evidence was identified to indicate an immunological or microbiological profile associated with socioeconomic status. Additionally, these were the first studies to attempt to investigate salivary cortisol concentrations as a surrogate measure of stress in children of this age. No evidence was identified for an association of salivary cortisol concentrations with socioeconomic status of children. The major limitations of these investigations were the small sample sizes, which resulted in low power to detect statistically significant differences. A much larger study is required to begin to delineate the important biological mechanisms which underlie socioeconomic disparities in dental health.

Since its establishment in 2006, Childsmile has since been rolled out as part of a national dental health service for all children in Scotland. The programme now encompasses a much larger target population and thus the potential to conduct large-scale studies has increased. Moreover, with the lessons learned from these studies, future clinical studies conducted within Childsmile have the potential to delineate the important biological mechanisms which lead to dental caries.

The methods used to collect both plaque and saliva samples from young children proved successful. The most important aspect to address in order to increase the potential of future Childsmile clinical studies is the recruitment methods. Childsmile dental practices would likely be an important resource for the recruitment of very young children to future studies. However, in the current studies dental practice participation was minimal. The reasons for this are unclear. The practices were selected from general Dental Practitioners who expressed an interest in the study and volunteered to participate. The incentives were agreed with the practices and deemed reasonable. Thus, successful future dental practice participation requires further investigation and perhaps a revision of the incentives and training. The most successful recruitment was obtained by extended duty dental nurses and the continued participation of these key staff will be required. Additionally, given the high rate of children who failed to attend clinic appointments and the relative success of domiciliary visits in this study, dental health support workers within Childsmile, will likely be instrumental in recruiting children who do not attend dental practices and those who encompass 'hard to reach' children. Using this approach it should be possible to recruit substantial numbers of children from across the socioeconomic spectrum. Additionally, the recruitment of three-year-old children from Childsmile nurseries participating in the NDIP programme as part of Childsmile monitoring could provide an excellent opportunity to longitudinally follow-up children. Indeed, previous large scale caries studies have been successfully performed in this age group and in this setting (McMahon et al. 2010). Moreover, there is immense potential for longer term follow-up via the NDIPs carried out in schools when children are aged five- and 11-years. Thus, the ability to simultaneously collect plaque and saliva samples for microbiological and immunological investigations may be feasible.

Another important aspect to consider for future studies is the study design. The detection of *S. mutans* in dental plaque is a valuable and accepted technique to identify children at increased risk of developing dental caries. However, recent metagenomic studies have provided evidence in support of a polymicrobial aetiology for dental caries. These studies have so far been performed only with small numbers of participants and studies conducted in young children are lacking (Aas et al. 2008; Alcaraz et al. 2012; Belda-Ferre et al. 2011). The

advancement of these technologies and the subsequent reduction in running costs makes this technology more accessible for use in large-scale population studies. A population-based study of the oral metagenome or transcriptome associated with health and disease in young children would likely yield important insights into the initiation of dental caries, and could also have far-reaching implications for associated diseases such as cancer, metabolic and cardiovascular diseases (Aida et al. 2011; Tremblay et al. 2011). Childsmile's young target age group and multiple visit interventions could provide a platform from which to longitudinally investigate the development of the oral metagenome in children through to adolescence and all stages of disease.

In the present studies salivary cortisol concentrations were measured only at a single time point. Ideally a basal cortisol measurement would be established for every study participant. In a recent study, basal cortisol measurements were found to be associated with an increased prevalence of dental caries and lower SES in children aged five- to six-years old (Boyce et al. 2010). This was the first study to provide evidence of the potential biological mechanisms which may underlie socioeconomic disparities in dental health. However, further research is required to corroborate these results and to identify the microbiological and immunological pathways involved. The collection of multiple saliva samples over consecutive days would likely be unattainable in a large-scale study of Childsmile participants. However, careful selection of a sub-sample of participants using a matched-case control design would likely yield important insights.

Further research is required to investigate whether the concentrations of salivary antimicrobial peptides influence the susceptibility to dental caries. As natural antibiotics, antimicrobial peptides are currently under intense investigation for their use as potential therapeutic agents for a myriad of diseases (Liu et al. 2010) Furthermore, there has been some suggestion that low concentrations of antimicrobial peptides, such as the HNPs 1-3 may provide an indicator of caries risk in young children (Tao et al. 2005). A large-scale clinical study of Childsmile participants, using a matched case-control design could identify whether the HNPs 1-3 have potential as therapeutic agents for the protection against dental caries. Furthermore, the data reported here indicated that sIgA antibodies specific for oral streptococci increased in children from one-

to three-years of age. It could not be determined from these studies whether increased antibody titres influenced the susceptibility of dental caries in young children and there was no evidence to support a role for sIgA antibodies influencing colonisation with Mutans streptococci. Thus, further research is required to elucidate the role of sIgA antibodies in dental caries aetiology. In this regard, understanding how the host initiates an adaptive immune response to *S. mutans* is crucial.

An additional aim of these studies was to investigate the initiation of adaptive immune responses to *S. mutans* using a series of *In vitro* and *In vivo* studies. The data reported in this thesis indicate that upon acquisition in the host, *S. mutans* does not elicit an inflammatory immune response and indeed may subvert immune responses to promote its persistence within the oral biofilm. However, additional studies have indicated that protective immunity can be generated and that the breadth of the secretory immune response to *S. mutans* is important (Nogueira et al. 2005; Parisotto et al. 2011). Further in depth investigations to establish the mechanisms which lead to the development of a protective immune response in some individuals are required. In this respect, the use of *In vivo* animal models of dental caries could provide important insights into the kinetics of antigen uptake and presentation in the oral cavity with the identification of the location of effector immune responses and the cells involved. These studies could have significant implications, for both understanding the immune response in the oral cavity and for identifying the components required to establish neutralising immunity to dental caries.

The final aim of this thesis was to develop and optimise the use of an *In vitro* *S. mutans* biofilm model. This biofilm model is arguably more representative of S. *mutans* biofilm formation *In vivo* compared with commonly used microtitre plate assays. The sucrose-dependent biofilm model described here represents a useful model to investigate potentially therapeutic molecules for their ability to inhibit Gtf-mediated adherence and accumulation of *S. mutans*. Using this model, the minibody denoted ‘SS2’ was demonstrated to have inhibitory activity against the formation of *S. mutans* biofilms. Thus this study represented an important first step in characterising the potential of SS2 for therapeutic intervention prior to its application in *In vivo* models of dental caries and possible subsequent use in human clinical trials. The *In vitro* biofilm model described here could represent

an important first stage in investigations of naturally occurring molecules, which may potentially be identified from future clinical Childsmile studies to inhibit *S. mutans* biofilm formation.

7.1 Conclusions

This thesis has documented evidence to suggest that *S. mutans* colonises the oral cavity of young children at a time when they are immunologically immature. As colonisation with *S. mutans* increases, salivary immune responses undergo maturation as indicated by increases in the concentrations of antimicrobial proteins and sIgA antibodies specific for oral streptococci in children aged one- to three-years. Additionally, *In vitro* and *In vivo* evidence suggest that *S. mutans* does not elicit an inflammatory immune response upon colonisation of the host (Figure 7-1). This was despite using infective doses of *S. mutans* far higher than would likely be encountered naturally. Thus, the early acquisition of *S. mutans* in a relatively immunologically immature host together with the absence of an inflammatory immune response likely aids the colonisation of *S. mutans* and its persistence within the oral biofilm and subsequent contribution to dental caries. Further in depth studies building on the observations reported here will significantly advance our understanding of the host-pathogen interactions within the oral cavity. These studies would ultimately provide a platform from which to investigate other common oral infections and thus, create an opportunity for novel therapeutic interventions.

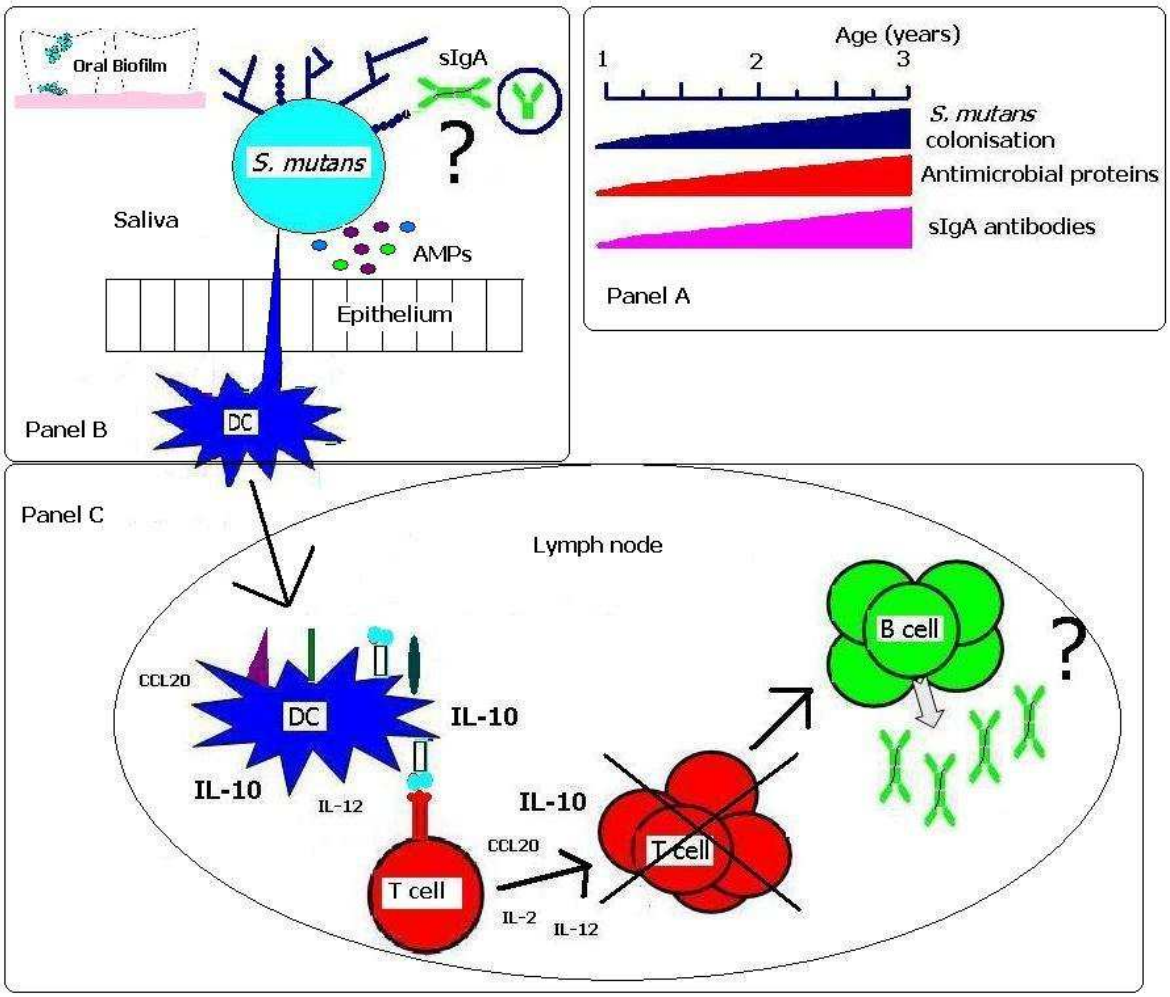

Figure 7-1: Initiation of an immune response to *S. mutans*

With increased microbial colonisation of the oral cavity from one- to three-years of age, salivary immune responses undergo considerable maturation as indicated by increases in salivary antimicrobial proteins and sIgA antibodies (panel A). *S. mutans* can become detached from the oral biofilm via the sheer mechanical forces of saliva, natural dissemination from the oral biofilm and from disruption through tooth-brushing. Once in saliva, *S. mutans* can presumably be detected by DCs, either in the gut or oral mucosa. However, the exact mechanisms of antigen uptake in the oral cavity have yet to be elucidated (panel B). Studies have shown that upon antigen uptake, DCs migrate to the draining lymph node where they direct effector immune responses. *In vitro* data indicated that DCs in the presence of *S. mutans* upregulated cell surface expression of Eα-MHCII peptide complexes and co-stimulatory molecules (CD40, CD80 and CD86). This was accompanied by high level expression of the anti-inflammatory cytokine IL-10 and low levels of pro-inflammatory IL-12 and the chemokine CCL20, which suggested that DCs were not functionally mature. Subsequently, T cells failed to proliferate in response to DCs activated in the presence of *S. mutans*. Once again this response occurred in an environment with high levels of IL-10 coincident with low level expression of pro-inflammatory markers, IL-12, IL-2 and CCL20 and an absence of IFN-γ or IL-17 (panel C). Furthermore, *In vivo* DCs failed to mature and migrate in response to *S. mutans* exposure. Despite these observations, sIgA responses directed against *S. mutans* can be generated and detected. The ultimate outcome of secretory immunity on *S. mutans* natural infection remains to be fully elucidated but is likely shaped by the chronicity of infection, antigenic load, the nature and potentially location of DC responses.

Appendix I: Parental information sheet and consent form

Parent Information Sheet (February 2009 V.3)

Saliva and dental plaque samples to better understand tooth decay

Short title: Collecting saliva and bacteria from children to better understand tooth decay.

WHY HAS YOUR CHILD BEEN CHOSEN?

Your child is invited to take part in this research study as you are part of the Childsmile Programme. Please take time to read the following information carefully.

WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of this study is to collect saliva and oral bacteria from children as it is hoped this will provide valuable information to help us understand dental decay.

We are interested to know how family circumstances might affect dental health in children, so we will be asking you questions on your education, your job, and your income. We ask these questions because they are very important for our study and we would like to assure you that this information will be kept completely confidential as with the rest of the questionnaire.

WHAT DOES YOUR CHILD HAVE TO DO? ARE THERE ANY RISKS?

1. We wish to collect a small volume of saliva by either placing a small plastic collection tube under the tongue allowing the saliva to collect, or by allowing your child to dribble saliva into a tube. It will take a few minutes to collect the saliva.

2. A small amount of dental plaque will be collected from the mouth, using a soft swab. The plaque will then be placed in fluid and the bacteria will be analysed at a later date.

Your child should not experience any discomfort and there are no risks associated with either procedure.

We wish to collect both these samples on two occasions, approximately 18 months apart, at a time when your child is attending for dental check ups. This will provide information about how saliva and oral bacteria change over time.

DOES YOUR CHILD HAVE TO TAKE PART?

It is up to you to decide whether or not your child should take part in this study and donate saliva and swabs dental plaque. If you decide you would like your child to take part in the study, you will be given this information sheet and consent form to sign and keep. You will have an opportunity to ask questions and have them answered to your satisfaction. If you choose for your child not to take part in the study, your child will not be disadvantaged in any way.

WHAT ARE THE BENEFITS OF YOUR CHILD TAKING PART?

Your child's participation in this study may help improve dental care for children in the future. We know that differences in saliva and oral bacteria can cause differences in how much tooth decay occurs, and this study may help understand why and may help prevent dental decay in the future. However, your child will not directly benefit from taking part in this study.

WHAT WILL HAPPEN TO MY SAMPLES AND PERSONAL DATA?

Your child's samples and associated personal medical data ("Study Data") will be stored securely, processed and used for investigations at the University of Glasgow. Any preserved

saliva samples and Study Data may be stored for up to 15 years. All samples will only be used for the purposes described.

The studies that we are doing on your child's saliva and dental plaque will not suggest any clinical diagnosis or treatment for any disease and it is not the purpose of this research to provide you or your child with test results.

By signing the Consent Form you consent to the Study Dentist and his or her staff collecting and using your child's personal data for the study. This includes your participant profile, your child's date of birth, sex, postcode and tooth decay score (the tooth decay score is collected as part of the normal dental exam). Your consent to the use of your child's Study Data does not have a specific expiration date. By signing this form you consent to the use of Study Data as described in this form.

HOW WILL MY CONFIDENTILITY BE PROTECTED?

Special precautions are taken to ensure the research study is carried out with a high degree of confidentiality. If you agree to participate in the study, a code that is specific to your child will be used to label your child's samples and Study Data and identify all results that are recorded at the University of Glasgow. This coding of all information is to ensure that the results are kept confidential by keeping your child's identity and the results separate. **Samples will not include your name, address or Hospital number.** Only the Study Doctor and health care professionals who are part of the Childsmile programme have access to the code key that will connect your Study Data to you. The Study Doctor is responsible for handling of your child's Study Data in accordance with applicable Data Protection law(s). Please note, the results of the study may be published in medical literature, but your child will not be identified.

CAN I WITHDRAW MY CONSENT?

If you decide to donate your child's saliva and plaque, you are free to withdraw your consent for use of the sample at any time. If you choose to withdraw your consent, your child will not be disadvantaged in any way, including dental treatment and care your child is entitled to receive. If you withdraw your consent, your child's sample(s) will be destroyed. The University may still use Study Data obtained before you withdrew your consent.

WHO SHOULD I CONTACT FOR INFORMATION OR HELP?

Dr Shauna Culshaw
Clinical Lecturer/ Hon SpR
Glasgow Dental School & Hospital
378 Sauchiehall Street
Glasgow, G2 3JZ
Tel: 0141 211 9703

Prof Lorna Macpherson
Professor of Dental Public Health
Glasgow Dental School & Hospital
378 Sauchiehall Street
Glasgow, G2 3JZ
Tel: 0141 211 9750

Thank you for taking the time to read this invitation.

CONSENT FORM-SALIVA AND MICROBIAL PLAQUE

Saliva and dental plaque samples to better understand tooth decay

(November 2008 version 2)

PATIENT LABEL

Patient Identification Number for this trial: ___ _ _ _ _ _ _ _

1. I confirm that I have parental responsibility for the child above and have read and understood this information. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. I have read and understand the information sheet dated August 2008 for the above study. ☐
2. I understand that my child's participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I consent to allow a microbial plaque and saliva sample to be taken from my child for research purposes. I agree to samples being taken for research purposes on two occasions, approximately 18 months apart ☐
4. I agree that my child taking part in the above study. ☐
5. I confirm that I have received a signed copy of this information and consent form to keep. ☐
6. I give permission for my child's health information to be used for the monitoring and evaluation of this research study and the Childsmile programme. ☐

Name of Patient's Parent	Signature	Date
Name of Research Team Member	Signature	Date

One copy to be retained by parent, one copy to be placed in the patient's notes, and one copy to be retained in study file.

Appendix II: West of Scotland Research Ethics Committee Approval Letter

WoSRES
West of Scotland Research Ethics Service

NHS
Greater Glasgow and Clyde
West of Scotland REC 1

Western Infirmary
West of Scotland Research Ethics Service
Ground Floor, Tennent Institute
38 Church Street
Glasgow
G11 6NT

Tel: 0141-211-6230
Fax: 0141-211-1847

Ref AHT/SAJ

14 December 2010

Dr Shauna Culshaw
Clinical Lecturer in Periodontology
University of Glasgow Dental Hospital and School
Level 7
378 Sauchiehall Street
Glasgow
G2 3JZ

Dear Dr Culshaw

Study title:	**Unravelling the relationship between the host response and the development of oral biofilms.**
REC reference:	**08/S0703/139**
Amendment number:	**Amendment no 2 November 2010**
Amendment date:	**22 November 2010**

The above amendment was reviewed at the meeting of the Committee held on 14 December 2010.

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
Questionnaire: Validated (trial version)	trial version	10 November 2010
Participant Consent Form	version 3	03 November 2010
Participant Information Sheet: Parent	version 3	03 November 2010
Protocol	version 2	03 November 2010
Notice of Substantial Amendment (non-CTIMPs)	Amendment no 2 November 2010	22 November 2010

7.2

Appendix III: NHS Greater Glasgow and Clyde R&D Management Approval Letter

Online Form

23. Authorisations required prior to R&D approval

This section deals with authorisations by managers within the NHS organisation. It should be signed in accordance with the guidance provided by the NHS organisation. This may include authorisation by clinical supervisors, line managers, service managers, support department managers, pharmacy, data protection officers or finance managers, depending on the nature of the research. Managers completing this section should confirm in the text what the authorisation means, in accordance with the guidance provided by the NHS organisation.

This section may also be used by university employees or research support staff to provide authorisation to NHS organisations, in accordance with guidance from the university.

S. F. Allan.
R&D Lead Children Services.
Yorkhill. 18/9/08.

Declaration by Principal Investigator or Local Collaborator

1. The information in this form is accurate to the best of my knowledge and I take full responsibility for it.
2. I undertake to abide by the ethical principles underpinning the World Medical Association's Declaration of Helsinki and relevant good practice guidelines in the conduct of research.
3. If the research is approved by the main REC and NHS organisation, I undertake to adhere to the study protocol, the terms of the application on which the main REC has given a favourable opinion and the conditions requested by the NHS organisation, and to inform the NHS organisation within local timelines of any subsequent amendments to the protocol.
4. If the research is approved, I undertake to abide by the principles of the Research Governance Framework for Health and Social Care.
5. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to the conduct of research.
6. I undertake to disclose any conflicts of interest that may arise during the course of this research, and take responsibility for ensuring that all staff involved in the research are aware of their responsibilities to disclose conflicts of interest.
7. I understand and agree that study files, documents, research records and data may be subject to inspection by the NHS organisation, the sponsor or an independent body for monitoring, audit and inspection purposes.
8. I take responsibility for ensuring that staff involved in the research at this site hold appropriate contracts for the duration of the research, are familiar with the Research Governance Framework, the NHS organisation's Data Protection Policy and all other relevant policies and guidelines, and are appropriately trained and experienced.
9. I undertake to complete any progress and final reports as requested by the NHS organisation and understand that continuation of permission to conduct research within the NHS organisation is dependent on satisfactory completion of such reports.
10. I undertake to maintain a project file for this research in accordance with the NHS organisation's policy.
11. I take responsibility for ensuring that all serious adverse events are handled within the NHS organisation's policy for reporting and handling of adverse events.
12. I understand that information relating to this research, including the contact details on this application, will be held by the R&D office and may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.
13. I understand that the information contained in this application, any supporting documentation and all correspondence with the R&D office and/or the REC system relating to the application will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

Appendix IV: Sociodemographic Questionnaire

Childsmile Study: **Saliva and dental plaque samples to better understand tooth decay** v2 December 2008

PARENT PROFILE

These questions are about YOU (not your child). We ask these questions because they are very important for our study but we would assure you that this information will be kept completely confidential.

Child Code (this will be supplied by the research team).....

1.1 What is your Gender (please tick): Male □ Female □

1.2 How old are you? (please write age in years in box) Age ☐

1.3 Do you smoke? (please tick) Yes □ No □

1.4 How many children do you have?.....

1.5 Ages and genders of children

Age of child	Gender of child

2. Infant feeding

2.1 Do you/did you breastfeed? (please tick) Yes □ No □

If yes for how many months? ..... ☐ .....months

2.2 At what age did you/do you plan to start to wean your child? ☐ months

Please complete the questions on the next page

Child Code (this will be supplied by the research team).....

3. Education

3.1 How many years of full time education did you complete? ☐ Years

3.2 What is the highest educational level you obtained? (please circle)

1 = Primary school

2 = Secondary school

3 = School or college sixth form

4 = College of Further Education

5 = Polytechnic or University

6 = Some other type of college_____ (specify)

4. Occupation questions

4.1 What is your current / or what was your last occupation? _____

4.1 What is your partner's / spouse's current occupation? _____

5. Income questions

5.1 Would you please look at this card and give me the letter for the group in which you would place total household income (including yourself) from all sources, before tax and other deductions.

(write letter in box) ☐

5.2 What proportion of your household income (including your own) would you say comes form benefits?

Please tick ONE box

None ☐ very little ☐ about a quarter ☐ about a half ☐ about three quarters ☐ all ☐

Appendix V: Food frequency questionnaire

UNIVERSITY OF ABERDEEN

Scottish Collaborative Group Food Frequency Questionnaire version C2

Diet questionnaire for children

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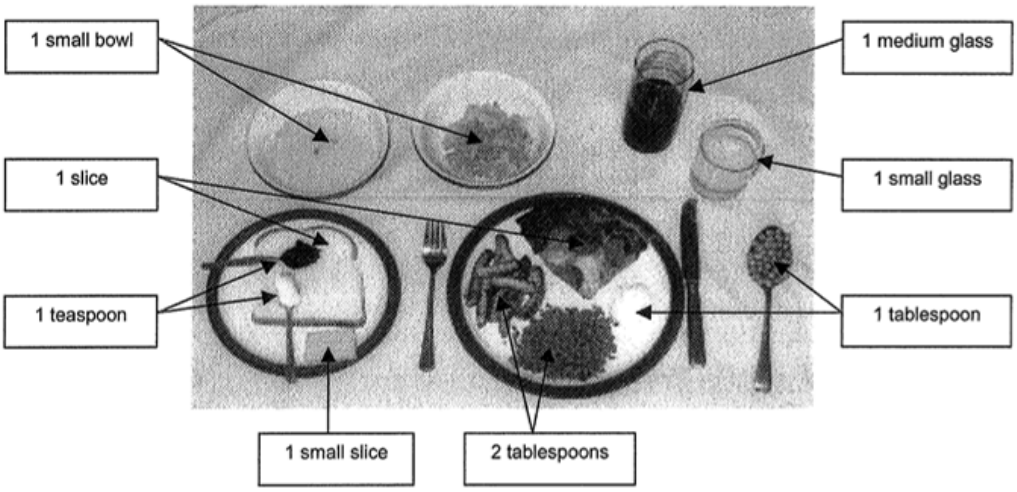
We would like you to describe your child's usual diet over the last 2-3 months. This should include all main meals, snacks, and drinks. You should also include any foods and drinks your child consumed outside your home, e.g. at school or nursery, at out of school clubs, at restaurants or cafes or with friends and other family members.

The questionnaire lists 140 types of foods and drinks. For each food or drink a measure is given which describes a small portion to help you estimate how much your child usually has. The photograph below gives examples of some of these measures.



How to complete the questionnaire

Please take a few minutes to read the instructions carefully.

Please use **black or blue pen** to complete the questionnaire: do not use pencil.

For **every line** in the questionnaire, you need to tick **one box** to say how many times your child usually has this food or drink.

- If your child does not usually have any of this food or drink, please tick the first box (rarely or never).
- If your child has the food or drink more than once a month but less than once a week, please tick the next box (one or two per month).
- If your child has the food or drink every week but not every day, please tick one of the weekly boxes to indicate how many measures of this food or drink he/she has in a typical week (1 per week, 2-3 per week or 4-6 per week).
- If your child has the food or drink every day, please tick one of the daily choices (1 per day, 2-3 per day, 4-6 per day or 7 or more per day).

For dishes that are made up of more than one food you may have to split it up into its separate parts e.g. a ham sandwich (2 slices of white bread, 1 teaspoon of butter and 2 slices of ham).

For a few foods, your child may have more than one measure on several days a week but not every day. For these foods please use the daily choices which give approximately the same total intake per week, e.g. for 8-10 measures per week please tick 1 per day (see example of white bread below).

Example:

If your child has a piece of Weetabix every day, three medium glasses of regular blackcurrant diluting juice every day, two slices of white bread 5 days a week, an apple twice a week, but never has peanut butter, your answers should look like this:

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
Unsweetened cereals (e.g. Cornflakes, Shreddies, Weetabix, Rice Krispies)	1 small bowl, 3 tablespoons or 1 piece						√			
Regular blackcurrant diluting juice	1 medium glass							√		
White bread or rolls	1 slice or roll						√			
Apple	1 small apple				√					
Peanut butter	1 teaspoon	√								

If you want to change an answer, simply cross out your first tick and add another one in the right box.

If your child has any foods or drinks which are not listed, or if you are not sure about where to add any foods or drinks, please use section 17 ('other foods') at the end of the questionnaire.

It is very important that you put a tick on every line.
If your child rarely or never has the food, it is very important that you tick the box for rarely or never.

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
1. Breakfast cereals										
Unsweetened cereals (e.g. Cornflakes, Shreddies, Weetabix, Rice Krispies)	1 small bowl, 3 tablespoons or 1 piece									
Sweetened cereals (e.g. Frosties, Sugar Puffs, Coco Pops, Honey Nut Loops)	1 small bowl or 3 tablespoons									
Ready Brek or porridge	1 small bowl or 3 tablespoons									
Muesli (all types)	1 small bowl or 3 tablespoons									
2. Bread *(including sandwiches and toast)*										
White bread or rolls	1 slice or roll									
Brown or granary bread or rolls	1 slice or roll									
Wholemeal bread or rolls	1 slice or roll									
Croissants, garlic bread or Aberdeen rolls	1 roll or 2 slices									
Other breads (e.g. pitta, naan, tortilla, bagel)	1 piece									
3. Milk *(in drinks and on cereals)*										
Full fat cow's milk	1 small glass or 1/4 pint									
Semi-skimmed cow's milk	1 small glass or 1/4 pint									
Skimmed cow's milk	1 small glass or 1/4 pint									
Soya Milk	1 small glass or 1/4 pint									
Flavoured milk (e.g. chocolate, strawberry)	1 small glass or 1/4 pint									
4. Yogurt, cheese and eggs										
Drinking yogurts (Actimel, Yakult)	1 bottle									
Flavoured yogurts (e.g. all fruit yogurts, Crunch Corners, Crunchie)	1 small pot									
Fromage frais (all flavours)	1 small pot									
Natural, low fat or low calorie yogurt	1 small pot									
Cream (all types)	1 tablespoon									
Full fat cream cheese (e.g. Philadelphia)	1 tablespoon									
Cheddar-type cheese (including Cheese strings)	1 small slice or 1 stick									
Edam, Brie or cheese spreads (e.g. Dairylea)	1 slice, 1 piece or 1 tablespoon									
Low fat hard or soft cheese	1 slice or 1 tablespoon									
Eggs (boiled, fried, scrambled or omelette)	1 egg									

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
5. Meat *(excluding Quorn and Soya)*										
Meat burgers or mince	1 small burger or 1 tablespoon									
Meat sauce (e.g. on pasta)	1 tablespoon									
Frankfurters	1 sausage									
Fried or grilled sausages	1 sausage									
Bacon or gammon	1 slice									
Cold ham or turkey	1 slice									
Salami or continental sausage	1 slice									
Stewed, fried, grilled or roast beef, pork or lamb	1 tablespoon or 1 slice									
Chicken nuggets	1 serving									
Casseroled, fried, grilled or roast chicken or turkey	1 tablespoon or 1 slice									
Meat or chicken pies, pasties or sausage rolls	1 individual pie or 1 roll									
6. Fish										
Fish fingers	1 finger									
Fish cakes or fish pie	1 fish cake or 1 tablespoon									
Grilled or poached white fish (cod, haddock, plaice)	1 small fillet									
White fish fried or cooked in batter or scampi	1 small fillet or 1 serving									
Grilled oily fish (fresh tuna, salmon, mackerel, herring)	1 small fillet									
Fried oily fish (fresh tuna, salmon, mackerel, herring)	1 small fillet or slice									
Smoked oily fish (kipper, mackerel, salmon)	1 small fillet									
Tinned tuna	1 tablespoon									
Tinned salmon, sardines, mackerel, pilchards	1 tablespoon or 1 small fillet									
Prawns	1 tablespoon									
7. Potatoes, rice and pasta										
Boiled, mashed or baked potatoes	1 tablespoon or 1 potato									
Potato croquettes or waffles	1 piece									
Roast or fried potatoes	1 potato or 2 tablespoons									
Oven chips	2 tablespoons									
Home-cooked chips	2 tablespoons									
Chips from a chip shop, café or restaurant	1 small bag									
Spaghetti and other pasta or couscous	2 tablespoons (cooked)									
Rice (all types)	2 tablespoons (cooked)									
Noodles (all types)	2 tablespoons (cooked)									

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
8. Savoury dishes, soups and sauces										
Pizza	1 small (6 inch) pizza or 1 slice									
Quiche	1 slice									
Quorn, Soya or Tofu products (all types)	1 serving									
Nut roast, nut burgers or vegetable burgers	1 serving									
Baked beans	1 tablespoon									
Other beans or lentils (excluding soups)	1 tablespoon									
Canned or dried soup	1 small bowl									
Home-made soup	1 small bowl									
Bottled sauces (e.g. tomato ketchup)	1 teaspoon									
Tomato sauce (e.g. for pasta)	1 tablespoon									
Other sauces (e.g. cheese, white, curry, sweet & sour)	1 tablespoon									
Gravy	1 tablespoon									
Mayonnaise or salad cream	1 teaspoon									
9. Vegetables *(fresh, frozen and tinned)*										
Mixed vegetable dishes (e.g. stir-fry, curry)	1 tablespoon									
Peas or green beans	1 tablespoon									
Sweetcorn	1 tablespoon or 1 small cob									
Broccoli	1 tablespoon or 2 pieces									
Cabbage	1 tablespoon									
Spinach	1 tablespoon									
Other green vegetables (e.g. leeks, courgettes)	1 tablespoon									
Cauliflower, swede (neeps) or turnip	1 tablespoon									
Raw carrot	1/2 carrot									
Cooked carrot	1/2 carrot									
Onions	1/4 onion or 2 teaspoons									
Tomatoes	1 tomato									
Peppers	1/4 pepper									
Other salad vegetables (e.g. lettuce, cucumber, celery)	1 small serving									
Coleslaw	1 tablespoon									
Potato salad	1 tablespoon									

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
***10. Fruit** (fresh, frozen and tinned)*										
Fresh fruit salad	1 tablespoon									
Tinned fruit (all kinds)	1 tablespoon									
Apples	1 small apple									
Oranges	1 small orange									
Bananas	1 small banana									
Grapes, melon, pear	1 small serving									
Kiwi	1 fruit									
Other fresh fruit (e.g. peaches, strawberries etc)	1 small serving									
Dried fruit (all kinds)	1 tablespoon									
11. Juice and other drinks										
Pure apple juice	1 small glass									
Other pure fruit juice (orange, pineapple etc.)	1 small glass									
High juice fruit drinks (Five Alive, Sunny Delight etc.)	1 small carton, medium glass									
Regular fruit juice drinks (e.g. Fruit Shoots, Capri Sun, Ribena cartons)	1 small bottle, pouch or carton									
Other fruit flavoured drinks including flavoured water (e.g. Calypso Cartoon)	1 carton, small bottle or medium glass									
Regular blackcurrant diluting juice	1 medium glass made-up									
No added sugar blackcurrant diluting juice	1 medium glass made-up									
Regular orange, lemon or other diluting juice	1 medium glass made-up									
No added sugar orange, lemon or other diluting juice	1 medium glass made-up									
Regular fizzy drinks (e.g. lemonade, Im Bru, Cola)	1 medium glass or 1/2 can									
Low calorie or diet fizzy drinks	1 medium glass or 1/2 can									
Drinking chocolate powder	2 teaspoons or 1 sachet									
Tea (excluding fruit, herbal or green)	1 cup									
Tap or mineral water (not in other drinks)	1 medium glass									
Smoothies (all kinds)	1 small bottle or carton									
12. Sugar, jam and other spreads										
Sugar (on cereals and in drinks but not in cooking)	1 teaspoon									
Jam, honey or marmalade	1 teaspoon									
Peanut Butter	1 teaspoon									
Chocolate spread	1 teaspoon									
Marmite	1 serving									
Butter or margarine	1 teaspoon									

Food	Measure	Rarely or never	One or two per month	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day
13. Crisps, nuts and savoury snacks										
Regular crisps (all types)	1 small bag									
Reduced fat crisps (all types)	1 small bag									
Other savoury snacks (Quavers, popcorn etc.)	1 small bag									
Peanuts and other nuts	1 small bag									
Savoury biscuits, crackers or breadsticks	1 biscuit or 2 sticks									
14. Biscuits and cakes										
Plain biscuits (e.g. Rich Tea, Digestive, ginger nuts)	1 biscuit									
Fancy biscuits (e.g. creams, iced biscuits)	1 biscuit									
Chocolate biscuits or cookies (all types)	1 biscuit									
Cereal bars or flapjacks	1 biscuit									
Scones or pancakes	1 piece									
Doughnuts, muffins or pastries	1 piece									
Fruit cake or malt loaf	1 small slice									
Plain cakes	1 small slice									
Cakes with icing	1 small slice									
Cream cakes or gateaux	1 small slice									
15. Desserts										
Mousse, blancmange or trifle	1 small pot or 2 tablespoons									
Jelly	1 tablespoon									
Milk puddings (e.g. rice, semolina)	1 tablespoon									
Sponge puddings (jam, steamed, syrup etc.)	1 tablespoon									
Fruit tarts, crumbles or pies	1 small slice or 1 tablespoon									
Custard	1 tablespoon									
Cheesecake	1 small slice									
16.Sweets, chocolates and ice-creams										
Boiled, chewy or chocolate sweets (e.g. toffee, chews, fruit gums)	1 small packet									
Chocolate bars (e.g. Mars, Milky Way, Dairy Milk)	1 small bar									
Wrapped ice creams (e.g. Solero, Cornetto, choc ice)	1 ice-cream									
Other ice cream (all flavours)	1 scoop or 1 small tub									
Iced lollies	1 lolly									

17. Other foods

Please enter details of any foods or drinks which your child has at least once a week which have not been included in the questionnaire above

Food or drink description	Amount usually consumed	1 per week	2 – 3 per week	4 – 6 per week	1 per day	2 – 3 per day	4 – 6 per day	7 or more per day

18. Brand details

Please give full details of the types (including brand name if possible) of any of the following foods which your child usually has

Butter or Margarine (e.g. Flora Buttery)

..... Office code ☐☐

..... Office code ☐☐

Oil or fat used for home cooking (e.g. Tesco corn oil)

..... Office code ☐☐

..... Office code ☐☐

19. Dietary supplements

Please give as full details as possible (including brand name and amount used) of any supplements

	Brand name and strength	Amount usually taken per week (e.g. 7 tablets, 2 teaspoons)
Vitamins or multivitamins		
Cod liver oil or other oil		
Other supplement		

20. Any other information on your child's diet

Date of completing questionnaire .....

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