A Method Development Study to Investigate the Efficacy of Different Frequencies of use of a Dental Cleanser

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Abstract

Optimal denture hygiene is key in the prosthesis wearing patient-population to avoid potentially harmful effects of pathogenic microorganisms that colonise the denture surface. Regular and effective cleaning of a denture is, therefore, believed to be essential for maintenance of healthy oral tissues. However, there is insufficient evidence to establish the most effective denture cleaning regimen.

The aim of this method development randomised controlled crossover clinical trial was to examine the effect of frequency of use of a denture cleanser on the microbial, clinical and patient-reported outcome measures of denture cleanliness. Its purpose was to establish an initial evidence base that would inform future larger scale studies in this area.

Nineteen patients were randomised and completed both seven-day treatment periods of the study. Clinical assessments, microbiological sampling and patient questionnaires were completed at selected time points to allow comparison between groups. A further *in vitro* study analysed the impact of different sampling methodologies on the number of microbes retrieved, to inform clinical protocols for future studies.

The results demonstrated that an increased frequency of use of a denture cleanser resulted in improved clinical and microbiological outcomes for denture cleanliness. These data can be used to adequately power future studies to confirm the findings of this initial study. The observation of the importance of moisture level at the sampling site will inform development of protocols for optimal sampling of denture plaque in future studies.

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Publications Based on This Thesis

1. Gordon Ramage, Lindsay O'Donnell, Leighann Sherry, Shauna Culshaw, Jeremy Bagg, Marta Czesnikiewicz-Guzik, Clare Brown, Debbie McKenzie, Laura Cross, Andrew MacInnes, David Bradshaw, Roshan Varghese, Paola Gomez Pereira, Anto Jose, Susmita Sanyal & Douglas Robertson (2019) Impact of frequency of denture cleaning on microbial and clinical parameters – a bench to chairside approach, Journal of Oral Microbiology, 11:1, 1538437, DOI: 10.1080/20002297.2018.1538437

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Author's Declaration

I declare that the work described in this thesis is my own work, unless otherwise acknowledged or referenced. This was completed under the supervision of Dr Laura Cross and Professor Jeremy Bagg.

This body of work has not been submitted as part of any other degree.

Definitions/Abbreviations

Colony-forming Units -	CFUs
GlaxoSmithKline -	GSK
Regional ethics committee -	REC
Glasgow Dental Hospital -	GDH
Serious adverse event -	SAE
Electronic case report form -	eCRF
Oral soft tissue -	OST
Analysis of covariance -	ANCOVA
Analysis of variance -	ANOVA
Species -	spp.
Patient reported outcome measures -	PROMs
Sodium hypochlorite -	NaOCl
Colony-forming equivalents-	CFEs
Polymethylmethacrylate -	PMMA
Subject assessment questionnaire -	SAQ

1 Introduction

1.1 Denture Cleanliness

Optimal denture hygiene is key in the prosthesis-wearing patient population to avoid potentially harmful effects of denture pathogens. The pathogenic effects of microorganisms that can colonise the denture-fitting surface has been established in the dental literature (O'Donnell and University of Glasgow, Budtz-Jørgensen *et al.*, 2000). Regular and effective cleaning of a denture is thought to be key in maintaining healthy oral tissues, in the same manner that effective toothbrushing is important in the prevention of dental and periodontal disease (Felton *et al.*, 2011b).

Although denture hygiene is a fundamental aspect of care for any dental prosthesis, there is insufficient evidence to establish the most effective denture cleaning regimen. Denture hygiene practices involve several important variables which will determine their anti-microbial and clinical effect. There is some agreement in the dental literature as to the superior effects of both a chemical and mechanical denture cleanser but an underexplored variable is the optimal frequency at which these should be used (de Souza *et al.*, 2009). Denture plaque is a complex microbial biofilm, which if left untouched following its initiation, will mature and develop on the relatively protected surface of the denture base. A mature plaque biofilm will increase the risk of denture stomatitis in a susceptible host (Kulak-Ozkan *et al.*, 2002).

Frequency of use of a denture cleanser may also be more pertinent in different patient groups. Elderly patients or those with a reduced capacity for self-care may be less effective in debridement and disinfection of a denture base therefore will require more regular episodes of denture hygiene to maintain a satisfactorily clean denture (Nishi *et al.*, 2012).

The aim of this pragmatic randomised controlled clinical trial was to establish the effect of frequency of use of a denture cleanser on the microbial, clinical and patient-reported outcome measures of denture cleanliness.

1.2 Demographics of Removable Denture Wearers

Globally the average age of the adult population is increasing, with an estimated 810 million people currently aged 60 or older and an increasing proportion of the population in this age group year on year (Guzman *et al.*, 2012).This trend is expected to continue and will inevitably have an effect on the dental healthcare needs of patients in the UK and internationally. It is predicted that due to the increasing numbers of elderly patients across adult age groups, in spite of the increased prevalence of tooth retention, the number of patients requiring complete dentures in developed countries will continue to increase (Douglass *et al.*, 2002). Within the UK, although the effect of an older population is offset by a decreasing incidence of edentulousness, as observed in the 2009 Adult Dental Health Survey, there is still a significant need for removable prostheses, with one in five of the UK adult population estimated to wear a partial or complete denture (Watt *et al.*, 2013).

Increasing levels of edentulousness are correlated with a decrease in oral function and can have a significant impact on social interaction and nutritional intake, compared to dentate individuals (Cousson *et al.*, 2012, Tsai and Chang, 2011). Wearing a removable prosthesis has been shown to improve oral function in this patient population, particularly in relation to social interaction, and may compensate for the functional deficit resulting from tooth loss (Mollaoglu and Alpar, 2005). Effectively, dentures are a foreign body within the oral cavity, which can cause unwanted sequelae as described below.

Increasing age is also a risk factor for poorer denture hygiene and may be associated with denture stomatitis (Baran and Nalçacı, 2009, Gendreau and Loewy, 2011). This is a significant factor to consider when attempting to provide evidence-based recommendations on denture hygiene for patients. Elderly patients may therefore require an increased frequency of use of a denture cleanser to achieve a satisfactory level of denture hygiene (Nishi *et al.*, 2012).

1.3 Denture Plaque

It is well understood that dentures aggregate a complex microbial plaque upon their surface which may be host to a variety of pathogenic microorganisms

(O'Donnell *et al.*, 2015a). The denture-fitting surface is an environment which is relatively protected from the oral cavity proper and is consequently predisposed to becoming a microbial reservoir for potentially pathogenic bacteria. This includes shielding from the clearance effect of saliva, which through mechanical flushing may cause disturbance to the forming biofilm, allowing the development and maturation of the microbial biofilm (Radford *et al.*, 1999). The structure of denture plaque as a biofilm is important to its pathogenicity, with microbial synergism and reduced susceptibility to anti-microbial agents increasing its pathogenic effect (Marsh, 2005).

Additionally, the rough, non-polished denture-fitting surface or that of a reline/rebase material may provide an irregular surface which can aid in the formation of the microbial biofilm by promoting its adherence to the prosthesis (Izumida *et al.*, 2014). It is accepted that the formation of denture plaque is intimately related to the effectiveness of the denture hygiene practices employed (Coulthwaite and Verran, 2007). It is also understood that the microbial biofilm of a denture wearer will be affected by a variety of other factors both within and out with the oral cavity. For example, a study by O'Donnell *et al.,* (2015) demonstrated that the oral microbiome is impacted by whether the patient wears a removable denture, if the denture is a complete arch prosthesis, or whether there are opposing natural teeth.

Furthermore, a greater prevalence of denture stomatitis has been observed in smokers compared to non-smokers in a prosthesis-wearing population (Morris *et al.*, 2013, Charlson *et al.*, 2010, dos Santos *et al.*, 2010). Patients' smoking habits are also known to impact on the microbial flora of the oral cavity (Wu *et al.*, 2016).

In addition to having significant local implications within the oral cavity, pathogens present in mature denture biofilms have been linked to systemic diseases such as bacterial endocarditis, aspiration pneumonia, respiratory tract infections and chronic obstructive pulmonary disease (Felton et al., 2011b).

Despite its importance in the pathogenesis of denture-related pathologies, there are no universally utilised standardised indices for quantifying plaque on denture surfaces (Paranhos *et al.*, 2010). There are several methodological approaches

to assessment of the quantity of dental plaque including: dry or wet weight, quantity of colony-forming units, clinical assessment of biofilm maturation, plaque quantity, or percentage area coverage of a denture base (Coulthwaite and Verran, 2009). There is little agreement in the literature as to the optimum method of plaque scoring and relationship of different images, although most commonly a ordinal scale is used and an index which scores the percentage coverage or visibility of biofilm is employed. Studies may utilise stained plaque scores and assess different areas of the polished, fitting, or tooth surface of the denture for assessment. Plaque virulence is also a key component of its clinical impact. Assessment methodologies to ascertain virulence include biochemical assays, analysis of oxygen metabolism from plaque bacteria, in addition to direct assessment of the clinical pathogenesis associated with plaque deposits (Coulthwaite and Verran, 2009). These heterogeneous methodologies limit the ability to compare results within the dental literature.

In this study, plaque assessment was carried out using a modified version of the clinical classification of denture cleanliness, as described by Blair *et al*. (1995). This index was utilised due to its simplicity, ease of use, reproducibility and its relative cost-effectiveness, as it did not require staining or image analysis.

1.4 Denture Staining

A marker of denture cleanliness is the presence or absence of denture staining. In an increasingly aesthetically aware patient population, denture staining is regarded as unacceptable. Therefore, assessment of the stain removal properties of a denture cleanser is a requirement from a clinical and commercial standpoint.

Several factors are involved in the susceptibility of dentures to staining. These include patient level factors such as gender, smoking and tea consumption and denture-related factors such as the presence of wear, ageing, overnight wear, duration of denture use (years in use) and hygiene method (Yang *et al*., 2014).

There is insufficient agreement in the dental literature regarding the best assessment method for dental staining. In this study, a modification of the Denture Cleanliness Index was used for stain assessment (Mylonas *et al*., 2014).

This semi-quantitative index was selected due to its simplicity and ease of use, although neither its sensitivity nor specificity have been validated within the dental literature.

1.5 Denture Stomatitis

Microbial biofilms on dentures have been strongly associated with the aetiology of denture stomatitis, a condition characterised by erythema and inflammation of the denture-bearing tissues. Denture stomatitis has a wide range of clinical presentations which are not reliably correlated with the level of disease evident. Denture stomatitis usually presents as clinically asymptomatic but may cause a burning sensation of the denture-bearing mucosa, varying degrees of pain or a bad taste (Ramage *et al.*, 2004, Budtz-Jörgensen, 1974).

Within the dental literature there are wide-ranging prevalence figures quoted for denture stomatitis with prevalence rates varying considerably depending on the patient population studied. One reason for this is the method of patient recruitment employed in each study. Conventionally, studies recruited patients from one of two groups: patients attending prosthodontic clinics or institutionalised patients. This may introduce a bias into the analytical study design as each individual population may have an antecedent condition predisposing them to denture stomatitis. For example, institutionalised patients may have a poorer level of oral and denture hygiene (Marchini *et al.*, 2006, Nishi *et al.*, 2012). It has been reported in the literature that the prevalence of denture stomatitis among denture wearers may range from 15% to in excess of 70% (Gendreau and Loewy, 2011). This condition has been demonstrated to be more prevalent in women and the elderly (Gendreau and Loewy, 2011, Mikkonen *et al.*, 1984).

Denture stomatitis has been described using various classifications. In the literature, the most commonly used is Newton's classification (Gendreau and Loewy, 2011). Erythematous candidosis may have a variety of clinical presentations: ranging from pinpoint/localised hyperaemic lesions associated with a denture base (Newton's Type 1), through to generalised erythema (Newton's Type 2) and, in its most severe form, papillary hyperplasia or the granular presentation of denture stomatitis (Newton's Type 3) (AV, 1962).

Denture stomatitis is known to be multifactorial in nature and to be influenced by such factors as candidal infection, salivary flow, age of prosthesis, denture base material, denture trauma, prosthesis wear patterns, smoking, nutritional intake, immunodeficiency and general denture cleanliness. The presence of *Candida* spp., particularly *Candida albicans* and *Candida glabrata*, play a clinically important role in the development of the disease (Jose *et al.*, 2010, Coco *et al.*, 2008, Ercalik-Yalcinkaya and Oezcan, 2015). A key factor in the prevention of denture stomatitis is good denture hygiene practices (Kulak-Ozkan *et al.*, 2002, Budtz-Jørgensen *et al.*, 2000). Poor denture hygiene, particularly in cases where *Candida albicans* infection is also present, has been demonstrated to be intimately related to the presence of denture stomatitis (Jeganathan *et al.*, 1996, Budtz-Jörgensen *et al.*, 1975).

Also evidenced to be a significant factor in the initiation and development of denture stomatitis is denture trauma which precipitates the inflammatory reaction in the presence of several other predisposing factors. This was demonstrated in a randomised controlled trial which evidenced that stabilisation of a denture base with limitation of associated denture-induced trauma significantly reduced the likelihood of denture stomatitis (Emami *et al.*, 2008).

The host response and ability to tolerate and respond to the predisposing factors related to denture stomatitis also play a clinically significant role in disease pathogenesis and virulence (Farah *et al.*, 2000). Patients with compromised host defence mechanisms or who have a degree of immunodeficiency are particularly prone to candidosis (Farah *et al.*, 2000). Furthermore, it is well reported in the dental literature that denture-wearing habits, age of prosthesis and attendance at dental appointments are important factors in the aetiology of denture stomatitis (Lombardi and Budtz-Jörgensen, 1993, Abelson, 1981, Marinoski *et al.*, 2014, dos Santos *et al.*, 2010). Average daily duration of continuous wear and overnight wear of a prosthesis are significantly related to the presence of denture stomatitis (Zissis *et al.*, 2006, Compagnoni *et al.*, 2007). In addition to overnight wear being an important factor in the development of denture stomatitis, Budtz-Jorgensen *et al* (2000) suggested that keeping dentures dry overnight may inhibit the growth of a denture biofilm.

Although not often regarded as a life-threatening condition, denture stomatitis has the potential to have a significant impact on the oral health of individuals as well as the oral health of a population due to the potentially high numbers of patients involved (Nevalainen et al., 1997, Espinoza et al., 2003).

1.6 Denture Hygiene Methods

Methods of cleaning dentures in relation to both clinical and microbial outcomes have been studied, although no clear evidence exists as to the gold standard of denture home care or denture disinfection in relation to the presence of denture stomatitis (Emami *et al.*, 2014). It has also been suggested that poorly maintained dentures and those which have been worn for a significant period of time are more likely to accumulate a microbial biofilm and be associated with denture stomatitis (Hoad-Reddick *et al.*, 1990). Similarly, ineffective or inappropriate denture cleaning regimens, such as the use of an abrasive denture cleanser and the subsequent rough surface it will create, may have a negative impact and promote biofilm development (Abelson, 1981, Saha *et al.*, 2014).

A significant contributing factor to the prevalence of denture stomatitis, and poor denture hygiene within the prosthesis-wearing population, is patient understanding and compliance with denture hygiene protocols (Barbosa *et al.*, 2008). Although it is well established that an effective denture cleaning regimen will help to prevent denture stomatitis, many patients remain unable or unwilling to clean their dentures effectively (Mikkonen *et al.*, 1984, Budtz-Jørgensen *et al.*, 2000). Kanli *et al*. suggested that only 16.7% of dentures they examined in their study were correctly cleaned and that there was a significant correlation between denture hygiene and the presence of *Candida albicans*, a key pathogen in the development of denture stomatitis (Kanli *et al.*, 2005, Coco *et al.*, 2008). Potential reasons for this may include: a lack of information given to patients on denture hygiene; patients' perceptions of the cleanliness of dentures; and that patients were less critical of the cleanliness of their dentures than dental professionals (Dikbas *et al.*, 2006, Barbosa *et al.*, 2008, Collis and Stafford, 1994). A lack of clarity within the prosthesis-wearing population regarding the best cleaning regimen available is mirrored within the available dental literature (Barbosa *et al.*, 2008, Jagger and Harrison, 1995). A recent survey of dental healthcare professionals and denture wearers, in both

developed and developing countries, reported wide variation in advice given to patients on the optimum cleaning method and also the reported patient cleaning regimens (Axe *et al.*, 2016).

The dental literature is bereft of high quality clinical evidence as to the most effective denture cleanser or denture cleansing regimen, partly due to the heterogeneous nature of studies into this topic (Nikawa *et al.*, 1999). Although there has been a significant effort to study the effectiveness of denture cleansers, non-standardised methodology and heterogeneity in outcome measurements studied have made the interpretation of the results of the literature close to impossible. A Cochrane Review by De Souza *et al.* (2009) concluded that there is a lack of evidence regarding the comparative effectiveness of denture cleansers and that pooling of the results of available randomised control trials was impossible due to wide ranging interventions and outcome variables assessed. From the limited randomised control trials that were of high enough quality for inclusion, the authors concluded that there is evidence to suggest that combined use of a chemical cleanser alongside mechanical cleaning is more effective than a placebo when interventions are aimed to reduce plaque coverage of a denture or the microbial burden of a denture base. It should be noted that, of the clinical trials reviewed, every trial was reported to be at a high or unclear risk of bias in several categories, particularly in relation to the risk of selective reporting (de Souza *et al.*, 2009). This finding is corroborated by another systematic review of the available dental literature, which concluded that the non-standard methodologies used to assess the efficacy of dental cleansers make comparison of the different cleansers and cleansing regimens impossible (Nikawa *et al.*, 1999).

1.7 Frequency of Denture Cleansing

Frequency of denture cleansing is often reported to be an influential factor in the cleanliness of a prosthesis and closely related to the clinical outcomes of poor denture hygiene. However, there is a limited evidence base to support this (Nishi *et al.*, 2012). Although it is well established that denture cleanliness has a positive correlation with the prevalence of denture stomatitis, it has yet to be established whether cleaning frequency has an impact on denture cleanliness (Kulak-Ozkan *et al.*, 2002). There is limited evidence that an increased

frequency of use of a chemical denture cleanser has a beneficial effect on the microbial burden on a denture, although no evidence-based recommendations of optimal cleaning frequency are available. Notably, Nishi *et al*. (2012) reported that use of a chemical denture cleanser at a frequency of three to four times per week produced similar results to daily use. Within the same study the authors also commented that, in an institutionalised population from a practical perspective, daily use establishes a more reliable cleansing routine (Nishi *et al.*, 2012).

Cleaning frequency may also selectively impact on the microbial population of a multispecies biofilm, with daily use of a dental cleanser selectively impacting specific microbial species and minimally impacting others. The clinical effect of this is yet to be understood (Lucena-Ferreira *et al.,* 2014).

1.8 Manual Mechanical Cleaning

Much of the available evidence published in the dental literature compares denture cleansers to manual cleaning alone, with selected studies also assessing the efficacy of manual cleaning against ultrasonic cleaning of dentures. Although no clear consensus is evident, a common conclusion identified is that chemical cleansers are superior to manual cleaning with water alone and that a combination of manual cleaning and chemical disinfection is the most effective method of cleansing (Cruz *et al*., 2011, Srinivasan and Gulabani, 2010, Lee *et al*., 2011, Gornitsky *et al*., 2002). This was supported by Jose *et al*. (2010) who concluded chemical denture cleansers exhibit an ability to disrupt and reduce the microbial biofilm on a denture base although they lack the ability to remove the microbial remnants of the biofilm. These act as foci for regrowth and recolonization of the denture with potentially pathogenic bacteria, and therefore mechanical cleaning is also advised. A controlled clinical trial reported by Chamberlain *et al*. (1985) demonstrated that, when tissue inflammation is the measured outcome, mechanical denture debridement can be an effective method of denture cleaning.

The literature is unclear regarding the impact of a dentifrice on mechanical removal of denture plaque. It is clear that brushing with toothpaste or water is less effective than when used in combination with a chemical cleanser (de Souza

et al., 2009). There is some evidence that use of a toothpaste, which commonly includes abrasive particles for a whitening effect on teeth, can cause abrasion of the denture base and the resultant scratches predispose the denture to increased plaque and stain retention (Kiesow *et al.*, 2016).

1.9 Chemical Cleansing

Chemical denture cleansers can be divided into five main categories based upon their mechanism of action or key component: alkaline peroxides, alkaline hypochlorite, dilute acids, disinfectants and enzymes (Moore *et al.*, 1984). Chemical denture cleansers are thought to be particularly effective in cases where conventional manual cleaning may be impaired, for example in an infirm elderly population or where the denture surface has been altered and manual cleaning may not be performed effectively (Hoad-Reddick *et al.*, 1990, Gornitsky *et al.*, 2002). An example of this is in relation to dentures utilising soft lining materials or tissue conditioners, as conventional manual cleaning may not be indicated due to the risk of damage to the base material. In contrast, chemical denture cleansers have been demonstrated to be effective in disruption of the microbial biofilm and do not pose such a risk to the denture base material (Moffa *et al.*, 2016). It has been shown that particular denture cleansers may also have a deleterious effect on soft lining materials, therefore care must be taken when selecting a chemical cleanser to consider the compatibility of cleanser and base material in addition to the anti-microbial properties of the denture cleanser (Nikawa *et al.*, 1994, Jagger and Harrison, 1995, Izumida *et al.*, 2014). Through evaluating microbial outcomes in a randomised controlled trial, Chan *et al.* (1991) demonstrated superior cleaning efficacy of chemical cleansers compared to mechanical debridement alone.

In addition to the beneficial effects of regular chemical disinfection of a denture to reduce the plaque and microbial burden on dentures, Sharp and Verran (1985) demonstrated a positive effect on inhibition of plaque formation when cleansers are used on clean denture bases .

Denture cleansers containing enzymes have been demonstrated to be effective in the prevention of plaque accumulation (Budtz-Jørgensen *et al.*, 1983). This is achieved through the lysis and removal of the fungal cells, evident in denture

plaque, depending on the type of enzyme present (Nakamoto *et al.*, 1991). De Souza *et al.* (2009) reported that longer soaking in an enzyme-containing denture cleanser is more effective than its effervescent counterparts when plaque removal is the outcome measure.

Hypochlorite denture cleansers are increasingly utilised for chemical disinfection of dentures. Hypochlorite has demonstrated superior cleaning efficiency to other chemical cleansers (Jose *et al.*, 2010). The main drawback of hypochlorite is the risk of damage to the denture base resulting in roughening of the acrylic surface or discolouration (Coco *et al.*, 2008). Due to this, the American College of Prosthodontists recommends that dentures should not be soaked in a hypochlorite-containing denture cleanser solution for periods of time which exceed 10 minutes (Felton *et al.*, 2011b).

Chemical denture cleansers may also be based upon alkaline peroxides. Compared to other chemical denture cleansers, these have a reduced detrimental impact on the surface characteristics or colour stability of acrylic prostheses (Kiesow *et al.*, 2016). Dissociation of the alkaline peroxide into hydrogen peroxide upon contact with water is the main antimicrobial action.

Alternative chemical cleansers are also evidenced in the dental literature including organic acids, such as humeric acid and citric acid, or disinfecting agents such as chlorhexidine (de Andrade *et al.*, 2012, Meriç *et al.*, 2016, Izumi *et al.*, 2016). Although promising, much of the literature on alternative chemical denture cleansers is produced through *in vitro* experiments and is difficult to translate to the clinical environment.

In this clinical trial an alkaline peroxide-based denture cleanser was used. The product was the GlaxoSmithKline denture cleanser Corega Tabs Dental Weiss für Raucher [Denture Whitening for Smokers], which was a German marketed product.

1.10 Ultrasonic Cleaning

Ultrasonic devices are also advocated as adjuncts to conventional manual and chemical denture cleaning and have been demonstrated to be more effective

than the use of denture cleansers alone (Abelson, 1981, Gwinnett and Caputo, 1983, Palenik and Miller, 1984). This is in part due to the ability of ultrasonic cleaners to disrupt the plaque biofilms and aid in plaque removal, a weakness of chemical denture cleansers (Sharp and Verran, 1985). Felton *et al.* (2011), whilst noting a weak evidence base, suggested in guidelines produced for the American Academy of Prosthodontists that patients should have an ultrasonic clean of their dentures carried out once per year at their dentist.

Ultrasonic devices have been demonstrated to be equally as effective as conventional manual debridement of a denture when removal of denture plaque is the outcome measured (Cruz *et al.*, 2011, Duyck *et al.*, 2016).

Ultrasonic cleaning also exhibits a high efficacy in bacterial killing, with the majority of viable bacterial cells being predictably destroyed in a short duration of time when submerged in an ultrasonic cleaning bath (Kawasaki *et al.*, 2016).

1.11 Microwave Disinfection

Microwave irradiation has been evidenced to be effective in disinfection, and potentially sterilisation, of denture base material both *in vitro* and *in vivo* (Sesma et al., 2013b, Brondani et al., 2012). It is not a widely adopted method of denture disinfection, possibly due to the risk of warping of the denture base through repeated sterilisation cycles (Polychronakis *et al.*, 2018, Wagner and Pipko, 2015).

1.12 Denture Hygiene Outcomes

An important caveat to the conclusions drawn from the studies assessing the efficacy of denture cleansers is the variability in outcome measures studied.

Microbiological outcomes are often the primary outcome measure for denture cleanser efficacy studies. In experimental studies which utilise microbial inoculation and subsequent assessment of colony-forming units (CFU) as the outcome measure, the specific microbial species chosen may act as a confounder and affect interpretation of the results. Paranhos *et al.* (2009) demonstrated, through an *in vitro* study, that different cleaning regimens will have a greater or

lesser effect on different microbes. Therefore, care must be taken when reviewing the current literature in order to determine the most effective denture cleaning method particularly focusing on the clinical effect on a pathogenic microflora.

There is also no agreed standard for assessment of plaque or staining of a denture base within the literature. Subjective, objective and patient-reported outcome measures (PROMs) are reported with little consensus on key parameters for assessment. Lack of intra- or inter-rater agreement scores also limit the consistency and cast doubt on the internal and external validity of data provided.

A recent trend within the published literature is a focus on patient reported outcomes and experiential outcome measures. This is under-reported in many studies evaluating the efficacy of use of denture cleansers. Although valuable when utilised correctly in a clinical trial, suboptimal blinding in much of the literature may result in response bias and introduce error into the results. Care therefore must be taken with the experimental design and interpretation of the data produced (Gosall and Gosall, 2012).

2 Methods and Materials - Clinical Trial

2.1 Study design

This study was conducted using an examiner-blinded randomised controlled trial. A cross-over design was utilised with two treatment periods and a washout period of seven ± three days between each arm of the trial. Examiners and microbiologists involved in the data collection and sample analysis phases of the trial were blinded to participant allocation. The clinical trial was completed in a single centre.

2.2 Aims

The aims of this clinical trial were to evaluate the effects of daily use of a chemical denture cleanser compared to a chemical denture cleanser used only once per week, in terms of:

(i) Clinical outcomes

(ii) Microbial outcomes

(iii) Patient satisfaction values

2.2.1 Primary Outcome

The primary outcome was to assess the number of Colony-forming Units (CFUs) cultured from denture disc sampling at one week following daily use of a denture cleanser and compare this to CFUs cultured from once weekly use of the denture cleanser. This was evaluated on day seven of each arm of the trial and compared to baseline levels recorded on day zero (pre-treatment).

2.2.2 Secondary Outcome

The secondary outcome was to assess the number of CFUs on day three cultured from denture disc sampling. CFU counts following daily use of a denture cleanser were compared to samples taken from the group using the cleanser once per

week. This was evaluated on day three of each arm of the trial and compared to baseline levels recorded on day zero (pre-treatment).

2.2.3 Exploratory Outcomes

As this was a pilot study the trial was not adequately powered to establish statistical significance for all outcome measures of interest. Therefore, exploratory objectives were defined prior to commencement of the trial with the purpose of hypothesis development for future research projects.

The exploratory objectives of the trial aimed to assess several clinical, microbial and patient satisfaction outcomes:

- Evaluation of the plaque levels on the denture-fitting, polished and teeth surfaces. Levels were compared to baseline values and assessments of plaque scores carried out on day three and day seven.

- Evaluation of the stain levels on the denture-fitting, polished and teeth surfaces. Levels were compared to baseline values and assessments of stain scores carried out on day seven.

- Evaluation of microbial counts from a denture sonicate on day seven of each arm. This aimed to assess the number of colony-forming units cultured following daily use of a denture cleanser and compare this to those from the once weekly use of the chemical cleanser arm of the trial. The denture sonicate was performed on day zero and day seven of each arm of the trial.

- Evaluation of the microbial composition of denture plaque samples collected via denture disc sampling. Levels were compared to baseline values and assessments of plaque composition carried out on day three and day seven.

- Evaluation of patient reported outcome measures of denture cleanliness through the subject assessment questionnaire.

2.3 Sample Size

Recruitment aimed to screen 30 patients with the intent to recruit and randomise 20 patients to the clinical trial. It was predicted this would allow 17 patients to complete both arms of the trial.

A lack of similar or appropriate studies in the dental literature meant that a formal sample size calculation was not possible. The Biostatistics unit at GlaxoSmithKline Consumer Healthcare, Weybridge, UK (GSK) estimated that a sample size of approximately 17 participants would be adequate to evaluate the effectiveness and safety of the different interventions within the clinical trial. Statistical significance was set at $p<0.05$.

2.4 Patient Recruitment

Participants were identified through a variety of pathways. Potential subjects consisted of patients treated at Glasgow Dental Hospital and School and patients who were informed of the trial through Regional Ethics Committee (REC) approved advertising. Approved posters (Appendix 1) were placed in patient waiting areas and noticeboards throughout Glasgow Dental Hospital. Approval was given by the REC for social media and newspaper-based advertising campaigns, but these were not utilised due to sufficient patient recruitment.

Patient treatment clinics that were likely to see a high proportion of edentulous or partially edentulous patients were identified. Appropriate clinicians were informed about the trial and encouraged to contact a member of the research team if a patient was interested in learning more information and potentially enrolling in the trial. Clinics identified as possible recruitment sites were new patient and review clinics in Fixed and Removable Prosthodontics and Oral Medicine Departments. Dental undergraduate prosthodontic treatment clinics were also identified as potential sites for patient identification. Clinical staff members involved in treatment/supervision on these clinics were asked to contact a member of the research team on site if a patient with a well-fitting, complete maxillary denture expressed an interest in participation in the clinical trial. Patients were subsequently provided with the approved Patient Information Sheet (Appendix 2) and given further basic information on the

clinical trial. If appropriate, and if a patient indicated they were keen to participate, they were invited to undertake a telephone screening interview. Patients were remunerated £320 (or £40 per visit) for participating in the clinical trial.

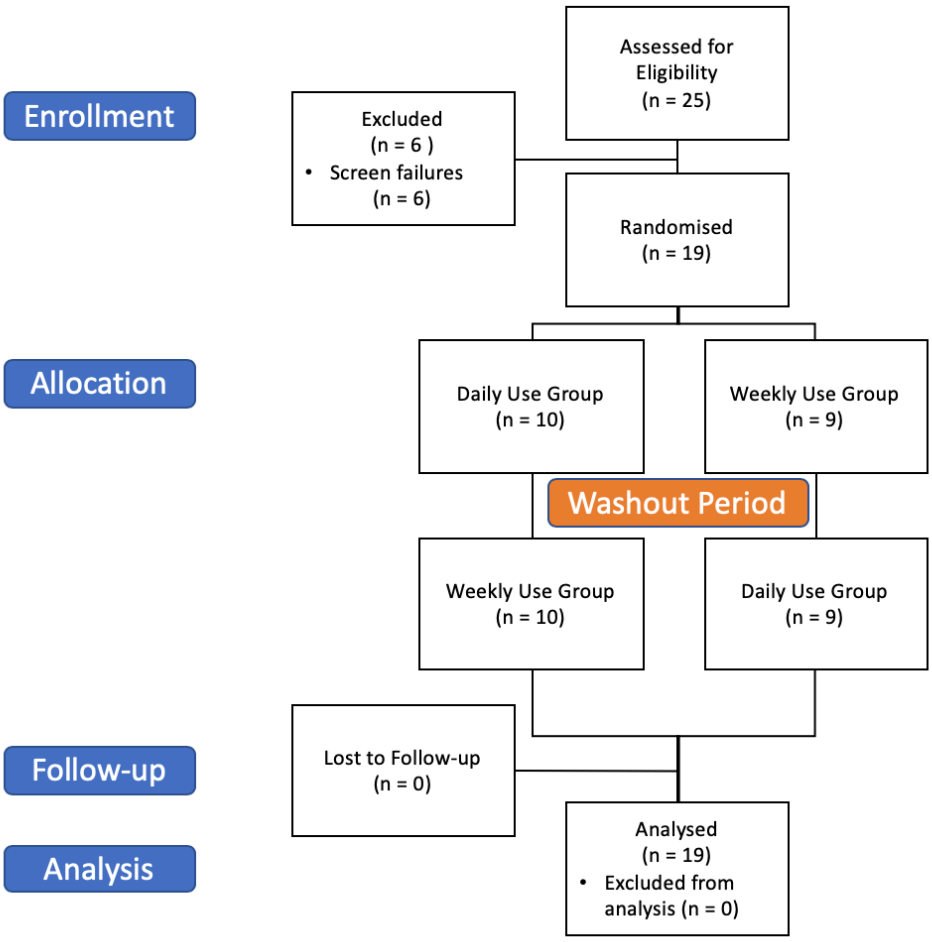

Figure 1. CONSORT Flow Diagram.

2.5 Patient Screening

2.5.1 Telephone Screening

If interested in participation in the clinical trial, potential subjects were screened initially using the approved telephone screening script.

Screening was carried out by one of the trained clinical research nurses and prospective participants were informed of the basic requirements and, in layman terms, the purpose of the research was explained. If given permission to

proceed, questions pertaining to a participant's suitability for inclusion were asked:

- Do you wear a complete upper denture?
- If so, is it a good fit?
- Are you fit and well enough to make it to Glasgow Dental Hospital?
- Do you take currently, or have you taken any antibiotics/inhalational steroids/antifungals or antiseptic mouthwashes in the last two months?
- Do you smoke?
- Have you had recent oral surgery, such as an extraction, or other oral operation?
- Are you currently experiencing any problems with your teeth or mouth?
- Are you aged between 18 and 84 years of age?
- Are you pregnant or currently breastfeeding (only if age appropriate)?

These questions were designed by GSK to relate specifically to inclusion and exclusion criteria and approved by the REC.

If the answers indicated that they may be suitable for inclusion in the clinical trial, patients were appointed to a formal screening visit. Patients who were deemed unsuitable for inclusion into the trial were informed and thanked for their time.

2.5.2 Clinical Screening

At the initial clinical visit patients were screened to determine suitability for inclusion in the clinical trial. Patient demographics were collected and recorded in the CRF. This included age, year of birth and gender.

Additionally, screening involved collecting a relevant medical history from prospective participants. The aim was to assess subjects' suitability for participation and to ensure exclusion criteria were not met (see section on exclusion criteria). Details of general health, recent medical/surgical histories (within previous 12 months), regular medications and allergies were recorded.

This was carried out by a medically or dentally qualified individual, usually the examiner, and utilised the Glasgow Dental Hospital and School standard medical history form.

A dental history, taken during the screening visit, focused on the history of denture wear, in addition to the age and wear patterns of the patient's current maxillary and, if applicable, mandibular prosthesis.

Clinical screening and subsequent assessments throughout the clinical trial were completed by a trained team of calibrated dental practitioners. A team of three experienced research nurses supported the clinical trial. The research nurses received training in the protocols for denture cleaning according to the trial protocol. Good clinical practice (GCP) training was completed by the clinical trial team and updated at appropriate intervals.

2.6 Oral Soft Tissue Assessment

An examination was carried out of the oral soft tissues, maxillary arch form and the denture bearing tissues at the screening appointment.

The oral mucosa was separated into anatomical areas and assessed for the presence of erythema, desquamation, ulceration and other relevant clinical observations, for example keratosis. GSK deemed the presence of any of these clinical findings to constitute "minor oral irritations". The areas assessed included:

- Mucogingival fold
- Maxillary edentulous gingival mucosa
- Hard palate
- Soft palate
- Mandibular edentulous gingival mucosa (if applicable)
- Mandibular gingival mucosa (if applicable)
- Labial mucosa
- Buccal mucosa
- Tongue
- Sublingual area
- Submandibular area
- Salivary glands
- Tonsillar tissue
- Pharyngeal mucosa

This examination was repeated at each subsequent visit and, where possible, carried out by the same examiner.

2.7 Assessment of the Denture-Bearing Tissues

Denture-bearing mucosa was assessed using the Kapur denture-bearing tissue score (Kapur, 1967). This assessed three key components of the denture-bearing mucosa and assigned a numerical score reflecting its suitability as denture-bearing tissue. Although the score can be applied to both the maxilla and mandible, for the purposes of this study only the maxillary tissue was assessed.

Shape of ridge was assessed as flat, v-shaped, between v- and u- shaped and u-shaped. These were scored one, two, three and four respectively. Similarly, tissue resilience was scored using this index as flabby (easily displaceable), resilient and firm. These were scored as one, two and three. Location of border tissue attachment was scored as low, medium or high and assigned a score of one, two and three. With each category a higher score indicated a better-quality denture-bearing tissue.

2.8 Denture Assessment

Assessment of the maxillary prosthesis, both independently and in function, was also carried out at the screening visit.

The examiner assessed the retention and stability of the complete denture using the Olshan modification of the Kapur denture stability and retention indices (Olshan *et al.*, 1992).

Retention was assessed by applying a vertical force away from the denture-bearing tissues and attempting to unseat the maxillary denture. The examiner attempted to displace the denture from the lateral incisor/canine region of the denture. The respective force required to displace the denture was recorded as zero, one, two, three, four or five as detailed in Table 1.

Score	Description
0	No retention- if denture is seated in place it dislodges itself
1	Poor- denture offers slight displacement to vertical pull and little or no resistance to lateral forces
2	Fair- denture offers moderate resistance to vertical pull and little or no resistance to lateral forces
3	Good- denture offers moderate resistance to vertical pull and lateral force
4	Very Good- denture offers very good resistance to vertical pull and lateral force
5	Excellent- denture offers excellent resistance to vertical pull and lateral force

Table 1. Retention Index

Stability, i.e. resistance to displacement of the denture in the horizontal plane, was assessed by the examiner through application of alternate forces to one premolar and the contra-lateral first molar tooth (Jacobson and Krol, 1983). The resulting displacement was scored using a stability index, Table 2.

Score	Description
0	No stability- denture base has extreme rocking under pressure
1	Poor- denture base has moderate rocking on its supporting structures under pressure
2	Fair- denture base has slight rocking on its supporting structures under pressure
3	Good- denture base has very slight rocking on its supporting structures under pressure
4	Excellent- denture base offers no rocking on its supporting structures under pressure

Table 2. Stability Index.

2.9 Inclusion Criteria

Participants were only considered eligible for inclusion into the clinical trial if they were deemed competent to consent for participation and expressed this by signing a voluntary written consent form. In order to facilitate inclusion of a wide range of potential participants 18 to 84-year olds were included in the study.

Participants had to be in good general health for inclusion, including an absence of clinically significant abnormalities which may impact on the trial or patients' suitability to enrol in the trial. Absence of factors which may impact on a patient's welfare, comprehension of trial obligations or adherence to trial processes were also necessary.

With regards to dental health, the patient must have had an edentulous maxilla restored with a conventional complete acrylic denture. The mandibular arch may have been edentulous, partially dentate, restored with a complete removable, partial removable, tooth or implant-supported prosthesis.

The maxillary complete denture, using the modified Kapur index, must have been scored as fair or above for retention and stability (Kapur score of ≥2).

Additionally, in the opinion of the examiner, the patient's denture (maxillary) must be considered to be adequately designed and constructed. Criteria for assessment of a well-made denture were defined as the denture having:

- Adequate vertical and horizontal occlusal relationships
- Acceptable border extension (i.e. not under- or over-extended)
- Satisfactory contour, colour, thickness and finish
- Absence of significant porosity or defects in fitting, polished or tooth bearing surfaces

2.10 Exclusion Criteria

Patients who were pregnant or those who were likely to become pregnant during the time period of the trial were not eligible for inclusion in the trial. Similarly, breastfeeding mothers were excluded from the trial.

Prospective patients were excluded from the trial if their medical history included:

- Current or relevant severe or unstable physical, psychiatric or medical illness which render the participant unlikely to complete the trial
- Illness or injury which may increase the risk to patient safety and well-being during the duration of the trial
- Pathology or medical condition which may act as a confounding variable for trial outcomes/adverse events, e.g. diabetes which may render a patient at increased risk of denture stomatitis
- Implanted cardiac pacemaker
- Current medication which may interfere with ability to adhere to the study protocol or affect efficacy evaluations
- Smoking/using tobacco products or e-cigarettes and they are unable to cease for the duration of the clinical trial

- Allergy or intolerance to any of the study products
- A history of substance abuse within the previous 12 months

Subjects who had recently participated, or were currently participating, in another clinical trial (including cosmetic studies) or those who were involved in an experimental/investigational drug trial within 30 days of the initial visit were not eligible for inclusion. Additionally, participants who were employed by, or those who had immediate family members who were employed by any toothpaste manufacturer, the trial sponsor (GSK), or the study site were excluded from the trial.

Based upon the findings of the screening questions and examination, suitable participants were invited to continue with the proposed visit schedule. Ineligible subjects were thanked and their participation in the trial ended.

2.11 Consent

Subsequent to formal clinical screening, patients were consented by the investigator, or their suitably trained deputy, for inclusion in the clinical trial. Patients were provided with the Patient Information Sheet (Appendix 2) and taken through the approved Consent Form (Appendix 3). Subjects were provided, as per protocol, with a copy of the signed Consent Form. A signed copy of each patient's consent was recorded in the case report form (CRF) which was stored securely in the Clinical Research Facility.

2.12 Randomisation

Following screening, a patient deemed suitable for inclusion in the clinical trial was randomised to the control or intervention arm of the trial. Participants were randomised using a computer-generated randomisation schedule, created by the Biostatistics Department at GSK, using validated software. Subjects were assigned a unique identification number in sequential, ascending order when screened for inclusion into the clinical trial.

If subjects were screen failures, i.e. excluded from the trial after consent to participate but prior to randomisation, a basic dataset was collected including demographics, details of screen failure, details of any serious adverse event

(SAE) and details of the patient's eligibility relative to the inclusion/exclusion criteria. If patients were deemed not suitable to be randomised, due to a temporary failure to meet the inclusion criteria, they were not eligible to be re-screened for inclusion at a later date.

2.13 Experimental Protocol

Subsequent to treatment allocation, participants were assigned to a treatment sequence order.

All participants were supplied with alkaline peroxide-based denture cleansing tablets (Corega Tabs Dental Weiss für Raucher [Denture Whitening for Smokers], German marketed product). Lower teeth or dentures were cleaned as per the participant's normal routine, excluding the use of mouthwash (which was not permitted). If dentures were present, these were soaked and cleaned in a separate container from the upper denture. Maxillary dentures were cleaned once per day, inclusive of the supervised product use at the trial site, according to the trial protocol.

In the daily use of a denture cleanser group, participants soaked their denture in a cup of very warm water (150ml) for 15 minutes with one tablet of denture cleanser in the water. The denture was then brushed for 30s using the solution and then rinsed under running water for 10 seconds. The once weekly use group utilised the same protocol but did not use the denture cleanser tablet in the water (only soaked and brushed using water alone) until their supervised product use on day seven at the trial site, at which point a denture cleanser tablet was used.

2.14 Assessment Methods

2.14.1 Plaque

At several points during the trial the presence and amount of denture plaque were assessed on the surface of the maxillary denture to allow evaluation of changes in denture cleanliness.

The modified Clinical Categorization of Denture Cleanliness Index, as described by Blair *et al* (1995), was used to assess any plaque deposits evident on the denture surfaces. This index assessed the fitting, polished and teeth surfaces of the denture independently and assigned a numerical value to each relating to the presence and amount of plaque present, Table 3.

Score	Description
0	No visible plaque; no matter adherent to the side of the dental probe on light scraping
1	No visible plaque; matter adherent to the side of the dental probe on light scraping
2	Deposits of plaque just visible on careful examination without need to confirm by scraping
3	Deposits of plaque clearly visible
4	Gross plaque deposits; “Velvet appearance”

Table 3. Modified Clinical Categorisation of Denture Cleanliness.

2.14.2 Stain

Similar to the assessment of plaque on the denture base, staining was assessed at several points throughout the clinical trial. The Modified Denture Cleanliness Index, first described by Mylonas *et al* (2014), was utilised to assess the staining present on the fitting, polished and teeth surfaces of the maxillary denture. The distribution of staining was assigned a numerical score relating to the proportion of denture surface covered in stain. This is described in Table 4.

Score	Description
0	No staining detectable
1	Little staining (less than 25% of denture surface stained)
2	Moderate staining (between 25-50% of denture surface stained)
3	Severe staining (greater than 50% of denture surface stained)

Table 4. Modified Denture Cleanliness index.

2.14.3 Disc Sampling

Denture disc sampling was carried out to collect data on the plaque levels and composition on the fitting surface of the denture base. To reproducibly collect denture disc samples the fitting surface of the denture was divided into four quadrants and marked with a denture marker. The dividing lines ran through the midline of the denture and intersected a line demarcating the rough denture surface, containing the rugae, and smooth posterior section of the denture (Figure 2). If a palatal relief chamber was present on the denture base this disc sampling was taken from lateral to this and the chamber was avoided.

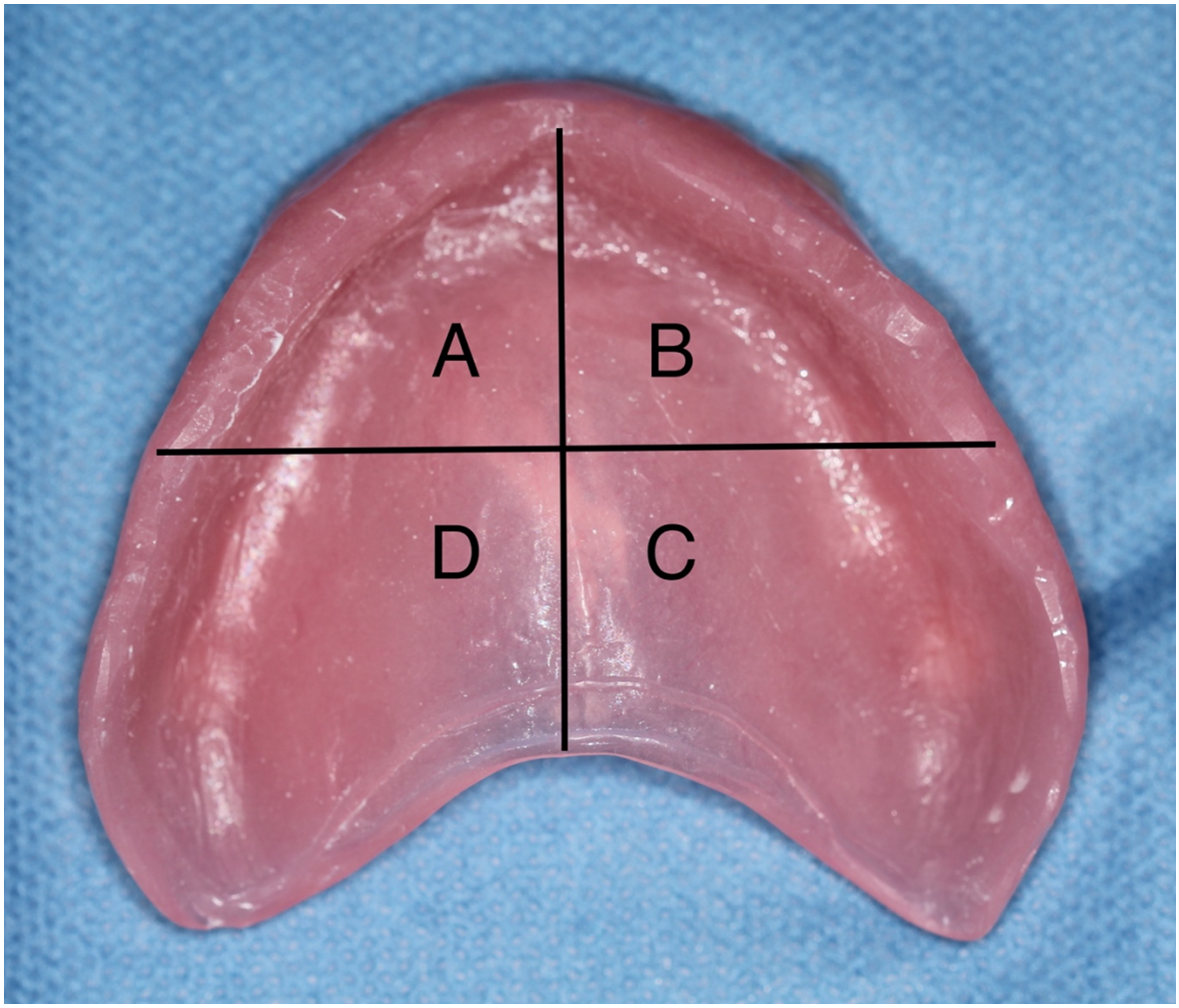

Figure 2. Demarcation of Denture

A pre-sterilised 10mm filter paper disc (Whatman Grade 1, Sigma-Aldrich Company Ltd., Dorset, England) was used to collect each sample. Pre-prophylaxis samples, prior to denture cleaning as per protocol, were taken from sites A and D; post-prophylaxis samples were taken from sites B and C.

Samples were collected by pressing discs with minimal pressure on the allocated denture base section using sterile tweezers for a period of 20 seconds. Discs were placed to allow two samples to be collected from each section, i.e. disc position to allow two discs to lie side by side without any overlap. One disc from each section was placed in a bijoux tube containing 5ml of sterile saline and the other placed in a cryotube containing 1.5ml of the cell storage reagent RNAlater®.

2.14.4 Sonicate

A denture sonicate was collected at the beginning and end of the clinical trial to evaluate the microbial burden on the denture base and to sample adherent microorganisms.

As per protocol, dentures were placed in a sterile sealed plastic bag containing 50ml of sterile saline. The sealed bag was placed in an ultrasonic bath and operated at 35 Kilohertz for a period of 15 minutes. The contents of the bag, including the microbes dislodged from the denture base during sonication, were transferred to a sterile 50ml tube and transported to the lab in anonymised form. Patient identification numbers were used during analysis to ensure blinding of the lab team remained intact.

2.14.5 Subject Assessment Questionnaire

The patient assessment questionnaire was carried out at the beginning and end of each trial arm to assess and evaluate patient perception of denture cleanliness (patient reported feel and look), denture freshness and freshness of their breath. The subject assessment questionnaire was provided to patients with a printed copy of the multiple-choice questions and answers. The responses were recorded on an ordinal scale as a categorical dataset. Additionally, the examiner instructed the patient when to insert their denture into their mouths

and read out the relevant questions in order and at an appropriate time. Answers were recorded in the Electronic Case Report Form (eCRF).

2.15 Oral Soft Tissue Check

Using an identical protocol to the screening visit, an oral soft tissue assessment was carried out at each trial visit. The aim was to ensure there were no adverse or serious adverse events noted. Any areas of oedema, erythema, ulceration or abnormal oral mucosa were recorded in the eCRF and re-examined at subsequent visits until they resolved.

2.16 Denture / Dental Prophylaxis

At the beginning and end of each trial arm, denture and dental prophylaxes were carried out on the denture(s) and mandibular teeth, if applicable. This was to ensure zero stain and plaque (score of zero) were present on the denture and therefore to reproducibly compare the changes during the duration of monitoring.

The protocol for prophylaxis of the dentures was:

- Placement of dentures in 50ml of NaOCl (10%) solution in a sealed plastic bag. This was then placed in an ultrasonic bath at 35 Kilohertz for 10 minutes
- Rinsing of the dentures under running water (mains water)
- Steam cleaning of the dentures to remove residual staining
- If applicable, at baseline, resistant stains were removed using a straight handpiece with acrylic bur. If this was used, on appropriate surfaces, polishing of the denture using pumice and a rotating brush at 1500rpm was carried out.

Dental prophylaxis of the mandibular teeth, if present, was carried out according to local departmental protocols. A supra-gingival scale was carried out using a Cavitron® scaler. A rotating rubber cup was subsequently used with pumice to remove staining and loosely adherent debris.

2.17 Repeatability Assessment – Intra-Rater Agreement

Repeatability assessments were carried out to assess the reproducibility of the assessment methodology for stain and plaque. Examiners were asked to repeat stain and plaque scoring, as described in the stain and plaque assessment section, at one visit per treatment arm. This was carried out at visit two and six for each patient as this visit permitted adequate time for repeatability assessment to be carried out.

Subsequent to the initial stain and plaque assessment, examiners were asked to wait for a period of 10 minutes then to repeat the assessment. Examiners were not permitted to refer to earlier scores prior to the repeatability assessment.

Weighted Kappa scores were utilised to assess reproducibility.

2.18 Appointment Scheduling

The trial consisted of two treatment periods separated by a wash out period of six days. Each treatment period included four visits. The scheduling of visits is outlined in Figure 3.

Day -1	Day 0	Day 3 ± 1 day	Day 7 ± 1 day	Washout Period 7 ± 3 days	Day -1	Day 0	Day 3 ± 1 day	Day 7 ± 1 day
Visit 1	Visit 2	Visit3	Visit 4		Visit 5	Visit 6	Visit 7	Visit 8

Figure 3. Visit Scheduling

Assessments carried out at each respective visit on each trial arm were identical, e.g. visit one was the same as visit five. Outcome assessments carried out at each visit are summarised in Table 5.

Visit	Assessments Carried Out
1 and 5 (Day -1)	Plaque (Pre-prophylaxis) Plaque (Post-prophylaxis) Stain (Pre-prophylaxis) Stain (Post-prophylaxis) Disc sampling (Pre-prophylaxis) Disc sampling (Post-prophylaxis) Denture sonicate (Pre-prophylaxis)
2 and 6 (Day 0)	Plaque (Pre-treatment - Baseline) Plaque (Post-treatment) Stain (Pre-treatment - Baseline) Disc sampling (Pre-treatment - Baseline) Disc sampling (Post-treatment)
3 and 7 (Day 3)	Plaque (Pre-treatment) Plaque (Post-treatment) Stain (Pre-treatment) Disc sampling (Pre-treatment) Disc sampling (Post-treatment)
4 and 8 (Day 7)	Plaque (Pre-treatment) Plaque (Post-treatment) Stain (Pre-treatment) Stain (Post-treatment) Disc sampling (Pre-treatment) Disc sampling (Post-treatment) Denture Sonicate (Post-treatment)

Table 5. Visit Assessments.

2.19 Microbial analysis

Microbial analysis was carried out in Glasgow Dental School, 378 Sauchiehall Street, Glasgow, G2 3JZ, and Academic Centre for Dentistry Amsterdam, Gustav Mahler Laan 3004, 1081 LA Amsterdam, The Netherlands.

When screened, participants were allocated a unique study number and a study pack was created with appropriate labelling for each sample. Study numbers were used for identification of samples and collection of data. After participant

allocation and randomisation, this number was used for analysis and reporting of outcomes.

For colony-forming unit analysis, the Miles and Misra method was used subsequent to serial dilutions of 10^0 to 10^{-5} for both disc sampling (imprint) and denture sonicate samples (Miles *et al.*, 1938). This involved triple plating on blood and *Candida* agar plates with 20μl drops (from a distance of 2.5cm from plate) on allocated sections. Samples were placed in an appropriate incubator, either aerobic or anaerobic, and incubated for a period of 96 hours at 37°C. Colonies were then counted and recorded to an appropriate log sheet. Retained samples or unprocessed biological materials were retained as per protocol and subsequently destroyed in a biological waste stream.

Molecular microbial analysis was carried out using quantitative polymerase chain reaction using species-specific DNA probes. QC standard DNA (Life Technologies, Paisley, UK) was used as the positive control and a blank template (no DNA) was used as the negative control. Specific probes were used to evaluate the presence of the following key oral microorganisms in addition to a 16s generic bacterial primer and a 18s generic fungal primer (O'Donnell et al., 2015a):

- *Candida albicans*
- *Streptococcus* species
- *Actinomyces naeslundii*
- *Veillonella dispar*
- *Lactobacillus casei*
- *Lactobacillus zeae*
- *Rothia denticariosa*
- *Fusobacterium nucleatum*

Retained samples or unprocessed biological materials were retained as per protocol and subsequently destroyed in a biological waste stream.

Microbiome analysis of the DNA collected using the imprint method (disc sampling) was carried out using the Illumina Miseq platform (O'Donnell *et al.*, 2015a). Hypervariable region 4 on the 16S rRNA gene was used due to its low error rate in species identification and also increased ability to detect species diversity (Kozich *et al.*, 2013). Due to the variable quantity of DNA in the samples collected (samples will be collected from the control and treatment arm) the whole sample was extracted for analysis to avoid bias. As per protocol, samples retained from qPCR were retained as back up in the event of sample loss. Subsequent to analysis, clinical samples were destroyed as per protocol.

2.20 Statistical Analysis

Statistical analysis was carried out by the Biostatistics Department, GSK. Mean, median and standard deviation summary statistics were reported. Minimum and maximum data points were reported for continuous data sets. Counts and percentages were reported for categorical data.

Analysis of anaerobic, aerobic and *Candida* species were analysed and reported separately.

Analysis of covariance (ANCOVA) was used to assess for differences in outcome measures of plaque, stain and microbial counts, for both disc sampling and denture sonicate. Period-level and subject-level pre-treatment outcome measures were considered as covariables and factors evaluated included treatment and period (from baseline to assessment visit).

Analysis of variance (ANOVA) was used to analyse post-treatment denture sonication samples. The dependent variable was sonication scores on day seven with the factors considered being treatment (trial/control), period and subject (random).

Where model assumptions are not met the non-parametric statistical hypothesis test, Wilcoxon, was used to analyse treatment differences for outcomes studied.

Intra-operator agreement was calculated using a weighted Kappa score for stain and plaque assessments once per trial arm for each patient.

Intra- and inter-operator agreements were evaluated using Cohen's coefficient of concordance (weighted Kappa score) during the inter-rater agreement exercise.

Responses provided during the patient assessment questionnaire were analysed and reported as counts and percentages of each response.

3 Methods and Materials – *In Vitro* Assessment of Denture Disc Sampling

3.1 Background

Subsequent to completion of the randomised controlled trial, primary analysis of the data revealed the microbial burden of denture samples to be inconsistent with expected values. Low numbers of microbes were detected, and differences were not explained through the investigated independent variables.

It was hypothesised that the disc sampling methodology may be affected by the moisture present at the time of sampling, therefore a subsequent laboratory-based experiment was designed to assess the impact of sampling of wet/dry acrylic discs using wet/dry sterile paper discs.

The impact of these clinical variables on the efficacy of microbial disc sampling was evaluated using an *in vitro* model.

3.2 Objectives

The primary objective was to evaluate if there was a statistical difference in microbes retrieved from a disc sampling method when the acrylic surface and/or paper disc is moistened.

3.3 Disc Preparation/Sampling Method

Pre-sterilised 10mm filter paper discs (Whatman Grade 1, Sigma-Aldrich Company Ltd., Dorset, England) were used dry and moistened with sterile phosphate buffered saline (PBS) to sample microbial growth from prepared sterilised heat cured acrylic discs (Lucitone 199®, Dentsply International Inc, York, England) that had been subsequently inoculated with a biofilm containing organisms commonly present in denture plaque. Discs were prepared using a standardised protocol and sterilised using a combination of chemical and UV irradiation (Appendix 5).

Independent variables assessed were the moistness of the filter paper discs and the moistness of the inoculated acrylic discs. The experiment consisted of four

experimental groups and a negative control for both the sterilised acrylic disc and sterile PBS sampling medium. Experimental groups were:

- Dry filter disc/dry acrylic disc
- Wet filter disc/dry acrylic disc
- Dry filter disc/wet acrylic disc
- Wet filter disc/wet acrylic disc

For each repeat and plate, an identical acrylic disc was placed into the PBS sampling medium and sonicated to act as a positive control (sonicate group). The purpose of this was to confirm that a biofilm was present on the acrylic disc samples and to act as a control to which other experimental groups would be compared.

Nine experimental repeats were carried out and each sample plated in triplicate.

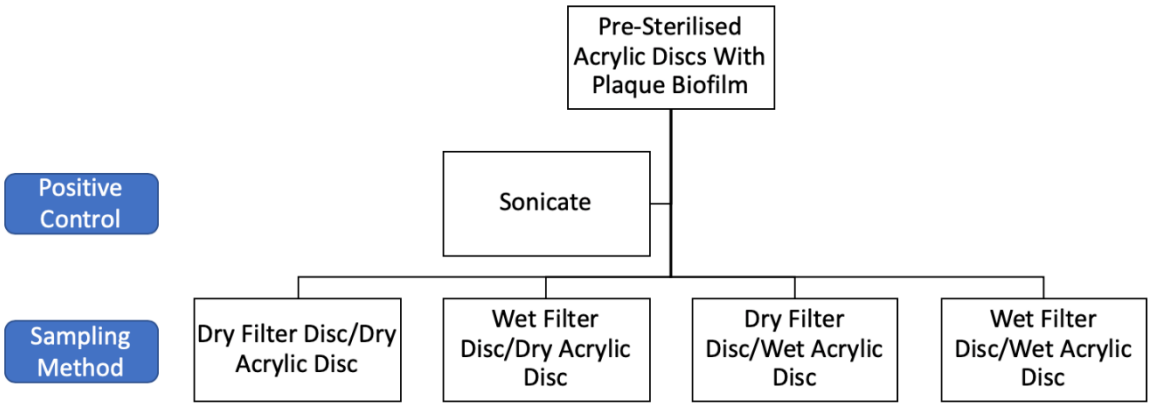

Figure 4. Sampling Flow Chart.

3.4 Acrylic Disc Inoculation

Eight species of bacteria (*Streptococcus species, Actinomyces naeslundii, Veillonella dispar, Lactobacillus casei, Lactobacillus zeae, Rothia denticariosa,*

Fusobacterium nucleatum) and one yeast species (*Candida albicans)* were grown in an artificial saliva medium.

The bacterial and candidal broth was grown in an artificial saliva medium, then inoculated onto the pre-sterilised acrylic discs (produced in GDH) and biofilm was allowed to develop through incubation in an appropriate atmosphere for a period of 72 hours.

3.5 Microbial Disc Sampling

Pre-sterilised paper discs were used for imprint sampling, using a similar method to that in the clinical trial for sampling of denture-fitting surfaces. Filter discs were placed onto inoculated acrylic discs for a period of 20 seconds prior to aseptic removal and storage in 1ml of PBS contained in a sterile bijoux tube.

Dry filter paper discs were used as packaged. Wet filter paper discs were moistened using sterile PBS prior to sample collection for “wet filter disc” samples.

Acrylic discs were sampled immediately after removal from artificial saliva/biofilm medium for “wet acrylic discs”. Acrylic discs were placed on a sterile surface for a period of 10 minutes prior to sampling for “dry acrylic discs”.

3.6 Processing of Disc Samples / Sonicate

Samples were sonicated in their bijoux tubes for 15 minutes in a room temperature sonic water bath.

Subsequent to sonication, samples were vortexed for 30 seconds and serial dilutions created in sterile micro tubes from 10^{-1} to 10^{-6}.

Each dilution was plated in triplicate on labelled agar plates. 10% blood agar was used for aerobic and anaerobic samples and Sabouraud’s Dextrose Agar was used for *Candida* samples. Plating was carried out using the Miles and Misra method with 3 * 20μl drops per half agar plate. Agar plates were incubated for 24 hours at 37^{o}C in appropriate aerobic or anaerobic cabinets/containers.

Following incubation, numbers of CFUs were counted on each sample under 2.5 times magnification and recorded in a culture log sheet.

Following recording of results, samples were destroyed following local departmental protocols.

3.7 Statistical Analysis

Microbial counts were recorded in an excel work sheet and analysis of the data carried out in SPSS statistical analysis software (IBM Corp. Released 2017. IBM SPSS Statistics for MacIntosh, Version 25.0. Armonk, NY: IBM Corp).

Non-parametric tests were used for statistical analyses as transformation of the data did not produce a normal data distribution. Medians and inter-quartile ranges were presented, and the Kruskal Wallis Test used for analysis between groups.

4 Results - Clinical Trial

4.1 Demographics and Baseline Characteristics

After telephone screening, 25 subjects were clinically screened for inclusion in the trial. A total of 19 subjects were randomised and all subsequently completed the study. All subjects received treatment as per the clinical protocol and no protocol deviations were recorded.

The mean age of the sample population was 68.7 years, ranging from 60-75 years (Standard deviation 5.1). The majority of participants were female (12, 63.2%) and 100% of the sample population was of white ethnic origin.

4.2 Denture Disc Microbial Sampling

4.2.1 Day Three

Day three demonstrated the first time point at which microbial samples were taken from subjects. Participants in the weekly group had not yet used the chemical denture cleanser and, at this stage, were using daily brushing with water in addition to a 15-minute soak in water. Participants in the daily group substituted the water for the chemical cleanser as per protocol.

4.2.1.1 Daily Group

A clear reduction in colony-forming units (CFUs) was seen post-treatment at day three in the daily use group for aerobic and anaerobic bacteria, compared to pre-treatment values on day zero. An adjusted mean reduction of -1.85 (SE 0.386) and -1.94 (SE 0.384) was seen for aerobic and anaerobic microbial counts, respectively. No *Candida* spp. were isolated at either baseline or day three for analysis.

4.2.1.2 Weekly Group

The weekly use of the denture cleanser demonstrated an increase in microbial burden at the day three time point compared to baseline values. Adjusted mean increases of 0.47 (SE 0.386) and 1.28 (SE 0.384) were seen for aerobic and anaerobic groups, respectively. Due to the low rate of recovery it was not

appropriate to carry out statistical analysis of changes in candidal counts over this time period.

4.2.1.3 Inter-group Analysis

Inter-group analysis demonstrated significant differences between the daily and weekly groups at day three compared to day zero. For aerobic and anaerobic bacteria, mean differences of 0.86 (-1.530, -0.196) (p 0.01) and -3.22 (-4.240, -2.193) ($p < 0.001$) were evident, respectively. No analysis was carried out on *Candida* counts due to the large number of samples which yielded no yeasts.

In the daily use group, a noteworthy number of samples retrieved no microbes post-treatment on day three (17 out of 19 for both aerobic and anaerobic groups) whereas fewer participants in the weekly group demonstrated no CFUs on sampling (seven subjects when assessing aerobic bacteria and four when assessing anaerobic bacteria).

4.2.2 Day Seven

Microbial sampling results from day seven demonstrated a similar trend to the results of the analysis of day three data. Microbial denture disc sampling was carried out pre- and post- treatment in both groups at this time point. Both groups used the chemical denture cleanser as per protocol.

A significant reduction in microbial counts was seen on the transformed dataset for the daily and weekly group post-treatment on day seven. No analyses were undertaken for the *Candida* spp. because of the large number of specimens that were culture negative for yeasts.

4.2.2.1 Daily group

The daily group demonstrated an adjusted mean reduction of -1.92 (SE 0.318) for aerobic bacteria and -1.8 (SE 0.327) for anaerobic bacteria post-treatment on day seven.

4.2.2.2 Weekly group

An adjusted mean reduction of -1.06 (SE 0.318) and -1.31 (SE 0.327) was seen in the weekly group for aerobic and anaerobic CFU counts, respectively.

4.2.2.3 Inter-group Analysis

Inter-group analysis revealed a statistically significant difference between the daily and weekly groups for aerobic bacteria only on day seven, -0.86 (C.I -1.530, -0.196) (p 0.0144). Differences in anaerobic microbial counts, although displaying a trend towards a greater reduction in CFUs for the daily treatment group, did not reach statistical significance, -0.48 (C.I. (-1.230, 0.261) (p = 0.1879).

Overall, at seven days a high proportion of the post-treatment disc samples demonstrated no microbial burden on the denture disc samples. For the daily test group 18 of the aerobic samples and 16 of the anaerobic samples yielded negative samples for microbes. For the weekly group, no microbes were retrieved on 13 occasions for both aerobic and anaerobic samples.

		Adjusted Mean Change from Baseline		Treatment Comparison	
		Daily Use (SE)	Weekly Use (SE)	Difference (95% C.I.)	P- Value
Aerobic Bacteria	Day 3	-1.85 (0.39)	0.47 (0.39)	-2.32 (-3.43, -1.20)	0.0002
	Day 7	-1.92 (0.32)	-1.06 (0.32)	-0.86 (-1.53, -0.19)	0.0144
Anaerobic Bacteria	Day 3	-1.94 (0.38)	1.28 (0.38)	-3.22 (-4.24, -2.19)	<0.0001
	Day 7	-1.80 (0.33)	-1.31 (0.32)	-0.48 (-1.23, 0.26)	0.1879

Table 6. Aerobic, Anaerobic and *Candida* spp. Mean Values on Day Zero and Day Seven.

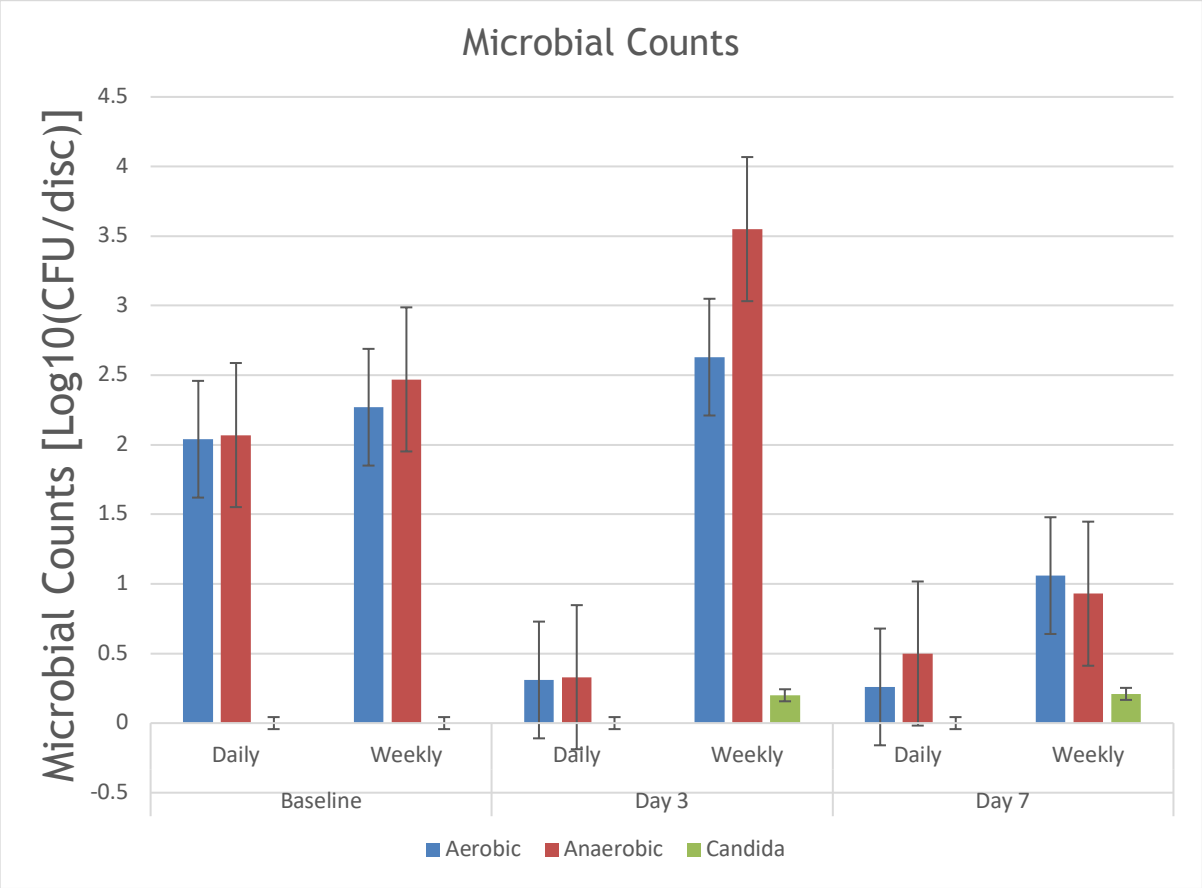

Figure 5. Aerobic, Anaerobic and *Candida* spp. Microbiological Counts on Day Three and Day Seven.

4.3 Denture Sonicate

Denture sonicate samples demonstrated a small reduction in microbial burden over the course of the experimental period for both arms of the trial.

4.3.1 Daily Group

The daily group demonstrated a reduction in adjusted mean values for transformed data (Log10 transformed) from baseline pre-screening to post treatment on day seven of 7.42 Log10(CFU/Denture) to 7.15 Log10(CFU/Denture), 7.46 Log10(CFU/Denture) to 7.22 Log10(CFU/Denture) and 1.65 Log10(CFU/Denture) to 0.68 Log10(CFU/Denture) for aerobic, anaerobic and *Candida*, respectively.

4.3.2 Weekly Group

The weekly group demonstrated a reduction in adjusted mean values for transformed data (Log10 transformed) from baseline pre-screening to post

treatment on day seven of 7.59 Log10(CFU/Denture) to 7.04 Log10(CFU/Denture), 7.76 Log10(CFU/Denture) to 7.19 Log10(CFU/Denture) for aerobic and anaerobic bacteria, respectively. *Candida* spp. samples evidenced a slight increase in microbial burden from 1.2 Log10(CFU/Denture) to 1.48 Log10(CFU/Denture) from pre-prophylaxis to post-treatment on day seven.

4.3.3 Inter-group Analysis

Inter-group analysis revealed no statistically significant difference between daily and weekly groups for aerobic, anaerobic or *Candida* spp. CFU counts. Adjusted mean differences of 0.12 (C.I. -0.257, 0.505) (p = 0.5118) for aerobic bacteria, 0.07 (C.I. -0.326, 0.460) (p = .7316) for anaerobic bacteria and, -0.60 (C.I. -1.863, 0.656) (p 0.3250) were observed.

4.4 Plaque Sampling

Low plaque scores were evident at all timepoints during the clinical trial, particularly on the polished surfaces of the dentures. Positive changes in denture cleanliness were seen at nearly all timepoints for both the daily and weekly group compared to baseline values, with consistent trends of reduced plaque scores for the daily group compared to the weekly group.

4.4.1 Daily group

For the daily group, at baseline the mean plaque scores on the tissue, polished and teeth surfaces were 0.63 (SE 0.191), 0.37 (SE 0.114) and 1.16(SE 0.279) respectively. At the three-day time point the mean plaque scores were 0.11 (SE 0.072), 0.05 (SE 0.053) and 0.32 (SE 0.154) for the tissue, polished and teeth surface respectively. At the seven-day time point the mean plaque scores were 0.16 (SE 0.115), 0.05 (SE 0.053) and 0.21 (SE 0.123) for the tissue, polished and teeth surface respectively. This represented a reduction in adjusted mean plaque scores for the tissue fitting surface of -0.58 (SE 0.201) and -0.52 (SE 0.179) at day three and seven compared to baseline, respectively. The polished surfaces demonstrated a reduction in mean plaque scores of -0.3 (SE 0.046) and -0.3 (SE 0.161) at day three and seven compared to baseline, respectively. The denture teeth plaque scores demonstrated a similar pattern with a reduction of -

0.65 (SE 0.197) and -0.79 (SE 0.154) in mean plaque scores at day three and day seven compared to baseline, respectively.

4.4.2 Weekly Group

For the weekly group, at baseline the mean plaque scores on the tissue, polished and teeth surfaces were 0.79 (SE 0.224), 0.32 (SE 0.134) and 0.79 (SE 0.237) respectively. At the three-day time point the mean plaque scores were 0.68 (SE 0.276), 0.05 (SE 0.053) and 0.68 (SE 0.217) for the tissue, polished and teeth surface respectively. At the seven-day time point the mean plaque scores were 0.79 (SE 0.224), 0.42 (SE 0.221) and 0.68 (SE 0.172) for the tissue, polished and teeth surface respectively. This represented a reduction in adjusted mean plaque scores for the tissue fitting surface of -0.05 (SE 0.201) and 0.05 (SE 0.179) at day three and seven compared to baseline, respectively. The polished surfaces demonstrated a change in mean plaque scores of -0.28 (SE 0.046) and 0.09 (SE 0.161) at day three and seven compared to baseline, respectively. The denture teeth plaque scores demonstrated a similar pattern with a reduction of -0.30 (SE 0.197) and -0.26 (SE 0.154) in mean plaque scores at day three and day seven compared to baseline, respectively.

4.4.3 Inter-group Analysis

Inter-group comparison revealed a general trend of increased denture cleanliness in favour of daily cleansing at most timepoints during the clinical trial. Statistically significant differences in plaque scores were identified, in favour of the daily cleanser use group, at day seven for tissue fitting and denture teeth surfaces of the prostheses, -0.57 (C.I -1.10, -0,05) (p=0.032) and -0.54 (C. I -1.99, -0.09) (p=0.021), respectively. A non-significant difference was seen on the polished surface, -0.39 (C.I. -0.86, 0.07) (p = 0.095), at day seven. Relative changes in plaque scores on day three were non-significant, although showed a directional trend towards lower plaque scores in the daily group with a difference in mean plaque scores of -0.52 (-1.11, 0.07) (p = 0.077) for the fitting surface, -0.02 (C.I. -0.15, 0.12) (p = 0.788) for the polished surface and -0.35 (-0.92, 0.23) (p = 0.231) for the denture teeth surface.

	Daily (Adjusted Mean, SE)	Weekly (Adjusted Mean, SE)	Difference (Adjusted mean, p value)		
			Difference	95% C.I.	P-value
Fitting	-0.52 (0.179)	0.05 (0.179)	-0.57	(-1.095, -0.052)	0.03
Polished	-0.30 (0.161)	0.09 (0.161)	-0.39	(-0.858, 0.073)	0.10
Teeth	-0.79 (0.154)	-0.26 (0.154)	-0.54	(-0.989, -0.086)	0.02

Table 7. Changes in Plaque Scores on Denture-fitting Surface, Polished Surface and on Denture Teeth Between Baseline and Day 7.

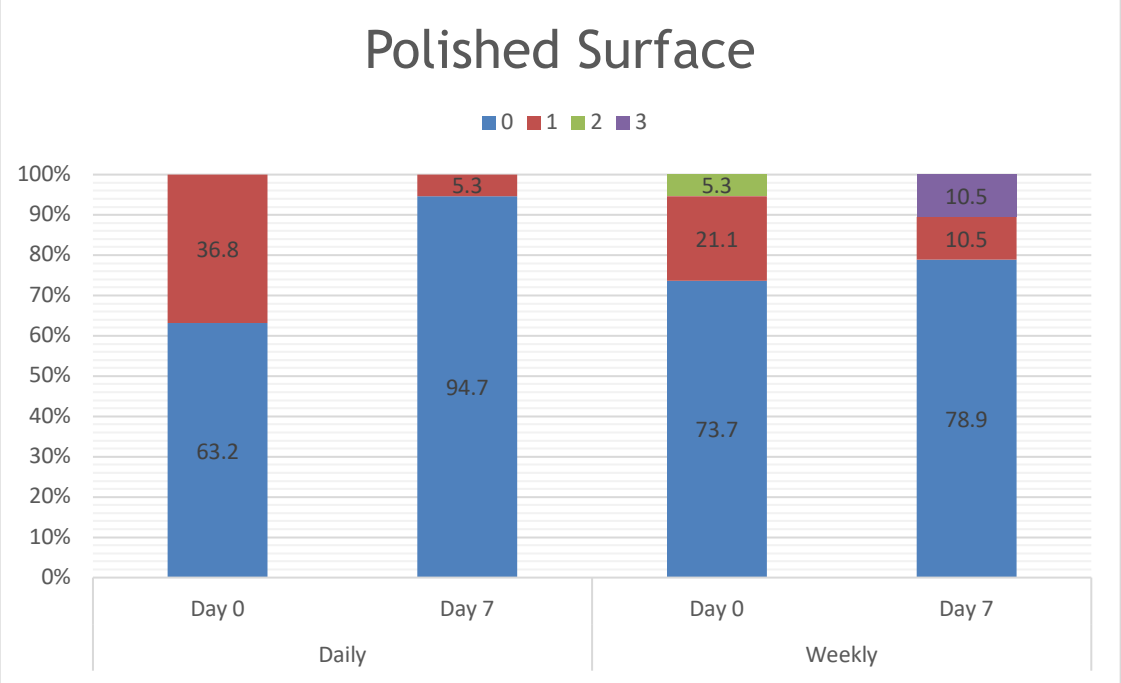

Figure 6. Changes in Distribution of Plaque Scores Between Baseline on Day Zero and Post-Prophylaxis on Day Seven for the Denture Polished Surface.

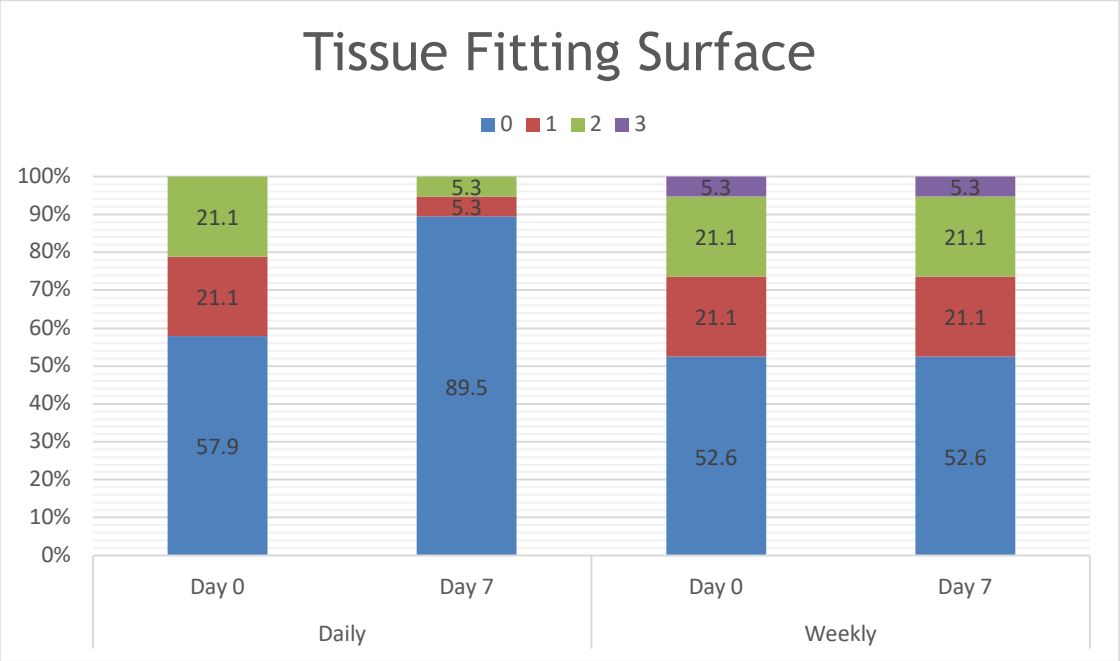

Figure 7. Changes in Distribution of Plaque Scores Between Baseline on Day Zero and Post-Prophylaxis on Day Seven for the Denture-fitting Surface.

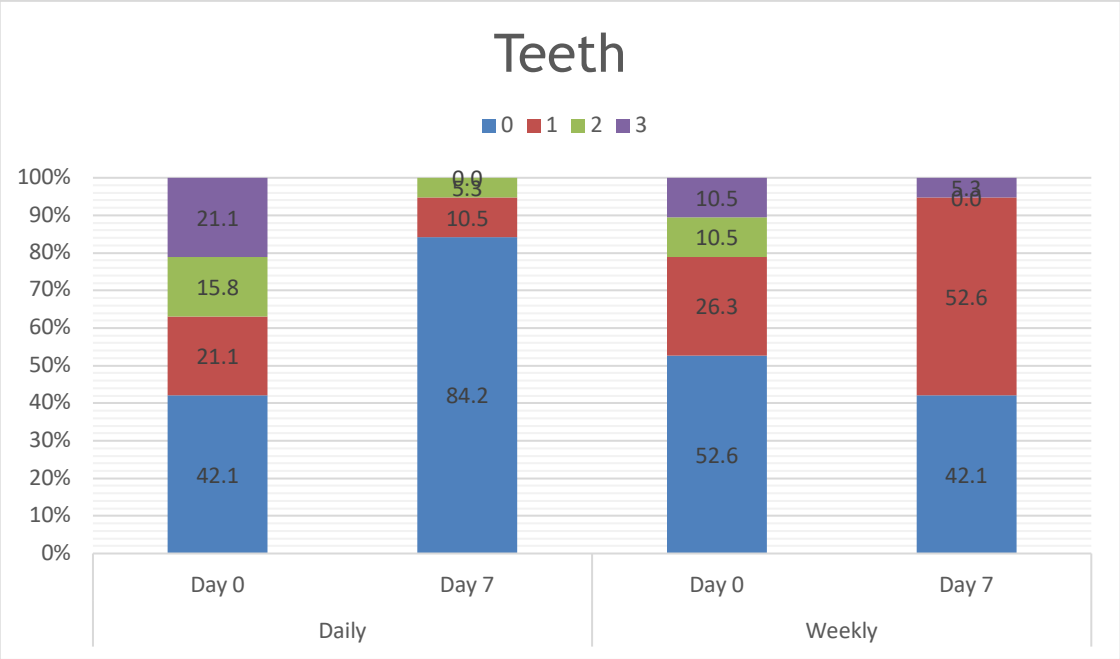

Figure 8. Changes in Distribution of Plaque Scores Between Baseline on Day Zero and Post-Prophylaxis on Day Seven for the Denture Teeth Surface.

4.5 Stain Sampling

Stain scores were low at all time points, including pre-prophylaxis on the initial screening visit, therefore intra- and inter-group differences in stain removal ability of each experimental arm could not be effectively assessed.

4.5.1 Daily Group

In the daily group, the mean baseline stain scores were 0.00 (SE 0.000) on the fitting and polished surfaces of the denture and 0.11 (SE 0.072) on the denture teeth surface. At day seven mean stain scores of 0.00 (SE 0.000), 0.05 (SE 0.053) and 0.11 (SE .072) on the fitting, polished and teeth surface of the denture surface were recorded. This resulted in differences in the adjusted mean stain score of -0.02 (SE 0.053), 0.03 (SE0.067) and -0.08 (SE 0.100) between day zero (baseline) and day seven (post-prophylaxis) on the tissue fitting, polished and denture teeth surface of the denture base respectively.

4.5.2 Weekly Group

In the weekly group, the mean stain scores at baseline were 0.05 (SE 0.053), 0.05 (SE 0.053) and 0.32 (SE 0.110) on the tissue fitting, polished and denture teeth surfaces of the complete dentures. At day seven the post-prophylaxis scores for the weekly group on the fitting, polished and teeth surface were 0.11 (SE 0.072), 0.11 (SE 0.072) and 0.37 (SE 0.14) respectively. Differences observed in adjusted mean stain scores on the fitting, polished and denture tooth surface were 0.08 (SE 0.053), 0.08 (SE 0.067) and 0.13 (SE 0.100) respectively at day zero and day seven.

4.5.3 Inter-group Analysis

Inter-group analysis revealed non-significant differences between the daily and weekly groups at day seven compared to baseline values. Differences, in favour of the daily group were -0.1 (C.I. -0.254, 0.054) ($p = 0.197$) on the fitting surface, -0.05 (C.I. -0.245, 0.145) ($p = 0.606$) on the polished surface and -0.20 (C.I. -0.506, 0.101) ($p = 0.183$).

	Daily (Adjusted Mean, SE)	Weekly (Adjusted Mean, SE)	Difference (Adjusted mean, p value)		
			Difference	95% C.I.	P-value
Fitting	-0.02 (0.053)	0.08 (0.053	-0.1	(-0.254, 0.054)	0.197
Polished	0.03 (0.067)	0.08 (0.067)	-0.05	(-0.245, 0.145)	0.506
Teeth	-0.08 (0.100)	0.13 (0.100)	-0.20	(-0.506, 0.101)	0.1831

Table 8. Changes in Stain Scores on Denture-fitting Surface, Polished Surface and on Denture Teeth.

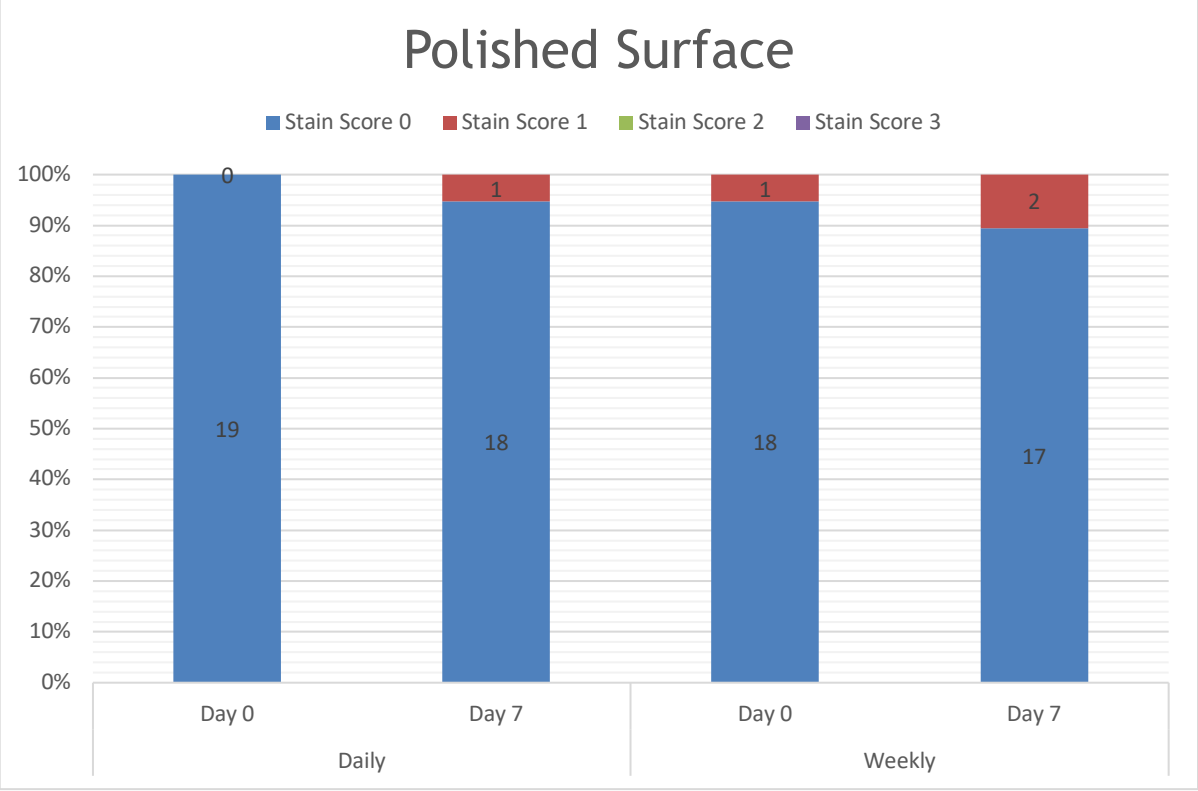

Figure 9. Changes in Distribution of Stain Scores Between Baseline on Day Zero and Post-Prophylaxis on Day Seven for Polished Surface of the Denture.

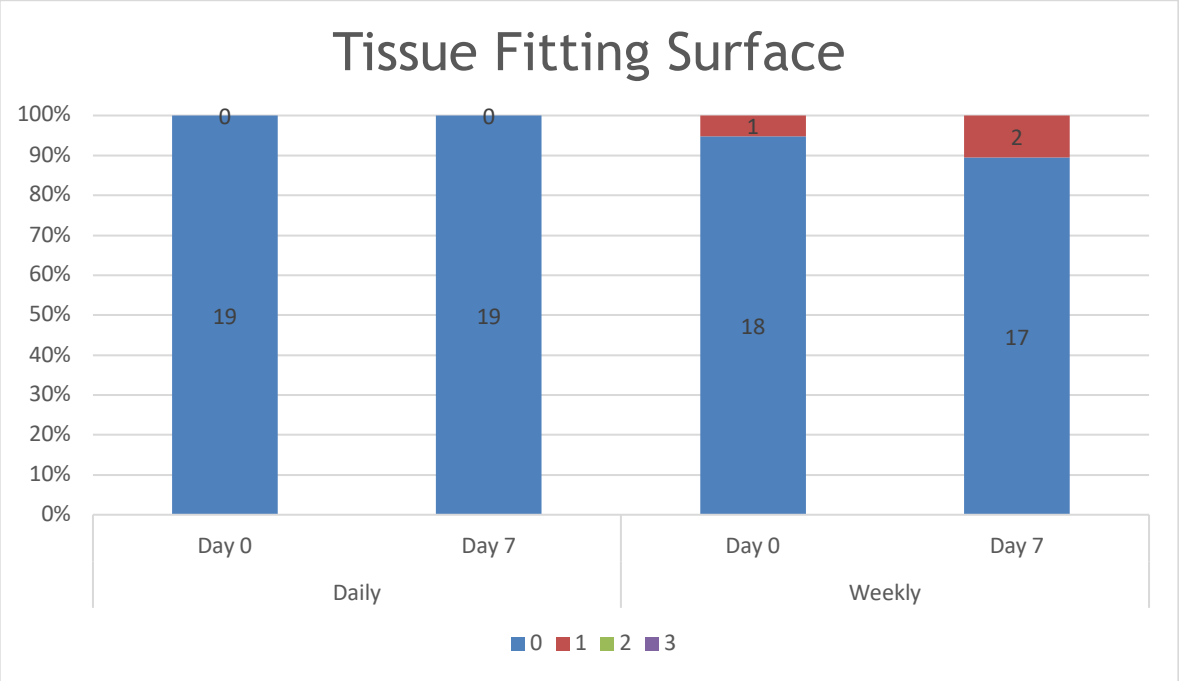

Figure 10. Changes in Distribution of Stain Scores Between Baseline on Day Zero and Post-Prophylaxis on Day Seven for Tissue Fitting Surface of the Denture

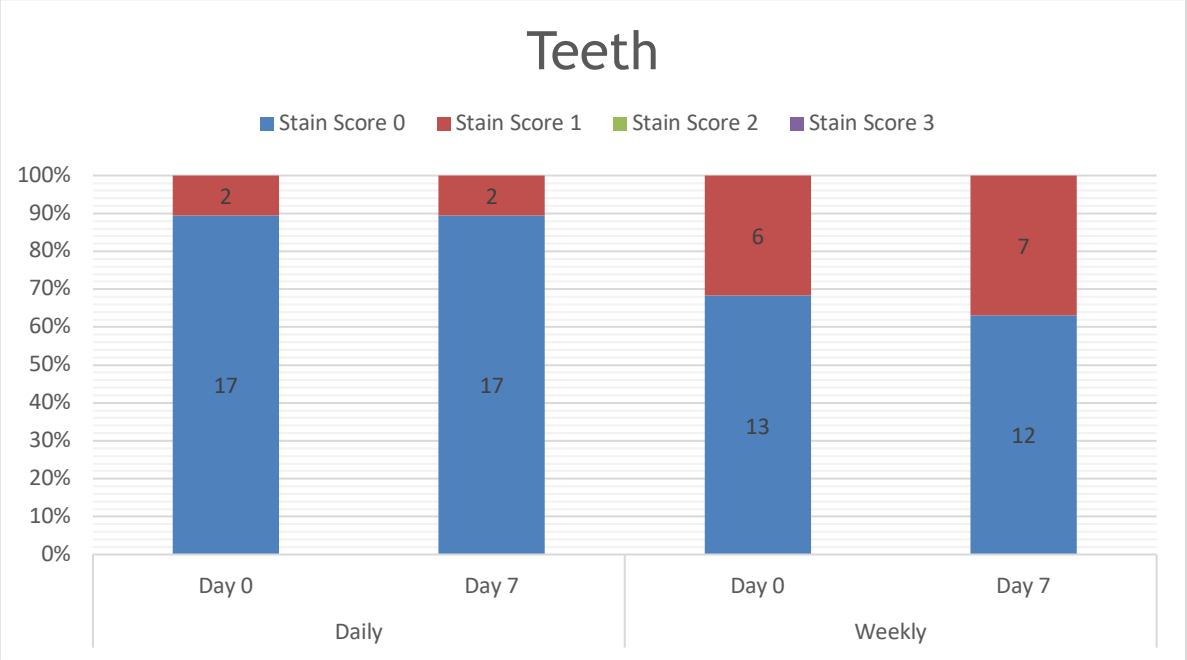

Figure 11. Changes in distribution of stain scores between baseline on Day zero and post-prophylaxis on Day seven for the Teeth Surface of the denture.

4.6 Microbial Composition

Descriptive statistical analysis was carried out on the microbial samples to define the relative percentages of key microbes in the denture plaque. No formal statistical analysis was conducted for inter-group analysis.

Figure 12 describes the number of colony-forming equivalents (CFEs) per disc at each time point for the daily and weekly groups. The diagram represents relative abundance of each of the eight key microbes identified in the exploratory analyses. Samples are presented as a percentage of the total number of microbes per disc, on the assumption that the total colony-forming equivalents of the eight samples tested constitute 100% of the microbial burden.

Quantitative PCR using specific primers identified four main categories of microbes based upon their relative prevalence: >10%, 1-10%, <1%, or not present. Two microbes in both the daily and weekly groups were more common throughout the trial: *Veillonella dispar* and *Streptococcus* spp. *Veillonella dispar* comprised 55-75% and *Streptococcus* spp. 15-41 %. *Actinomyces naeslundii* represented 1-10% of the microbial burden for each group and *Rothia dentocariosa*, *Lactobacillus casei*, *Fusobacterium nucleatum* and *Candida albicans* each contributed less than 1% of the total microbes identified on the disc samples. *Lactobaccilus zeae* was not identified in any of the samples.

Although no formal statistical analysis was appropriate between groups, a trend of increased relative prevalence of *Streptococcus* spp. was observed in both groups over the duration of the trial. Other than this, no other microbes significantly increased their relative prevalence in the samples.

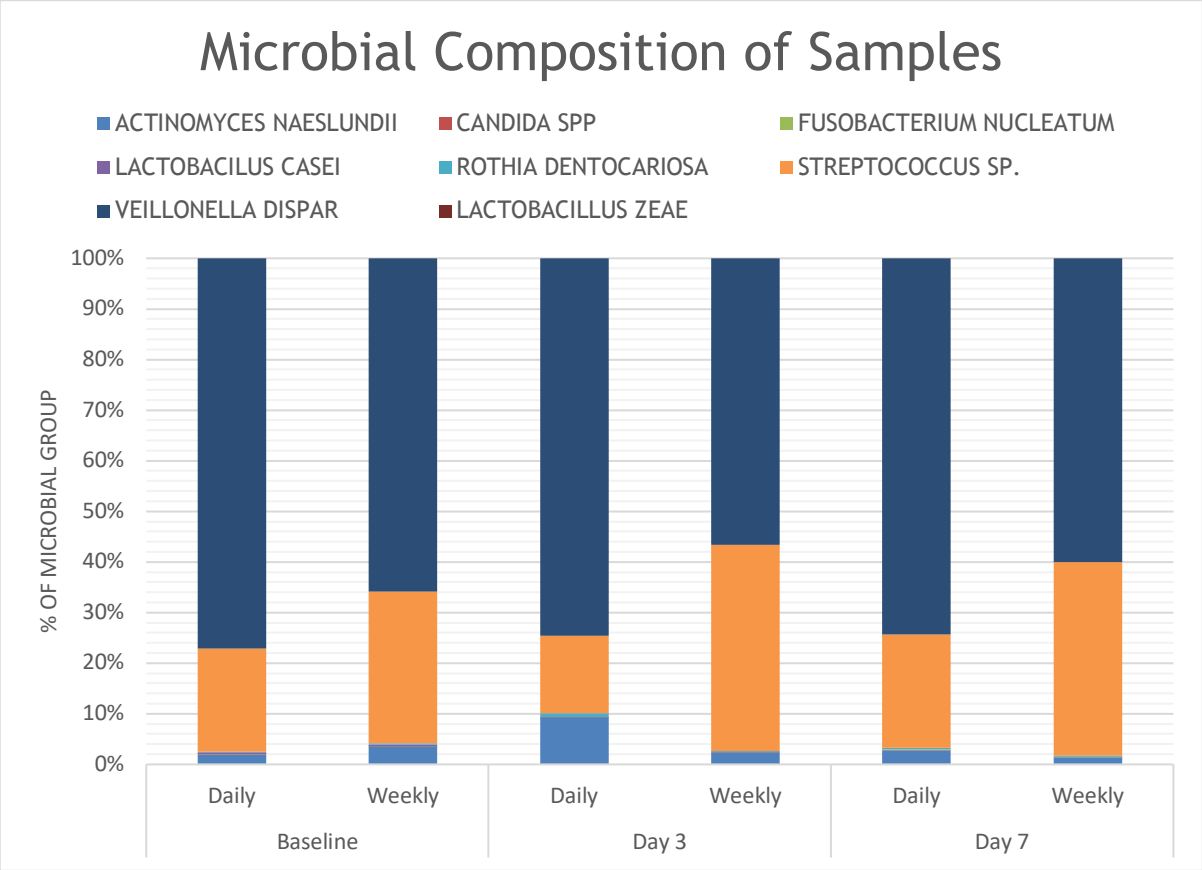

Figure 12. Microbial Composition of Denture Disc Samples at Days Zero, Three and Seven for Daily and Weekly Treatment Groups.

4.7 Subject Assessment Questionnaire

Subject assessment questionnaire (SAQ) responses provided a valuable insight into patient perceived outcome measures of denture cleansing.

A clear improvement was seen in the patient reported outcome measures (PROMs) for both the daily and weekly group at day seven compared to the screening appointment. In both groups 53% (10) participants felt their denture was “clean” at the initial screening visit. This increased to 95% (18) in the daily denture cleanser use group and 84% (16) in the weekly use group.

This improvement in PROMs is reflected in patient perceived improvement in appearance of cleanliness, opinion of denture freshness and perception of breath freshness. With regards to appearance of cleanliness 37% (7) of participants in the daily group and 53% (10) in the weekly group perceived their denture to be “clean” at the screening visit. This increased to 89% (17) for the daily group and 79% (15) in the weekly group at Day seven (post-prophylaxis). In the daily group 37% (7) of participants noted their denture to feel “very fresh”

at their initial visit, the respective figure for the weekly group was 58% (11). This improved to 90% (17) of participants claiming their dentures felt very fresh in both groups at the end of the trial. The patient-reported sensation of “very fresh” breath also increased from 21% (4) to 63% (12) in the daily group and from 26% (5) to 42% (8) in the weekly group from screening to post-prophylaxis on day seven.

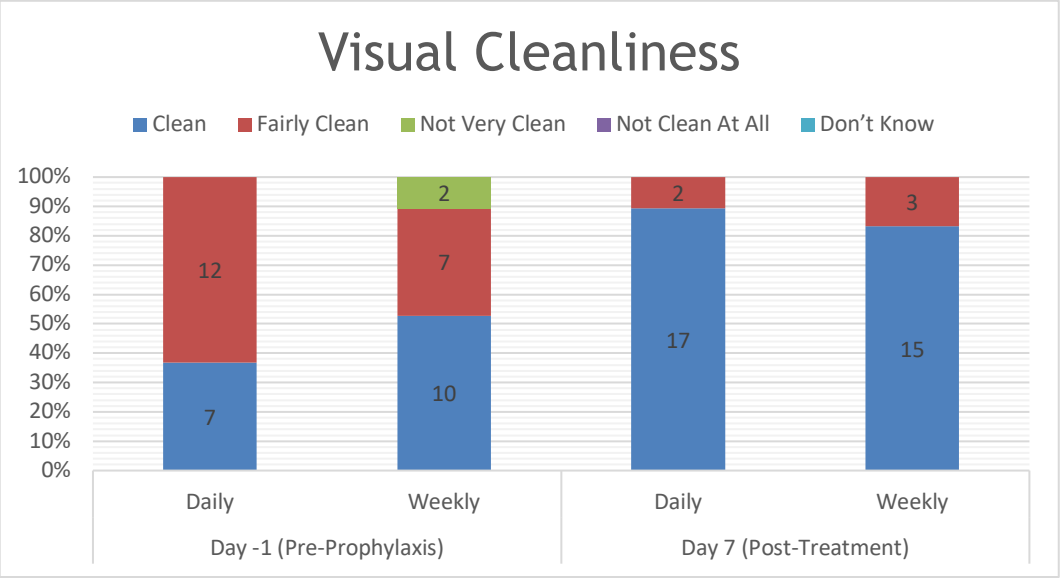

Figure 13. SAQ - Visual Cleanliness.

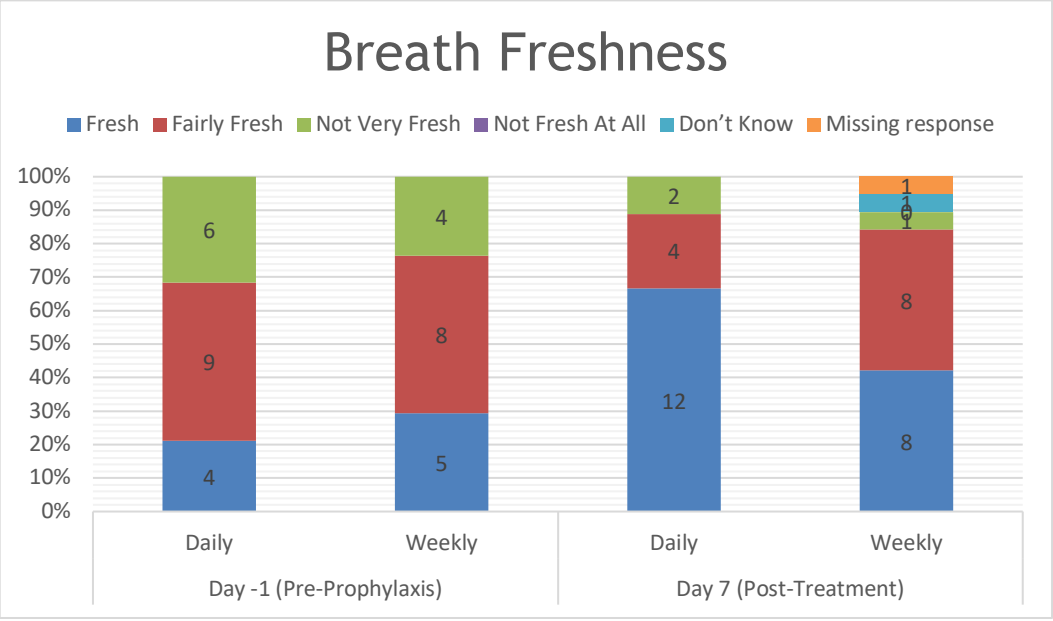

Figure 14. SAQ - Breath Freshness

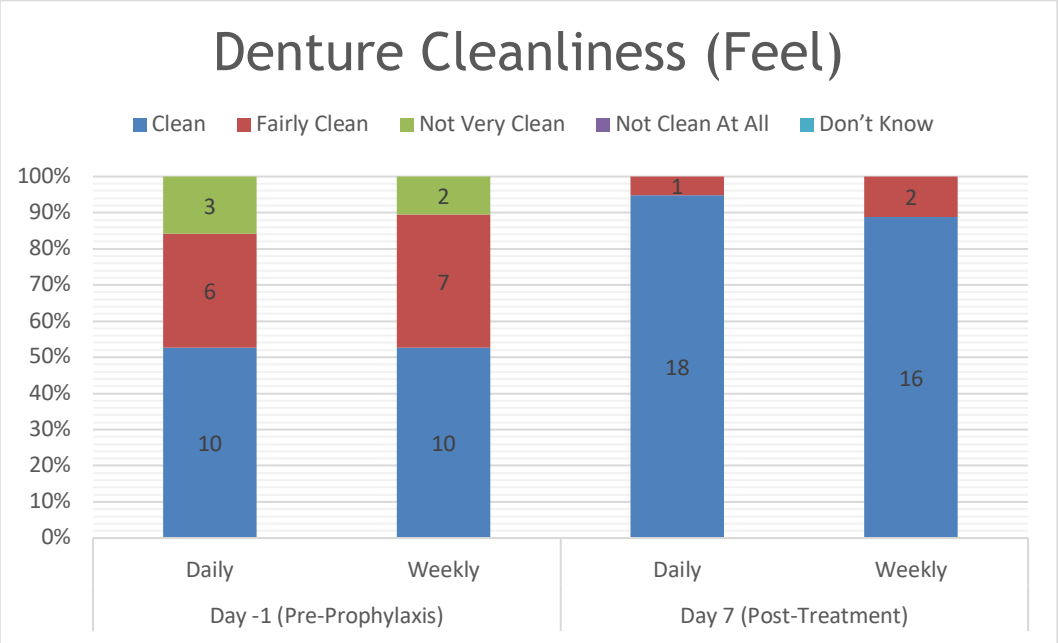

Figure 15. SAQ - Denture Cleanliness (Feel).

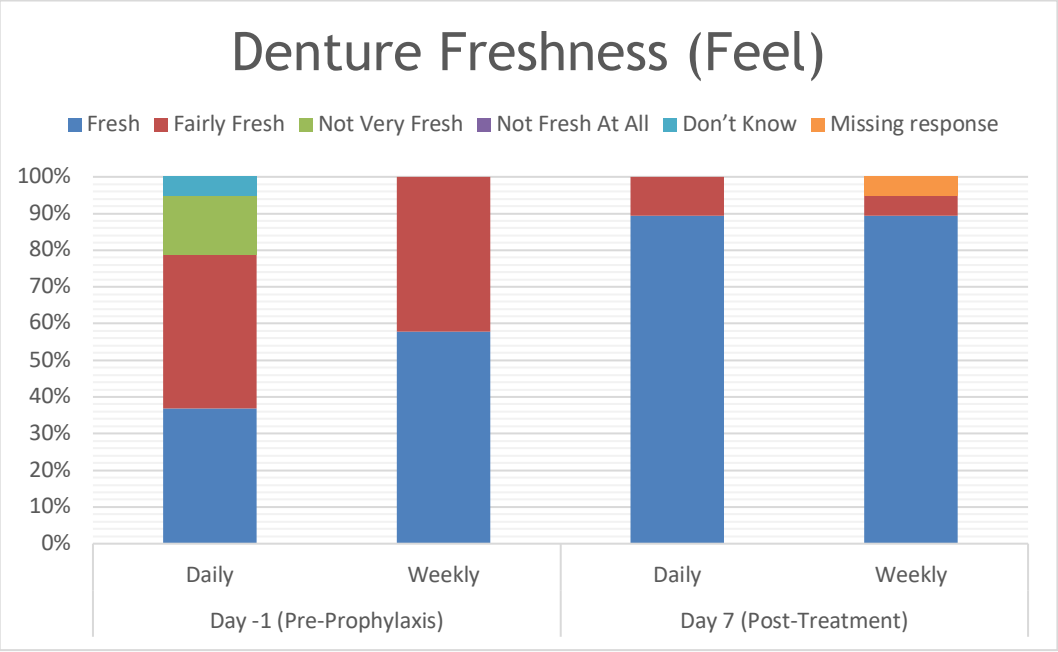

Figure 16. SAQ - Denture Freshness (Feel)

4.8 Repeatability Scoring

Intra-rater agreement scores were consistently high for all variables assessed across the clinical trial. Scoring of stain and plaque levels on each of the fitting, polished, and denture teeth surfaces was repeated once per arm of the trial, ten minutes after the initial score without referencing. From this intra-rater agreement scores were calculated.

For stain scoring, weighted kappa scores of 0.655 (C.I. 0.028, 1.000), 1.000 (C.I. 1.000, 1.000) and 1.000 (C.I. 1.000, 1.000) were found for the tissue fitting, polished and denture teeth surface of the denture, respectively. This represented excellent agreement for the polished and denture teeth surface and good agreement for the denture-fitting surface.

For plaque scoring, weighted kappa scores of 0.969 (C.I. 0.922, 1.000), 1.000 (C.I. 1.000, 1.000) and 1.000 (C.I. 1.000, 1.000) were found for the tissue fitting, polished and denture teeth surface of the denture, respectively. This represented excellent agreement for each variable.

	Stain	Plaque
Fitting	0.655	0.969
Polished	1.000	1.000
Teeth	1.000	1.000

Table 9. Intra-rater Agreement Scores for Clinical Trial.

5 Results – *In Vitro* Assessment of Denture Disc Sampling

The *in vitro* assessment of the disc sampling method was carried out over a period of four days, incorporating a 24-hour incubation period for each experimental repeat.

The five groups analysed were: dry filter disc and dry acrylic (DD), wet filter disc and dry acrylic (WD), dry filter disc and wet acrylic (DW), wet filter disc and wet acrylic (WW) and denture sonicate (S). Three experimental repeats were carried out utilising different inoculated acrylic discs. Each repeat included three samples from each experimental group. Each sample was plated in triplicate as per the method outlined in the Methods and Materials chapter. This produced 27 data points per experimental group.

Six samples were damaged in the *Candida* spp. group during storage therefore only 129 data points were available for analysis. Missing data points were spread across experimental groups.

The numbers of Colony-forming Units (CFUs) were counted for all groups and data assessed to ascertain the most appropriate statistical tests to carry out. As data were non-normally distributed, the median and inter-quartile range of CFUs were used for analysis, Table 10.

Counts (CFUs)		Aerobic	Anaerobic	*Candida* spp.
Median		6×10^6	7×10^6	19×10^5
Percentiles	25	19×10^4	28×10^4	515×10^2
	50	6×10^6	7×10^6	19×10^5
	75	23×10^6	24×10^6	43×10^5

Table 10. Median and Inter-Quartile Range for Aerobic, Anaerobic and *Candida* spp. Counts.

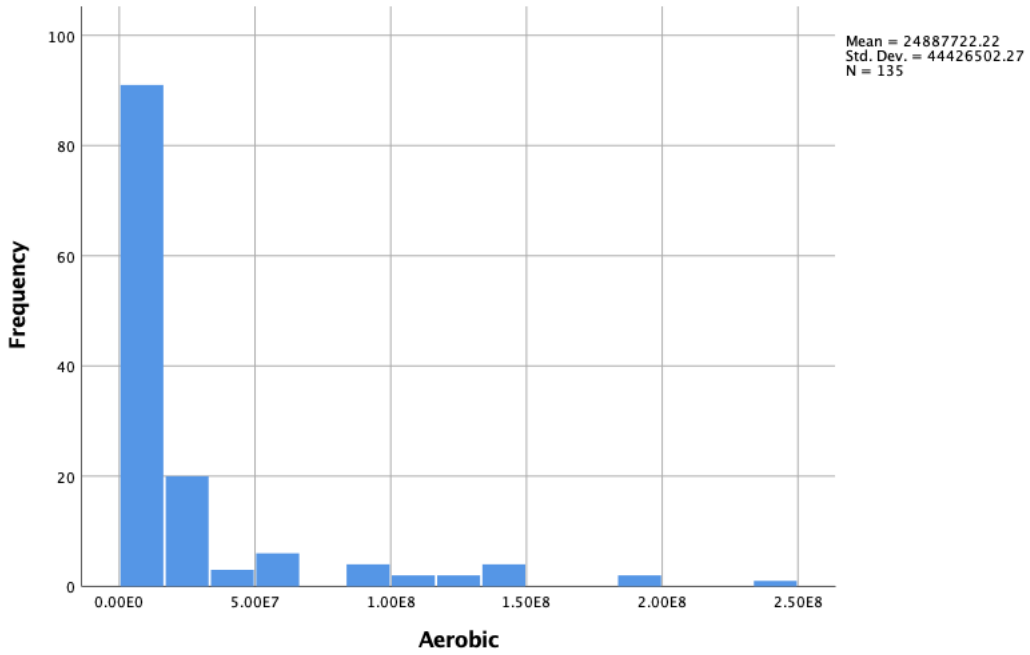

Figure 17. Aerobic Counts and Data Distribution.

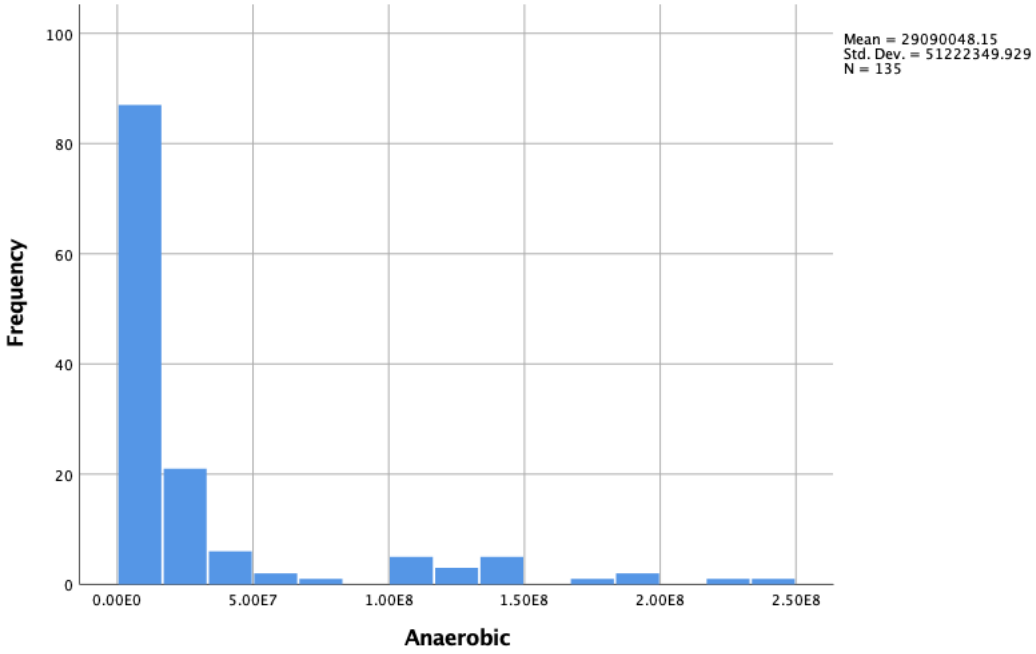

Figure 18. Anaerobic Counts and Data Distribution.

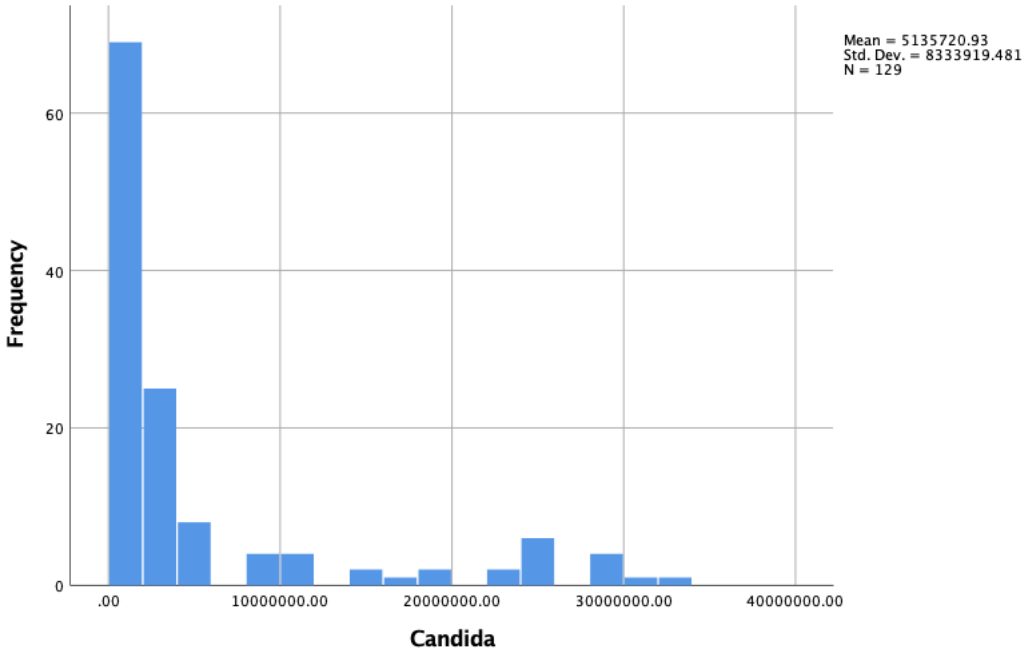

Figure 19. *Candida* spp. Counts and Data Distribution.

Data were non-normally distributed, as demonstrated in figures 17, 18 and 19. Transformation did not result in a normally distributed dataset, therefore non-parametric statistics were used to analyse results. Non-normal distribution was confirmed using the one-sample Kolmogorov-Smirnov test and visual assessment of raw and transformed data.

	Aerobic	Anaerobic	*Candida*	LogA	LogAn	LogC
Mean	2.49×10^7	2.91×10^7	5.14×10^6	14.58	14.70	13.25
Standard Deviation	4.44×10^7	5.12×10^7	8.33×10^6	3.34	3.30	2.97
Assymp. Sig.	0.000[c]	0.000[c]	0.000[c]	0.000[c]	0.000[c]	0.000[c]

[a]Test distribution is Normal.
[b]Calculated from data.
[c]Lilliefors Significance Correction.

Table 11. Normality Testing Results for Raw and Transformed Data.

Plotting of the data using boxplots representing median and inter-quartile range demonstrated a clear difference between experimental groups

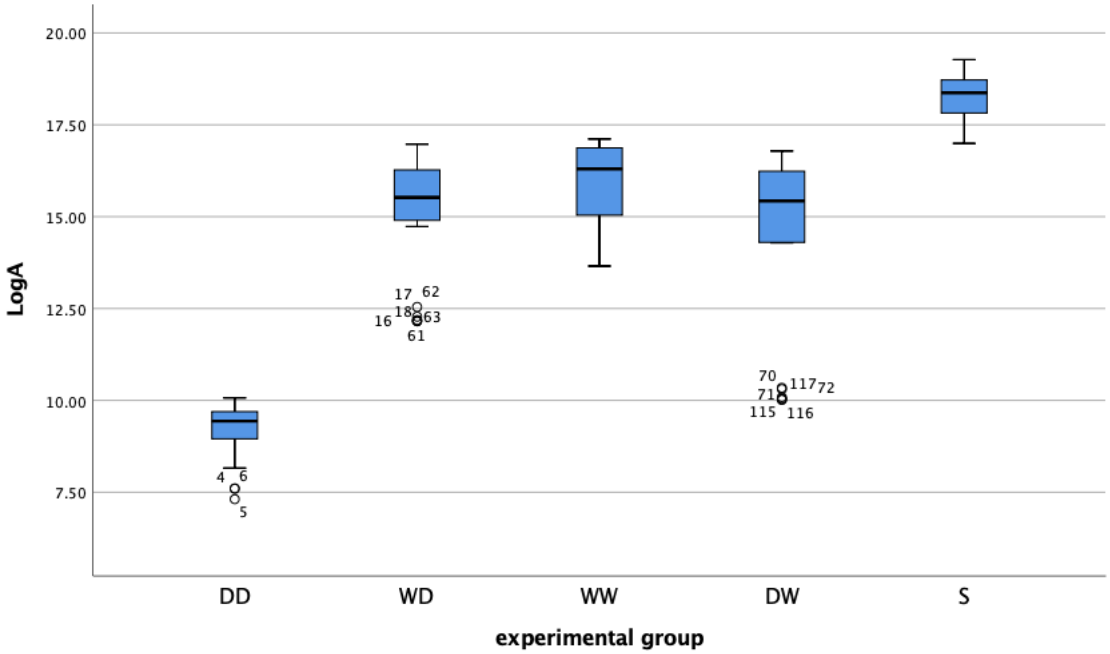

Figure 20. Data Distribution for Aerobic Microbial Counts.

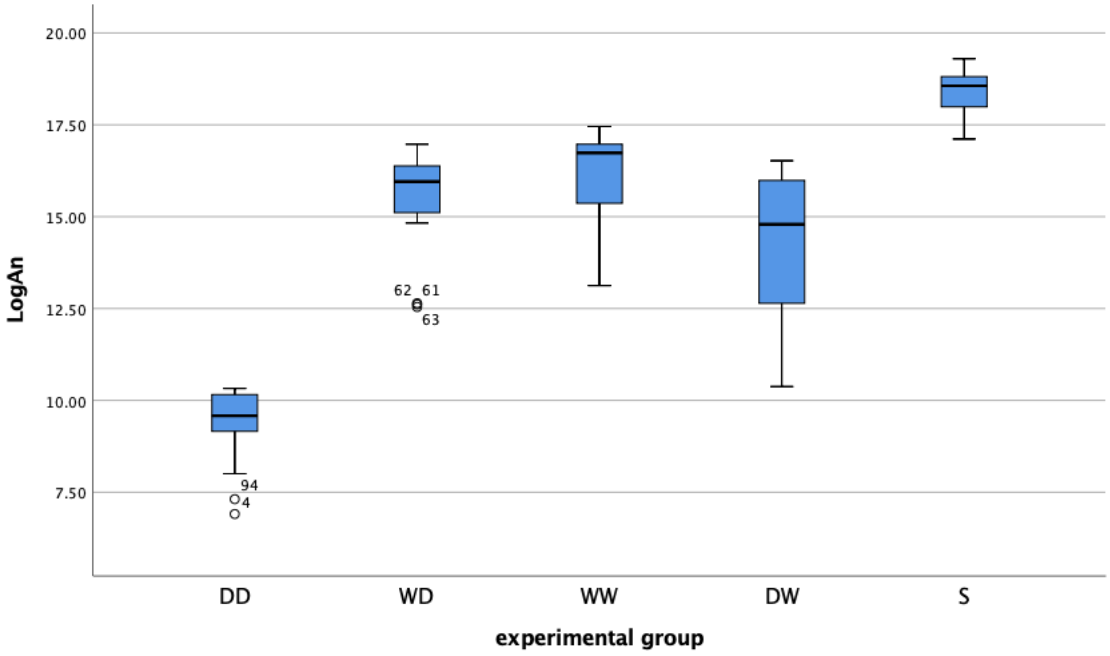

Figure 21. Data Distribution for Anaerobic Microbial Counts.

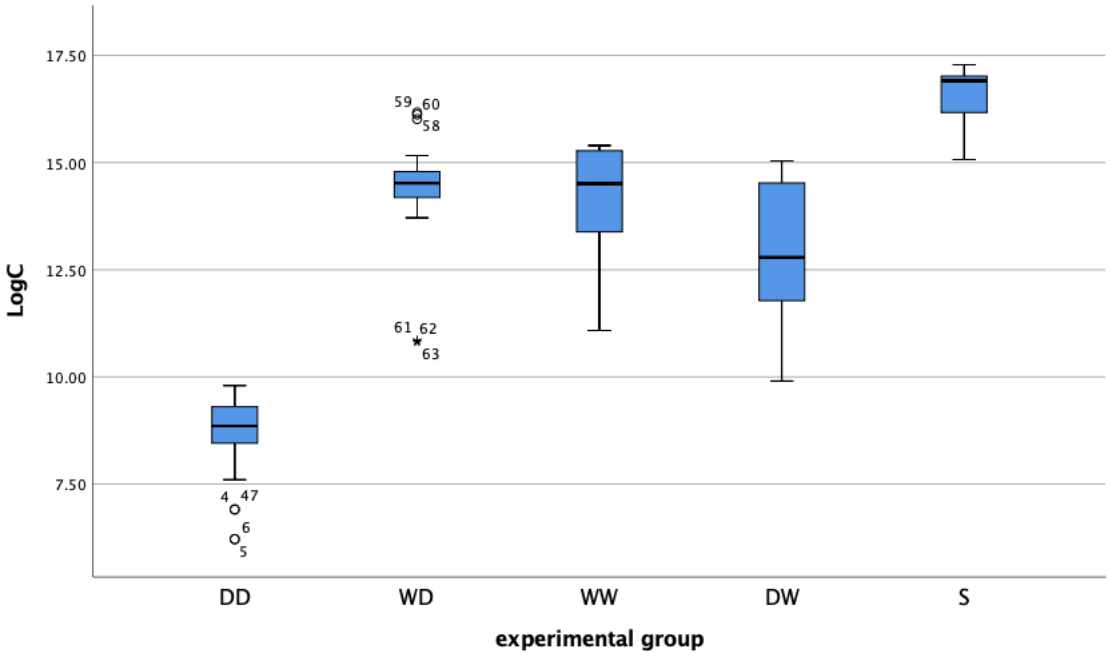

Figure 22. Data Distribution for Candidal Microbial Counts

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Inter-group analysis demonstrated statistically significant differences between experimental groups. As the difference between disc sampling methods was the primary outcome of interest, the sonicate was excluded from statistical analysis.

	Aerobic	Anaerobic	*Candida* spp.
Kruskal-Wallis H	65.27	70.96	63.71
df	3	3	3
Asymp. Sig.	0.000	0.000	0.000

[a]Kruskal Wallis Test
[b]Grouping variable: Experimental Group

Table 12. Statistical Results of Analysis of Data for DD, WD, DW and WW Experimental Groups.

Statistical testing involving denture sonicate values in addition to disc sampling methods were consistent with these results, demonstrating a statistical significance between groups.

	Aerobic	Anaerobic	*Candida* spp.
Kruskal-Wallis H	106.01	109.30	100.23
df	4	4	4
Asymp. Sig.	0.000	0.000	0.000

[a]Kruskal Wallis Test
[b]Grouping variable: Experimental Group

Table 13. Statistical Results of Analysis of Data for DD, WD, DW, WW and S Experimental Groups.

6 Discussion

This method development randomised controlled cross-over trial was designed to assess the clinical and microbiological outcomes of frequency of use of a denture cleanser. Somewhat surprisingly, this is a question that has not been the subject of a significant amount of research and remains largely unanswered. Although it seems plausible and intuitive that more frequent cleaning episodes would result in fewer viable microbes on the denture, in addition to a visually cleaner denture, this has not been scientifically addressed or confirmed. The opportunity to draw conclusions or compare outcomes of different frequencies of use of a denture cleanser on microbial and clinical outcomes within the peer-reviewed literature is also limited by the significant variation between studies.

One challenge in evaluating the current literature is that studies investigating denture cleansing have often used a sample population of convenience, typically institutionalised patients. This is particularly interesting in relation to frequency of use of a denture cleanser. Nishi et al., (2012) demonstrated that adults in a residential care facility may benefit from daily use of a denture cleanser due to the regular routine and subsequent improved compliance. For example, this can confound the results and clinical relevance of the findings as the presence of lifestyle and patient related factors in a care setting may not be reflective of the average edentulous patient living independently at home.

In spite of the large number of denture wearers and significant body of literature focused on denture cleansers, no ‘gold standard’ denture cleanser regimen can be identified. Chemical and mechanical cleansing in combination appear to be the most effective cleansing regimen from the available heterogenous literature (de Souza *et al.*, 2009).This study utilised mechanical daily cleansing regardless of which treatment arm the patient was participating in. Comparison of the different frequencies of use of a denture cleanser when combined with daily mechanical cleaning is particularly valuable as it replicates the most commonly utilised denture cleaning routine among patients attending general dental practices. This comparison, within a pragmatic clinical trial, ensures the clinical relevance of the trial results to the average edentulous patient population. A

drawback of the existing clinical studies in the dental literature is the heterogeneity in hygiene protocols used and their resulting low external validity.

Interestingly, although there is little high-quality evidence on the best denture cleansing routine, there is a significant amount of evidence on the harmful effects of different cleansing regimens on a variety of denture base materials. Heat-cured acrylic (polymethylmethacrylate (PMMA)) is the most widely used denture base material for complete dentures. Depending on a variety of factors, including curing cycle and surface treatments, PMMA will present varying degrees of surface roughness which will have an impact on susceptibility for a biofilm to develop on the denture surface. Prolonged exposure to chemical or mechanical denture cleansing will result in an increased surface roughness and create a favourable environment for biofilm growth (Izumida *et al.*, 2014). There is a clear consensus within the literature as to the potentially harmful effects of an incorrect cleansing regimen, e.g. immersion in boiling water may result in warping of the denture base and prolonged exposure to sodium hypochlorite (NaOCl) of a metal-based denture may have deleterious effects (Kiesow *et al.*, 2016). Curing protocols and technical skill in construction will also affect the acrylic surface and have an impact on the susceptibility to biofilm accumulation and ease of disinfection, e.g. incorporating porosity in the denture base through suboptimal processing which can act as a microbial niche. The use of well-made and functioning complete dentures in this *in vivo* trial is also valuable as it improves the external validity of the results, particularly when assessing a clinical outcome. A significant number of studies in the literature have used processed acrylic discs in an artificial environment, which may not adequately replicate a denture in clinical use when assessing the impact of interventions on denture cleanliness outcomes.

This lack of evidence and comparable studies made a formal sample size calculation impossible. GSK Biostatisticians calculated that 17 participants completing both arms of the clinical trial would provide adequate data for hypothesis testing for the primary microbiological outcomes and allow valuable exploration of other microbiological, clinical and patient-reported outcome measures. Clinical differences seen between groups would also allow future studies to perform robust and reliable sample size calculations to produce

statistically significant results, if present. Experimental and observational analytical studies assessing similar outcomes recruited and analysed a similar number of patients to this study. It was predicted that a total of 30 subjects would have to be screened for 20 participants to be randomised and, following dropouts, for 17 participants to complete both arms of the trial. In practice, only 25 subjects were screened which allowed 19 to be randomised to each arm of the study and all participants completed the trial without any dropouts. Recruitment was initially slow due to several screen failures as a result of the strict exclusion criteria, particularly the need for participants to have no pre-existing oral irritations. The absence of minor oral irritations was very uncommon in a complete denture-wearing population as a large proportion of patients with an edentulous arch will have at least a small area of non-clinically relevant irritation. This was addressed through a protocol amendment which removed the strict exclusion criterion and provided examiners with scope for including patients with non-clinically relevant oral irritations which would not impact on the outcome of the trial. Additionally, as participants could not be re-screened, those participants who were temporarily not suitable for inclusion due to reversible oral irritations, for example trauma from hot foodstuffs which would rapidly resolve, could not be included in the trial at a later stage.

The use of a cross-over trial design also provided an increased number of participants in each arm of the clinical trial, relative to a conventional parallel arm trial. This resulted in exact matching of participants within the limitations of the clinical trial. When utilising a crossover trial design, care must be taken to ensure adequate experimental period length for expression of the outcomes of interest, demonstration of clinical differences between interventions and an adequate washout period to ensure there are no residual effects of the previous treatment period. In this clinical trial, the strict prophylaxis and assessment protocols ensured each denture, at the start of a treatment period, was ‘clinically clean’ and as ‘microbiologically disinfected’ as practical. The length of each treatment arm was also adequate to allow microbiological outcomes (primary outcome of interest) and plaque build-up on the denture surfaces to be assessed. Throughout the trial low stain scores were recorded for all denture surfaces. This is probably related to the inadequate time for stain to develop on a well-made denture base, but also the exclusion criteria may have minimised

the potential impact of some of the causes of staining of a denture base, for example, smokers were excluded from this trial.

The washout period between experimental arms was deemed to be adequate, particularly when combined with the prophylaxis visit, as both arms start at a baseline of a disinfected denture with zero plaque or stain. Due to the nature of the intervention there will be no residual chemical effect of the previous treatment period.

This study was conducted over two different experimental periods, assessing linked components of the research project. The method development clinical trial was a combination of hypothesis testing for the primary microbiological outcomes and hypothesis generating for exploratory objectives. The *in vitro* experiment, assessing the impact of the assessment methodology on the microbiological outcomes, was devised in response to the clinical trial results. This was developed due to the variable and often low numbers of microbes retrieved from sampling within the clinical trial. Although a robust assessment methodology was used for microbial analysis, there were inevitable clinical variables within and between patients which may have impacted on the microbial sampling. An example of this is the timepoints in the clinical trial where repeatability assessments were indicated. These were standardised across the clinical trial and were performed on visit three and seven. At each of these visits, prior to the microbial sampling, a repeatability exercise was performed 10 minutes apart of stain and plaque assessment of the denture surfaces. Dentures were usually left extra-orally at the chairside for this time period, potentially resulting in drying of the denture surface prior to microbial sampling (after subsequent repeat of the stain and plaque scores). It was theorized that this may impact on the viability of microbes retrieved from denture bases and may have been an unaccounted-for variable in the clinical trial. Alternatively, some dentures may have been placed back into the patients' mouths, maintaining a continued moist denture base, which may have had the opposite impact on the viability of microbes retrieved from subsequent sampling. The *in vitro* assessment of this sampling methodology aimed to assess whether the presence of moisture on the acrylic or the sterile paper disc used for sampling would impact on the microbial outcomes. This produced statistically significant

differences between sampling groups and demonstrated the presence of moisture when sampling, whether on the acrylic surface or via a moistened paper disc for sampling, will retrieve significantly more viable microbes than a dry environment.

The sequencing of the experimental periods for the *in vivo* and *in vitro* experiments were unavoidable as the hypothesis for the *in vitro* study was developed due to unexpected variation in the clinical trial microbial results. This will help to inform the experimental and clinical protocols of future studies designed to assess denture microbial outcomes.

Although a randomised, controlled, cross-over trial was the optimal way to assess this experimental interventional trial, some limitations in the study design need to be considered when reflecting upon the results. Due to the nature of the self-performed intervention, in addition to supervised product use, it was impossible to blind the patients to the treatment allocation. Therefore, a bias may be introduced into the study design which must be considered when clinically applying the results of this trial. This is particularly relevant for the subjective participant assessment questionnaire responses, where it may be most impactful. Lack of patient blinding may also introduce bias into the experiment and have an effect on the objective microbial and clinical outcomes. The Hawthorne effect must also be considered when assessing the external validity of the clinical and microbiological results of the trial. Purely by being included in a denture cleansing trial, participants are likely to improve their denture hygiene which may be a factor in low stain and plaque scores throughout the trial. Alternative methods to blind both patient and examiner to treatment allocation were considered but were not feasible due to the resources required and frequency of visits needed. An example of a considered treatment period and experimental intervention to blind study participants was daily patient attendance at the research facility with blinded denture cleansing being carried out by a trained researcher, independent of the patient. This was judged to be impractical for both patients and the research team.

The primary objective of this study was to investigate the efficacy of daily use of a denture cleanser at day seven compared to once weekly use. At day seven, consistently lower numbers of microbes were retrieved from the daily use group

compared to the weekly use group. The results demonstrated statistically significant differences in aerobic microbes at this timepoint between groups in favour of the daily use group. Although directional trends were seen for anaerobic bacteria, the differences were not statistically significant. This could be due to inadequate powering of the trial and therefore, the information provided by these results could be used to adequately power a similar study in the future to demonstrate a significant difference, if present. It is possible that the sampling method may also have impacted on the relative differences between anaerobic and aerobic microbes retrieved. Exposure to oxygen during the sampling process may reduce the number of surviving obligate anaerobes. Additionally, due to their properties of growing in an oxygen depleted environment, anaerobes may be more likely to be incorporated into deeper layers of the microbial plaque biofilm and therefore be less likely to be sampled by the relatively superficial imprint sampling method.

Most interestingly, at day seven post-treatment, where both groups had received the same treatment immediately prior to sampling, there was still a statistically significant difference between groups (for aerobic bacteria). This would suggest that a more mature biofilm, allowed to proliferate further without daily use of a chemical denture cleanser, is more resistant to combined chemical and mechanical cleaning than a less established biofilm disrupted on a daily basis. Statistically significant differences in aerobic and anaerobic microbes retrieved were seen at day three when comparing daily and weekly chemical cleanser use groups. An increase was seen in both aerobic and anaerobic microbial counts for the weekly group, who had not yet used the chemical cleanser, but a decrease was seen for the daily use group. This is in agreement with previous studies, demonstrating that combined therapies for denture hygiene are most effective, but interestingly suggests that brushing with water alone, in the absence of the antimicrobial activity of a chemical denture cleanser, is inadequate to control microbial growth on a denture surface (Cruz *et al.*, 2011, Srinivasan and Gulabani, 2010, Lee *et al.*, 2011, Gornitsky *et al.*, 2002, de Souza *et al.*, 2009). Consistently low numbers of *Candida* spp. were retrieved throughout the duration of the study, which is surprising as this is known to be a clinically important yeast commonly associated with denture stomatitis (Ramage *et al.*, 2012). A potential explanation for the low numbers of *Candida* spp. retrieved is

that these form part of an established biofilm embedded in the plaque matrix. The *Candida* species may therefore be late colonisers and the experimental period, and efficacy of denture cleansing, may not allow proliferation of *Candida* spp. within the biofilm for sampling. This should be considered in future studies and a longer experimental period may be required to allow assessment of *Candida* counts as an outcome measure.

Plaque scores again demonstrated mainly non-significant directional changes in favour of the daily use group. Statistically significant differences between groups were seen for the samples taken from the fitting surface of the denture at day seven. Clinically this is the most important surface for plaque control due to the risk of erythematous candidosis as a result of inadequate denture hygiene. The length of the trial may also impact on the non-significant findings on the polished and tooth surfaces. Seven days is a relatively short period in the use of a denture and a mature plaque biofilm may not have had time to form on the teeth and polished surfaces due to the impact of oral clearance. These surfaces are subject to increased surface abrasion and are exposed to the oral environment to a greater degree, in function and at rest, than the relatively protected fitting surface of a denture. This will inevitably result in a greater likelihood of plaque biofilm development within the protected niche of the denture-fitting surface.

Stain scores displayed a similar pattern to plaque scores with no statistically significant differences between groups. Conclusions which can be drawn from the stain data are limited due to the low stain scores throughout the trial. As discussed, the length of the trial was not conducive to stain formation on a denture base and the design of future trials should consider incorporating a longer period of time to adequately assess this outcome.

The statistical analysis of the stain and plaque data was complicated due to the cross-over study design. Cross-over trials are limited to analysis using parametric statistics or dichotomous categorical statistics. As an ordinal categorical dataset with greater than two categories, a decision was made to utilise parametric statistics which are appropriate for use with a large number of data points for each group. Graphical representation of the data also demonstrated clear differences between weekly and daily groups in changes in distribution of

categories of plaque levels, favouring daily groups. It was not appropriate to carry out further *post-hoc* analysis on this exploratory outcome, although this may be used to adequately power future studies for hypothesis testing of plaque levels as a primary outcome variable.

The sensitivity and specificity of the plaque and stain assessment methods has also not been established from the literature. Although logical in the differentiation between groups, there is little established clinical significance of the different categories. Within the clinical trial, an intra-rater agreement exercise was completed at regular timepoints within each experimental period. These demonstrated excellent intra-rater agreement scores across stain and plaque assessment.

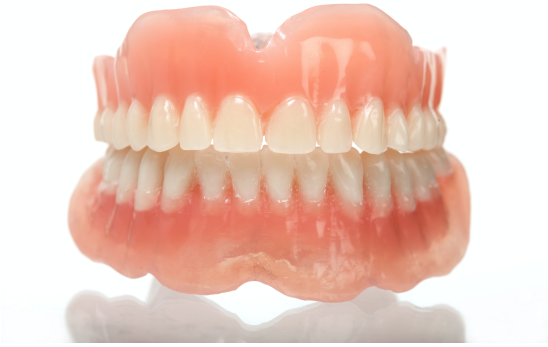
As previously discussed, the low and sometimes variable numbers of microbes retrieved from the disc sampling methodology raised the question of the reliability of this microbial sampling method and how common clinical variables may impact on the numbers of colony-forming microbes retrieved. It was hypothesised that the moisture of the denture base surface may impact on the number of microbes recovered and that a prolonged extra-oral dry time for the denture, which was the case at several points during the clinical trial, may impact on the microbial sampling data. The *in vitro* assessment of this question aimed to assess if there are significant differences in microbial sampling between groups depending on the dryness of the acrylic surface or sterile paper disc used for sampling. This experiment demonstrated significant differences between groups for microbes retrieved, highlighting the importance of controlling the relative wetness of the acrylic or paper disc used. This was a valuable addition to the clinical trial results and the stark difference between a dry sampling methodology and a moist environment was highlighted. In keeping with the ethos of a method development clinical trial, this information can be used to further standardise sampling methodology for future studies.

This study has evidenced the positive impact of an increased frequency of use of a denture cleanser in important clinical and microbiological outcomes for denture cleanliness. This is in keeping with the findings of previously published literature (Nishi *et al.*, 2012). The clinical differences seen can be used to adequately power future studies to identify statistically significant results, if

present, and observations on the impact of clinical sampling methodology can be used to develop protocols for optimally effective sampling of dentures in future studies. Further high-quality studies are required to fully ascertain the microbiological, clinical and patient reported outcomes of an increased frequency of denture cleansing.

Appendices

Appendix I – Patient Recruitment Poster



Do you wear a full upper denture?

Would you be willing to attend Glasgow dental hospital as part of a clinical study?

You will be compensated for your time.

We are recruiting to a clinical study which aims to identify the best way to keep dentures clean.

If you would be willing to participate then please call this number where one of the team will check to see whether you are eligible and give you more information.
0141 201 9322
or e-mail [insert study e-mail address]
and say “Denture care study”

GlaxoSmithKline

Appendix II – Patient Information Leaflet

PARTICIPANT INFORMATION SHEET

A method development clinical study to investigate the efficacy of the different frequencies of use of a denture cleanser

Please read this document carefully.
Please ask if you do not understand or would like more information.

1. Invitation to Participate

You are being invited to participate in a research study conducted with the approval of a national research ethics committee. The sponsor company for this study is GlaxoSmithKline Consumer Healthcare (GSK CH). You have been selected as a potential volunteer because you wear a full upper denture. The following information is provided so that you can make an informed decision regarding your willingness to participate. You do not need to decide today. If you feel that it would help please discuss with family and friends and ask us if there is anything that is not clear or if you would like more information.

2. Background and Purpose

This research study is aimed at evaluating how often people with dentures should use a denture cleanser to keep their dentures clean.

3. Voluntary Participation and Right to Refuse or Withdraw?

It is up to you to decide whether you would like to take part in this study or not. You do not have to take part in this study. You can take as much time as you need to decide upon taking part in this study. You may refuse to participate or may discontinue participation at any time without any negative effects. The study investigator or clinician, the study sponsor, or a competent authority has the right to stop your participation in the study at any time with or without your consent, for any reason. If you decide you no longer wish to take part in this study, all the previously collected samples submitted for analysis before you withdrew from the study will still be analysed and reported. If after withdrawal from the study you wish any of your samples not analysed to be destroyed prior to the analysis, please inform us to this effect in writing.

4. Study Procedures

A total of 20 healthy volunteers will be selected for this study with a view to at least 17 completing all of the visits. There are 2 treatment groups and you will be randomly (randomisation is like flipping a coin) allocated to which one you start the study on: One group will first clean their upper dentures with a denture cleanser once in the first week. They will then clean them as normal for a week. Then change to using the cleanser daily for a further week. The second group will do the same but in reverse order. You then swap to the other treatment group after a break of a week.

If you participate, you will be asked to attend 8 visits in total over the duration of up to 3-4 weeks. Assessments will take place at Screening, Baseline, Day 3 and Day 7. The second treatment period will follow the same pattern.

You will need to complete a daily product use diary throughout the study.

Study visits and procedures are listed in sequence below:

Treatment Period 1:

Visit 1 Screening Visit (Approximately 2 hour):

This initial visit is to determine whether or not you are suitable to participate in the study and will include the following: Informed consent will be obtained. You will be shown the list of study products' ingredients to confirm you are not allergic to any of them. If female, you will be asked to confirm that you are currently using an acceptable method of birth control and are not knowingly pregnant. You will be asked to confirm that you are willing to maintain this method of birth control throughout the study. You will also need to confirm that you are not breast-feeding.

A medical/dental health history will be taken including medications or non-medication therapies taken normally. You will be asked to fill in a questionnaire about your opinion on denture care and how clean your dentures feel. A clinical examination of your mouth, remaining teeth and dentures will be performed.

If you are suitable to continue, the clinician will take a sample of plaque from your dentures and record any staining of the denture. Your dentures, and where necessary your teeth also, will be cleaned. The denture will be placed in a sonic bath (a high pressure water based cleaning bath) for 15 minutes to remove all bacteria. After cleaning, a second sample of plaque will be taken. The fluid that the dentures are cleaned in will be kept to test the amount of bacteria present. You will be instructed on how and when to use the denture cleanser and given a diary to record your use of the denture cleanser..

Visit 2 Baseline Visit (Day 0) (Approximately 1 hour):

Changes in medical or dental history (since the last visit) are recorded and eligibility for the study confirmed. The clinician will check the health of your mouth and assess any staining or plaque accumulation on the denture and will take a sample from the underside of the upper denture before and after supervised cleaning of the denture. Throughout the study, samples will be stored and analysed as noted in "Plaque and Sonication Samples" below.

You will be given instructions on the use of the denture cleanser product and will clean the dentures under supervision. In one arm of the study you will be cleaning only with water while in the other arm it will be with the product under investigation. You will be provided with your allocated study product(s) and diary card (to record when you clean your dentures).

You will not be cleaning your denture again at home in the evening on this day.

Visit 3 (Day 3) (Approximately 1 hour):

Medical history and eligibility criteria will be reconfirmed noting any changes. Your completed diary card and product use will be checked. The clinician will check the health of your mouth and assess any staining or plaque accumulation on the denture and will take a sample from the underside of the upper denture before and after supervised cleaning of the denture. Your product and diary card will be returned to you.

You will not be cleaning your denture again at home in the evening on this day.

Visit 4 (Day 7) (Approximately 1 hour):

Changes in medical or dental history (since the last visit) are recorded. You will be required to return all your empty and partly used denture cleanser if applicable at the end of this phase of the trial. Your completed diary card and product use will be checked.

The clinician will check the health of your mouth and assess any staining or plaque accumulation on the denture and will take a sample from the underside of the denture before and after supervised cleaning of the denture. The denture will also be taken and placed into a sonic bath for cleaning. The fluid from the cleaning will be retained to test for the amount and type of microbes are present. Your teeth and dentures will then be cleaned if necessary. You will be asked to fill in a further questionnaire about your experience.

No Study Treatment for a week (7 days):

You can clean your dentures and teeth as normal during this period

Treatment Period 2:

Visit 5 (Approximately 2 hours):

Changes in medical or dental history (since the last visit) are recorded and eligibility for the study confirmed. A clinical examination of your mouth (including those supporting the teeth) will be performed. Your dentures, and where necessary your teeth also, will be cleaned. Plaque samples will be taken from the upper denture before and after cleaning, an assessment of any staining of your dentures will be carried out and the denture will be placed in a sonic bath for 15 minutes to remove all bacteria.

Visit 6 (Day 0) (Approximately 1 hour):

Changes in medical or dental history (since the last visit) are recorded and eligibility for the study confirmed. A clinical examination of your mouth (including those supporting the teeth) will be performed. Plaque samples will be taken from the denture before and after cleaning, an assessment of any staining of your dentures will be carried out and the denture will be placed in a sonic bath for five minutes to remove all bacteria.

You will be given instructions on the use of the denture cleanser product and will clean the dentures under supervision. In one arm of the study you will be cleaning only with water while in the other arm it will be with the product under investigation. You will be provided with your study product(s) according to the allocation schedule and diary card (to record when you clean your dentures).

Visit 7 (Day 3) (Approximately 1 hour):

Medical history and eligibility criteria will be reconfirmed noting any changes and your completed diary card and product use will be checked. The clinician will check the health of your mouth and assess any staining or plaque accumulation on the denture and will take a sample from the underside of the denture before and after supervised cleaning of the denture. Your product and diary card will be returned to you.

Visit 8 (Day 7) (Approximately 1 hour):

Changes in medical or dental history (since the last visit) are recorded. You will be required to return all your empty and partly used denture cleanser if applicable at the end of this phase of the trial. Your completed diary card and product use will be checked.

The clinician will check the health of your mouth and assess any staining or plaque accumulation on the denture and will take a sample from the underside of the denture before and after supervised cleaning of the denture. The denture will also be taken and placed into a sonic bath for cleaning. The fluid from the cleaning will be retained to test for the amount and type of microbes are present. Your teeth and dentures will then be cleaned if necessary. You will be asked to fill in a further questionnaire about your experience before and after supervised cleaning of the denture.

General Practitioner (GP) Contact:

You will be asked to provide details of your GP and dentist. With your consent we will inform your dentist of any dental treatment required. Your GP will not be informed or contacted unless you become seriously ill whilst taking part in the study.

Plaque and Sonication Samples

Plaque samples will be taken from your dentures at each visit and the sonication sampling (the water left from the denture cleaning bath) will take place on visits 1, 4, 5 and 8. The samples will be used to determine the amount and type of bacteria in your mouth. The samples will not be used for genetic testing or to test for any unknown diseases.

The samples collected will be frozen at -70°C and initially stored in the University of Glasgow Dental School for up to 12 months following completion of the study. After initial processing, the samples will be transferred to a University in Amsterdam (Academisch Centrum Tandheelkunde Amsterdam ACTA) for processing to assess the levels of bacteria. The samples will be destroyed once the analysis has been completed (no later than 12 months after completion of the study).

The samples will be taken and handled following the relevant laws or guidelines covering the collection, use, storage, transportation and disposal of human tissue and protection of data privacy (UK Human Tissue Act). To ensure that you are not identified by name, your plaque and sonication samples will be identified by a code that preserves your anonymity. Anyone who works with your samples will hold the information and results in

confidence. The location of your samples will be traceable using this unique code at any time from sample collection through to sample destruction.

If you decide you no longer wish to take part in this study, all the previously collected samples submitted for analysis before you withdrew from the study will still be analysed and reported. If after withdrawal from the study you wish any of your samples not analyzed to be destroyed prior to the analysis, please inform us to this effect in writing.

5. Possible Side effects of the Product or Risks from the Study Procedures.

The denture cleanser used in this trial is a marketed product but it is important to understand that some risks are involved in clinical research, just as in routine medical care and activities of daily living. It is anticipated the risks of participating in this study are minimal. There is always a possibility you will experience unexpected side effects.

We do not expect you to experience any significant side effects from using the study products. If you do experience a problem after leaving the site or up to 5 days after your last use of your study products, please inform a member of the study team.

Emergency contact details:
Joanna Mcgrory 0141 2119739

6. Possible Benefits to Volunteers

Your dentures will be cleaned thoroughly at the beginning and end of each phase of the study. There are no other expected benefits to participants but your involvement in the study will contribute to our knowledge of denture care.

7. Sponsor Interest in Study

Funding for this project is provided by GlaxoSmithKline (GSK) Consumer Healthcare. The sponsor company (GlaxoSmithKline Consumer Healthcare) will own the information resulting from the analyses, similar to information collected in the study. None of this information will identify you, and you will not benefit financially aside from the compensation you receive for taking part in the study.

8. If New Information Becomes Available

If new information about the denture cleanser becomes available during the course of the study, staff will tell you about it and discuss with you whether or not you want to continue in the study.

9. Are there any reasons why my participation in this study could be ended?

You are free to withdraw from participating in this study at any time and for whatever reason, specified or unspecified, and without prejudice. The Investigator or sponsoring company may discontinue your participation at any time without your consent. The following are reasons why you may be asked to withdraw from the study:

- If you are, or become pregnant.
- The Investigator's request.
- Your safety (such as an adverse reaction).
- At your request.
- If you don't comply with the study procedures.
- Your use of or need for other medications or therapy that could interfere with the study results.

10. Alternative procedures/products?

Alternative products to reduce bacteria in the mouth are available on the market within the EU. You will be asked not to use any other oral care products while participating in the study.

11 Reimbursement to Volunteers

Upon successfully completing all of the study procedures, you will receive a financial remuneration in the amount of £320. If you withdraw from the study early, you will receive £40 per visit completed. The amount of compensation you receive is a nominal sum for any inconvenience you may have experienced whilst participating in the study.

12. What if something goes wrong?

Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of the Association of the British Healthcare Industries (ABHI). Broadly speaking the ABHI guidelines recommend that 'the sponsor', without legal commitment, should compensate you without you having to prove that it is at fault. This applies in cases where it is likely that such injury results from giving any new drug or any procedure carried out in accordance with the protocol for the study. 'The sponsor' will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol for the study. Your right at law to claim compensation for injury where you can prove negligence is not affected. Copies of the ABHI guidelines are available upon request.

13. Confidentiality?

If you consent to take part in this study, NHS Greater Glasgow and Clyde, the University of Glasgow and GSK, in accordance with international regulatory guidelines, will store the information collected during the study. The Investigator is responsible for safety and security of the data. Handling, processing, storage and destruction of personal data will be in accordance with the UK Data Protection Act 1998 and the University of Glasgow's data Protection policy. The information may also be made available within and outside the EU to monitors, auditors (both from the research organisation and Sponsor company), members of the Ethics Committee and staff from regulatory authorities, for the purposes of data verification.

Only NHS Greater Glasgow and Clyde and University of Glasgow staff and the monitors will know that the information is related to you and this information is kept separate and confidential. Some study documents may also be looked at by authorised representatives from the NHS Greater Glasgow and Clyde or University of Glasgow to check that the study is being carried out in accordance with Good Clinical Practice research guidelines. Professional

standards of confidentiality will be followed by the authorised representatives. A representative from the sponsor company may observe the study procedures at one or more study visits.

The data from this study will be stored on a password protected electronic database called 'InForm' and as anonymised laboratory samples. Only data needed to meet the study objectives will be collected. Throughout the study, you will be identified only by a unique identification number. Your name is never entered into the electronic database. Data will be transferred outside of the EU (to the USA and India) for data processing and analysis. Procedures will be put in place to protect your information even in countries whose data privacy laws are less strict than those of this country.

A description of this clinical study will be available on the GSK Clinical Study Register: http://www.gskclinicalstudyregister.com and may also appear in clinical trial/study registries in countries in which the clinical study is conducted. GSK may publish study results for medical journals, meetings and on the internet for other researchers to use; your name will not appear in any publication.

14. Intellectual Property

The information and any materials or items that you are given about or during the study should be considered the confidential business information of the study sponsor. You are; of course, free to discuss with your friends and family while considering whether to participate in this study or at any time when discussing your present or future healthcare.

15. Complaints

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. Contact details are provided at the end of this information sheet. If you remain unhappy and wish to complain formally you can do this through the Research Governance Office, quoting reference 205202.

Please write to:

All communication will be dealt in strict confidence. Every care will be taken to ensure your well-being and safety are not compromised during the course of the study. However, special insurance arrangements are in place (no-fault compensation) in the event that something unforeseen happens and on the balance of probabilities, harm is attributed to your participation in this study.

16. Study results

The results of this study may be presented at meetings and may be published. No participants will be identified in any report. They will also be accessible on www.clinicaltrials.gov.

17. Contact to Ask Questions Regarding this Study or in Case of Emergency

You have the right to ask questions about this study at any time. You will be informed about any change to the study that might concern you. Should you have questions, please contact Joanna Mccory at the research site on 0141 2119739 during normal office hours.

In case of emergency you can call Mobile phone number TBC available 24 hours.

A copy of this information sheet and a signed consent form will be given to you.

Appendix III – Study Consent Form

Site Address: University of Glasgow
Dental School
378 Sauchiehall St.
Glasgow, G2 3JZ
United Kingdom
Phone number: 0141 2119739

Version 3.0, 30/03/2016
Study Number: 205202
Subject Identification Number for this study: _____

CONSENT FORM

Title:	A method development clinical study to investigate the efficacy of the different frequencies of use of a denture cleanser
Sponsor:	GlaxoSmithKline Consumer Healthcare
Principal Investigator:	Dr Douglas Robertson

Please initial box_ ____

1. I confirm that I have read and understood the Participant information sheet dated 30.03.2016 (version 3.0) for the above study and have had the opportunity to consider the information and ask questions. I understand that by signing this consent form, I do not waive any of my legal rights. ☐

2. I understand that my 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.
I understand that compensation for my time on the study will be pro-rata should I choose to withdraw. ☐

3. I understand that sections of any of my records may be looked at by the researchers and responsible individuals from regulatory authorities, representatives from the Sponsor company, a designated quality assurance function, or from the ethics committee, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I give permission to the investigators to pass clinical data collected from my examination to my Dentist, and to inform them of my participation in the study. ☐

5. I agree to provide the contact details of my Doctor / General Practitioner (GP) and Dentist / General Dental Practitioner (GDP). I agree to my doctor and dentist being informed if deemed necessary – for example if I fall seriously ill whilst taking part in the study or any relevant medical/dental condition is diagnosed – and understand that I will be informed of any incidental findings identified throughout the course of the study. ☐

6. I give permission that the data collected from me will be transferred to countries outside the European Economic Area to be processed. ☐

7. I confirm that I have been shown the list of ingredients of the products to be used in this study and am not aware of having had a previous adverse or allergic reaction to any of the ingredients listed. ☐

8. I agree to take part in the above study. ☐

_____ _____ _____

Name of subject Date Signature

_____ _____ _____

Name of Person taking consent Date Signature

Appendix IV – Subject Assessment Questionnaire

Subject Assessment Questionnaire (SAQ)

- Complete before prophylaxis on Day -1 [Visit 1 & Visit 5]
- Complete before supervised product use on Day 7 [Visit 4 & Visit 8]
- Complete after supervised product use on Day 7 [Visit 4 & Visit 8]

Ask the subject to take a look at the upper denture outside the mouth. Then, ask the subject to rate Question 1. (Select only one)

1. How clean does your denture look?

- Very clean
- Fairly clean
- Not very clean
- Not at all clean
- Don't know

After placing the upper denture back in the mouth, ask the subject to rate questions 2 and 3. (Select only one)

2. How fresh does your denture feel?

- Very fresh
- Fairly fresh
- Not very fresh
- Not at all fresh
- Don't know

3. How does your breath feel?

- Very fresh
- Fairly fresh
- Not very fresh
- Not at all fresh
- Don't know

Ask the subject to run the tongue along the surfaces of the upper denture including the teeth. Then ask the subject to answer the following question. (Select only one)

4. How clean does your denture feel?

- Very clean
- Fairly clean
- Not very clean
- Not at all clean
- Don't know

Appendix V - Protocol for Acrylic Disc Preparation

Construction

1. Generously apply separating medium to moulds
2. Mix acrylic as per manufacturer's instructions
3. Wail until dough stage
4. Will not stick at this stage
5. Wait till separating medium set
6. Small amount of acrylic- pea sized amount- into mould
7. Hydraulic press to 2 (on adjacent machine to working area) or 100 Bar for 5 minutes
8. Flask as per setting on dry bath
9. 3 hours on, 1 hour off, 5 hours on
10. 3 hours bench cooling time
11. Immerse in water for 5-7 days

Sterilisation

12. Discs added to 500 mL of ddH2O containing 1 NaOCl tablet for 24 hr
13. Discs were then sonicated for 15 min in ddH2O
14. Treated with 100% ethanol for 2 hr
15. Sonicated once more for 15 min in ddH2O
16. Each side of the disc UV treated for 15 min
17. Discs kept in dry sterile tubes until use

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Impact of frequency of denture cleaning on microbial and clinical parameters – a bench to chairside approach

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ORIGINAL ARTICLE

Impact of frequency of denture cleaning on microbial and clinical parameters – a bench to chairside approach

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ABSTRACT

Objective: Robust scientific and clinical evidence of how to appropriately manage denture plaque is lacking. This two-part study (i) developed an *in vitro* model of denture plaque removal, and (ii) assessed effectiveness of these approaches in a randomised clinical trial.

Method: (i) a complex denture plaque model was developed using the dominant microbial genera from a recent microbiome analyses. Biofilms formed on polymethylmethacrylate were brushed daily with a wet toothbrush, then either treated daily for 5 days or only on Days 1 and 5 with Polident® denture cleanser tablets (3 min soaking). Quantitative and qualitative microbiological assessments were performed. (ii), an examiner-blind, randomised, crossover study of complete maxillary denture wearers was performed (n = 19). Either once-daily for 7 days or on Day 7 only, participants soaked dentures for 15 min using Corega® denture cleansing tables, then brushed. Denture plaque microbiological assessment used sterilized filter paper discs.

Results: The *in vitro* model showed daily cleaning with denture cleanser plus brushing significantly reduced microbial numbers compared to intermittent denture cleaning with daily brushing ($p < 0.001$). The clinical component of the study showed a statistically significant reduction in denture plaque microbial numbers in favour of daily versus weekly treatment (aerobic bacteria $p = 0.0144$). Both *in vitro* and *in vivo* studies showed that denture plaque biofilm composition were affected by different treatment arms.

Conclusions: This study demonstrated that daily denture cleansing regimens are superior to intermittent denture cleansing, and that cleansing regimens can induce denture plaque compositional changes. Clinicaltrials.gov registration: NCT02780661.

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Denture; plaque; microbiology; denture cleansing; oral microbiome; antimicrobials

Introduction

As the elderly population expands to a predicted two billion by 2050, the number of denture wearers will continue to rise. Edentulousness (loss of all teeth) is an irreversible clinical condition that can be described as an ultimate marker of oral disease burden [1]. Currently, around 20% of the UK population wear removable dentures of some form, with 70% of UK adults older than 75 years old wearing dentures [2]. Denture wearing is also associated with socio-economic deprivation and is more common in women [3]. Many of these individuals have oral diseases related to their denture wearing including denture-induced stomatitis (DS), an inflammation of the denture bearing mucosa [4]. Poor oral hygiene is frequently observed within this group and several factors can impact the onset of DS such as salivary pH, smoking, sugar consumption, oral *Candida*, age of denture, and, importantly, denture cleanliness [5].

The high prevalence of edentulousness and associated DS highlight the importance of having consistent effective denture care regimens which patients can follow with confidence. However, the clinical and laboratory evidence to support one regime over another is not yet clear. To date, two systematic reviews featuring a total of six randomised controlled trials (RCTs) have been published [6,7]. These reviews concluded that there was lack of evidence on which to base guidelines and that further RCTs are required. Recent guidelines based on the available evidence suggest that removal of 'bacterial biofilm' is of paramount importance to sustaining good oral and systemic health and preventing DS [8]. These guidelines also advocate the reduction and maintenance of low levels of microbial denture plaque through daily soaking and/or brushing with an effective, non-abrasive cleanser, but there is a lack of clarity as to how this is best achieved. Subsequent meta-analyses indicate that in addition to existing methods, antiseptic

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mouthwashes, disinfection agents, natural antimicrobial substances, photodynamic therapy and microwave disinfection could all be effective adjunctive strategies for the management of denture hygiene [9].

Dentures are colonised when placed in the mouth by a complex microbial plaque biofilm, which contains numerous species of bacteria and fungi [10,11]. Plaque development and microbial retention are aided and enhanced by the irregular topographical surface including cracks and crevices which can exist within denture acrylic surfaces [12]. This environment also provides protection from chemotherapeutic agents and mechanical disruption methods, meaning that some denture surfaces can carry up to 10^{11} microbes per milligram of plaque [13,14]. Denture plaque biofilm also represents a reservoir for potential opportunistic respiratory pathogens [15].

There is a lack of consensus around suitable cleaning agents, with many denture wearers opting to use toothpaste to mechanically clean their dentures. However, this has been shown to induce abrasions, resulting in physical defects on the denture acrylic that may lead to enhanced microbial adhesion through altered surface topography [14,16–18]. Guidance on the frequency of cleansing is also lacking, although laboratory and clinical studies report that the sporadic use of denture cleansers facilitates the build-up of mature denture plaque biofilms [19–21]. Many chemotherapeutic interventions recommended are effective against planktonic oral bacteria, but unfortunately live intact biofilms are able to persist even after treatment with sodium hypochlorite [22]. These studies taken collectively suggest that denture cleansing is important, but more difficult to achieve than previously thought.

To shape and design an effective clinical trial, appropriate laboratory models are needed to assess *in vitro* the effect of novel approaches to denture cleansing on the biofilm. Unfortunately, progress here has been hampered by the fact that many denture plaque treatment studies have focussed on *Candida albicans*, primarily due to its role in denture-related disease [4, 21–25]. Available data conservatively estimates that at least 10-fold more bacteria than yeasts colonise the surface of dentures [10], clearly indicating that denture plaque has a polymicrobial and interkingdom composition [11]. Denture plaque biofilm models, such as a recently described 11 species interkingdom model, are likely to be more representative of the polymicrobial nature of the clinical situation [26]. In this study, we sought to adopt a bench-to-chairside approach to test the appropriateness of routine daily denture cleansing methods compared to intermittent methodologies. For this study we have used denture cleanser tablets that are based on generating hydrogen peroxide and peracetic acid. Due to the chronology of the entire study two different brand names were used (Corega and Polident) based on the countries in which they are marketed, and these products were used as per pack instructions and were chosen to represent the lower end of soaking practises that consumers typically use.

Material and methods

In vitro denture cleansing study

A denture plaque cleansing study and quantitative analysis of remaining viable cells was performed as previously described [26]. It was the aim to investigate whether a sequential denture cleansing technique was more advantageous than one treatment over the course of a 5-day treatment regimen. Briefly, laboratory strains were used to create a polymicrobial denture plaque biofilm model based on the most dominant genera/species identified from our recent denture microbiome study [10]. Polymethylmethacrylate (PMMA) discs were manufactured as described [27], providing the physical substrates on which biofilms were formed. The biofilms included *Streptococcus mitis* NCTC 12,261, *Streptococcus intermedius* ATCC 27,335, *Streptococcus oralis* ATCC 35,037, *C. albicans* 3153A, *Actinomyces naeslundii* ATCC 19,039, *Veillonella dispar* ATCC 27,335, *Rothia dentocariosa* DSMZ 43,762, *Lactobacillus casei* DSMZ 20,011 and *Lactobacillus zeae* DSMZ 20,178. Initially, *S. mitis, S. intermedius, S. oralis* and *C. albicans* were grown and standardised in artificial saliva to 1×10^7 cells/mL. These were added to each well of a 24 well plate (Corning Inc, New York, USA) containing 13 mm^2 PMMA discs (Chaperlin and Jacobs Ltd, Southend-on-Sea, UK) and incubated aerobically at 37°C for 24 h. Next, standardised (1×10^7 cells/mL) *A. naeslundii, V. dispar, R. dentocariosa, L. casei* and *L. zeae* were added to the preformed 24-h biofilm and incubated at 37°C in 5% CO_2 conditions for a further 4 days. Spent supernatants were removed and replaced with fresh artificial saliva daily.

Treatment regimens were either combinational daily treatment of brushing with hard water, followed by a daily 3 min soaking with a denture cleanser (Polident®3 min denture cleanser; GSK Consumer Healthcare, Weybridge, UK) (DC) for 5 consecutive days (DT group), daily brushing with hard water intermittent treatment (IT group) with DC on Day 1 and Day 5 only, or they were left untreated and were maintained in hard water corresponding to each treatment arm, serving as positive controls (UT group). Figure 1 shows a schematic of the treatment regimens.

Following each treatment, PMMA discs were incubated in Dey-Engley neutralising broth (Sigma-Aldrich, Gillingham, UK) for 15 min. PMMA discs were then sonicated in 1 mL phosphate-buffered saline (Sigma-Aldrich, Gillingham, UK) at 35 kHz for 10 min to remove the biomass, as previously

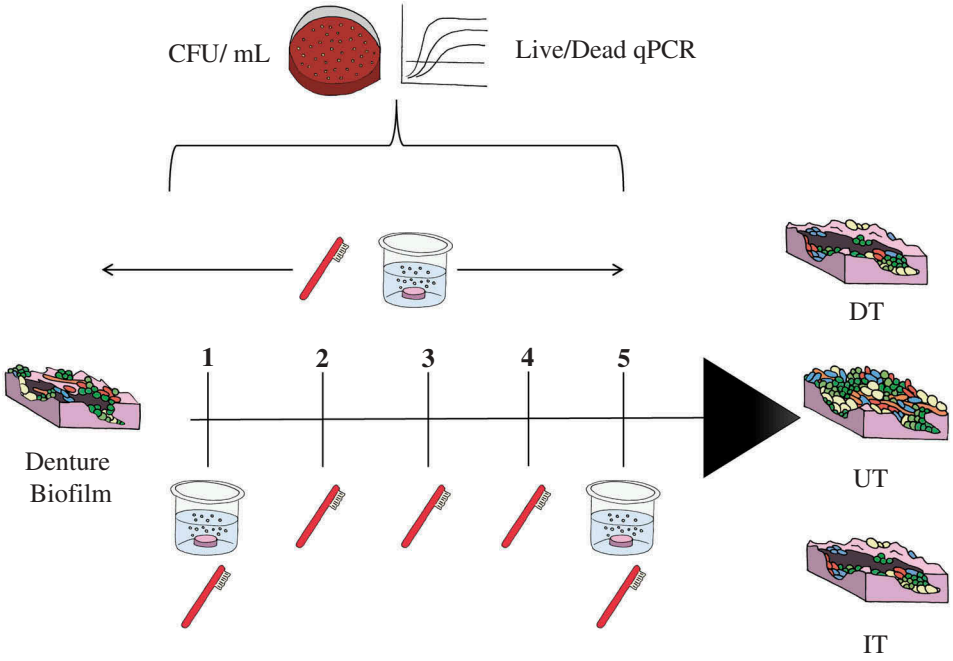

Figure 1. Sequential treatment of denture biofilm protocol.

described [21]. For quantitative analysis, both colony-forming unit (CFU) and quantitative live/dead PCR were performed, as described previously [26].

For the former, 20 µL denture plaque sonicate was transferred to a fresh microcentrifuge tube and serial log_{10} dilutions were performed in phosphate buffered saline, then 20 µL of each serial dilution was plated in triplicate on brain heart infusion + 10% blood agar plates (E&O Laboratories, Bonnybridge, UK), which were incubated aerobically and anaerobically at 37°C for 48 h [28]. The samples were also plated on Sabouraud dextrose agar and incubated at 30°C for 48 h for yeasts. Following incubation, the number of colonies was counted and represented as total viable aerobes, anaerobes and yeasts.

Viability of the treated biofilms was also assessed using live/dead PCR to enumerate the definitive and relative composition of the biofilms, a technique that has been shown to differentiate viable and dead cells from multispecies oral bacteria biofilm models. Samples were prepared as previously described, with some modifications [26]. In brief, 50 µM propidium monoazide (PMA) was added to each sonicated sample and incubated in the dark for 10 min to allow uptake of the dye. Samples were then exposed to a 650 W halogen light for 5 min before DNA was extracted using the QIAamp DNA mini kit, as per manufacturer's instructions (Qiagen, Crawley, UK). No PMA controls were included for each sample to determine total biomass. The primers used were previously published and are listed in Table 1. Three independent replicates from each parameter were analysed in triplicate using a MxProP Quantitative PCR machine and MxPro 3000P software (Stratagene, Amsterdam, Netherlands). Samples were quantified to calculate the colony-forming equivalent (CFE) based upon a previously established standard curve methodology of bacterial CFU ranging from 1×10^3 to 10^8 CFU/mL [15]. Melting curve analysis was performed for all primer sets to ensure a single peak, which was indicative of primer specificity.

Table 1. Primer sequences for denture biofilm species.

Target	Primer sequence (5′-3′)	Reference
16S	**F** – CGCTAGTAATCGTGGATCAGAATG **R** – TGTGACGGGCGGTGTGTA	[26]
18S	**F** – CTCGTAGTTGAACCTTGGGC **R** – GGCCTGCTTTGAACACTCTA	[26]
Streptococcus spp.	**F** – GATACATAGCCGACCTGAG **R** – CCATTGCCGAAGATTCC	[52]
A. naeslundii	**F** – GGCTGCGATACCGTGAGG **R** – TCTGCGATTACTAGCGACTCC	[52]
R. denticariosa	**F** – GGGTTGTAAACCTCTGTTAGCATC **R** – CGTACCCACTGCAAAACCAG	(53)
V. dispar	**F** – CCGTGATGGGATGGAAACTGC **R** – CCTTCGCCACTGGTGTTCTTC	[52]
L. casei	**F** – TGCACTGAGATTCGACTTAA **R** – CCCACTGCTGCCTCCCGTAGGAGT	(54)
L. zeae	**F** – TGCATCGTGATTCAACTTAA **R** – CCCACTGCTGCCTCCCGTAGGAGT	(54)

Data analysis

Data distribution, graph production and statistical analysis were performed using GraphPad Prism (version 5; La Jolla, CA, USA). After assessing whether data conformed to a normal distribution, One-way Analysis of Variance (ANOVA) and *t* tests were used to investigate significant differences between independent groups of data that approximated to a Gaussian distribution. A Bonferroni correction was applied to the p value to account for multiple

comparisons of the data. Non-parametric data were analysed using the Mann-Whitney U-test or the Kruskal–Wallis test with a Dunn's post-test to assess differences between independent sample groups. Statistical significance was achieved if $p < 0.05$. Principal Component Analysis (PCA) of the log (n) of CFUs of bacterial and yeast growth and CFEs of live bacteria and yeast after treatment were performed with R using in-built functions. Clustering (three clusters) was performed using the partitioning around mediods (pam) algorithm, a more robust version of k-means clustering, using the R package 'cluster'. Visualisation by the package 'ggplot2' was utilised to provide figures.

In vivo denture cleansing study

To assess the impact of daily or weekly DC on denture microbial count, composition, plaque and stain accumulation, a clinical trial was designed and carried out. This was a single-centre, randomised, controlled, examiner- and analyst-blind, crossover study conducted at Glasgow Dental Hospital and School, UK. The protocol was approved by an Independent Ethics Committee (West of Scotland Research Ethics Committee 3; Ref:16/WS/0092) and the study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use and local laws and regulations. All participants provided written informed consent prior to screening, demonstrated understanding of the protocol and were considered willing, able and likely to comply with all study procedures. This study was registered at ClinicalTrials.gov: NCT02780661. There was one amendment to the protocol to widen inclusion criteria to aid recruitment, this was not predicted to influence study outcomes.

Participants

Participants in general good health, aged between 18 and 84 years inclusive, were recruited through self-referral and identification at treatment clinics at Glasgow Dental Hospital and School. Participants were required to have a completely edentulous maxillary arch restored with a conventional, full acrylic based, complete denture. The mandibular arch could be dentate, partial or full edentulous and could be restored with a stable complete, partial or implant supported denture. Maxillary dentures needed to be of a well-made design and construction, as assessed by the study examiner and moderately well-fitting at the screening visit according to the Kapur Index [29], Olshan Modification [30]: retention score > 2, stability score > 2. Exclusion criteria included pregnancy; breastfeeding; known/suspected intolerance or hypersensitivity to study materials or ingredients; a serious, severe or unstable medical condition that would make the participant unlikely to fully complete the study; an implanted cardiac pacemaker; taking a daily dose of medication that might interfere with the participant's ability to perform the study or might affect efficacy assessments. Specific dental exclusion criteria included: a clinically significant or relevant oral abnormality that, in the investigator's opinion, could affect study participation; recent (within 30 days) gingival/oral surgery.

Study design and treatment

Study flow is detailed in Figure 2. At screening, participants provided written informed consent and eligibility was assessed. They received a dental prophylaxis and a denture prophylaxis of the maxillary complete denture; zero plaque and stain scores were confirmed by post-prophylaxis assessments. At the first study visit (Day 0) participants were assigned to a study treatment sequence order (1:1) in accordance with the randomisation schedule provided by the Biostatistics Department of GSK Consumer Healthcare. Randomisation numbers were assigned in ascending numerical order as each participant was determined to be fully eligible and consented for inclusion. All participants used supplied alkaline peroxide-based denture cleansing tablets (Corega®

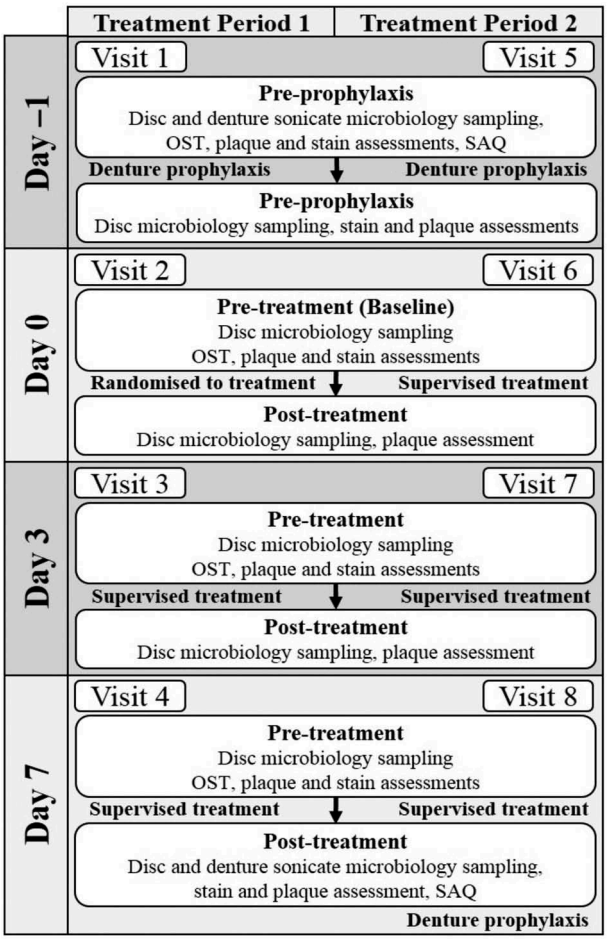

Figure 2. Study flow.

Tabs Dental Weiss für Racher [Denture Whitening for Smokers], German marketed product). The Daily Use group used one tablet per day (with supervised use at the site on Days 0, 3 and 7). The Weekly Use group used one tablet on Day 7 (supervised use at site on Day 7).

Dentures were soaked in a cup of 150 mL warm water with a cleanser tablet for 15 min, brushed for 30 s using the solution, then rinsed under running water for 10 s. At home, the Daily Use group repeated this procedure for the upper denture in the evening while the Weekly Use group carried out the procedure in warm water only. Both groups left dentures to soak overnight in 150 mL water. The lower denture, if present, was cleaned as usual but separately from the upper denture. Cleaning of the upper denture was not permitted in the morning; the lower denture could be cleaned as usual. Participants returned on Days 3 and 7. Following a washout period (7 ± 3 days) participants returned for treatment period 2, as described above including an initial denture and dental prophylaxis. Participants refrained from smoking, including e-cigarettes and the use of chewing tobacco or other tobacco products for the duration of the study. They could not use any other denture cleaners or regimens to clean their upper dentures. Participants were asked not to use denture fixative, xylitol-containing or oral-care type chewing-gum for the duration of the trial as these could impact hygiene parameters.

Examiners (clinical and laboratory scientists) and data analysts were blinded to treatment allocation. Examiners were not allowed to be in the room where test products were stored or allocated. Additionally, dispensing staff were not involved in any effectiveness assessments. Examiners were calibrated prior to commencement of the trial.

Microbial sampling

To collect samples from the tissue-fitting surface of the maxillary denture for microbiological analysis, the denture was sampled in four quadrants lateral to the midline and corresponding to the palatal rugae (Figure 3). Pre-prophylaxis and pre-treatment samples were taken from the left rough (A) and left smooth (D) denture side. Post prophylaxis and post-treatment samples were taken from the right rough (B) and right smooth (C) denture surface.

A pre-sterilized 10 mm filter paper disc (Sigma-Aldrich, Gillingham, UK) was lightly pressed against the allocated quadrant for 20 s prior to aseptic removal. Two discs, of the allocated quadrants (depending on the time point), were pooled and processed appropriately for microbiology cultures (CFU/disc) to assess microbial counts (aerobic bacteria, anerobic bacteria and *C. albicans)*, and for qPCR analysis to assess denture microbial composition, as described previously [26]. To investigate the microbial counts and microbial composition on the whole maxillary denture, on Day 1 (pre-prophylaxis) and Day 7 (post-treatment) the maxillary denture was placed in a sterile bag with 50 mL PBS and placed in a sonic bath for 15 min at 35 kHz. This procedure removed microbes adhered to all areas of the denture. The resulting samples were processed appropriately for microbiology cultures (CFU/denture) and for molecular microbial analysis by qPCR (CFE/denture), as described previously [26].

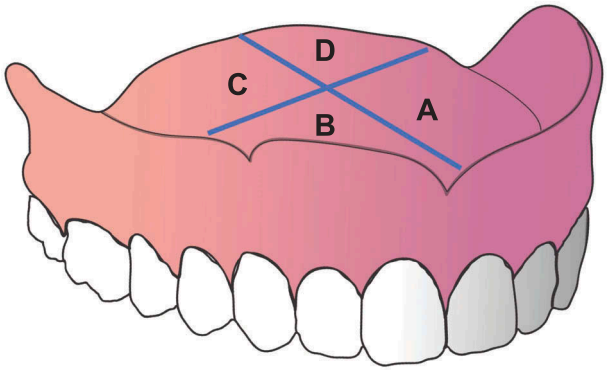

Figure 3. Quadrants used for sampling.

Clinical assessments

Denture plaque was assessed on three areas separately: fitting surfaces, polished surfaces and denture teeth (facial/buccal and palatal) based on the modification of the Clinical Categorization of Denture Cleanliness Index [31], where 0 = No visible plaque; no matter adherent to the side of the dental probe on light scraping; 1 = No visible plaque; matter adherent to the side of the dental probe on light scraping; 2 = Deposits of plaque just visible on careful examination without need to confirm by scraping; 3 = Deposits of plaque 'clearly visible'; 4 = Gross plaque deposits ('velvet appearance'). For a given denture area under examination, the highest score of that area was recorded.

Denture stain was also assessed on these areas, by modification of the Denture Cleanser Index [32]. The stain scale was related to the percentage of the surface covered in stain where 0 = No staining detectable; 1 = Little staining (<25% of surface stained); 2 = Moderate staining of surface (25–50% of surface stained); 3 = Severe staining of surface (>50% of surface stained).

Safety

Adverse events (AEs) were collected from the start of the denture and dental prophylaxis at the Screening Visit until 5 days following last administration of the

study product. Incidents were documented from the Baseline visit (Visit 2). Oral soft tissue examinations were performed at all baseline visits pre-prophylaxis or pre-treatment. The safety population was defined as all participants who were randomised and received at least one dose of study treatment during the study.

Evaluation criteria and data analysis

As variation and treatment effect were unknown, a formal sample size calculation was not possible. A total of 17 participants was determined to be suitable to assess effectiveness and safety of treatment products. The primary population for efficacy assessment was the intent-to-treat (ITT) population, defined as all participants who were randomised, received at least one dose of study treatment and provided at least one post-baseline assessment of microbial count from disc sampling. The per protocol (PP) population was defined as all participants in the ITT population who had at least one assessment of efficacy considered unaffected by protocol violations.

The primary objective was to evaluate and compare change from baseline in microbial count from denture disc samples of the Daily Use and Weekly Use groups on Day 7, with comparison on Day 3 as a secondary objective. Exploratory objectives included evaluation and comparison of the denture sonicate microbial count on Day 7 and, on Days 3 and 7 plaque levels, microbial composition from disc samples and stain levels on the maxillary denture. All endpoints were tested under the general hypotheses of a treatment difference between Daily and Weekly product use. All statistical analyses were conducted using Statistical Analysis System (SAS) version 9.2.

The three microbial counts (aerobic bacteria microbial count, anaerobic bacteria microbial count and *Candida* microbial count) were analysed separately for both treatment regimens at Days 3 and 7 compared to pre-treatment Day 0. Microbial counts were log transformed (base 10) prior to any analysis being performed. To be able to analyse all samples, if no microbes were retrieved ('0′ values), a constant (+1) was added to all values prior to log transformation. Changes from baseline were analysed using an analysis of covariance (ANCOVA) model with treatment and period as fixed effects, participant-level (mean across treatment periods) and period-level pre-treatment (on Day 0) baseline scores (with the same transformation) as covariates. To allow model estimates to be representative of the studied population, participant was included in the model as a random effect. Confidence Intervals (CIs) and p-values were calculated for the difference between the treatments (Daily use vs Weekly use). Model assumptions were investigated, no assumptions were violated.

Microbial counts at Day 7 and denture plaque levels at Days 3 and 7 compared to Day 0 pre-treatment were calculated with each endpoint using an ANCOVA as above. Model assumptions were investigated, no assumptions were violated. Microbial composition at Days 3 and 7, assessed by qPCR from disc samples, was represented for both treatments in a stacked bar chart as percentage of each microbial group. The sum of the eight oral microbial groups analysed was considered as 100%. Denture stain levels at Days 3 and 7 on tissue fitting surfaces, polished surfaces and denture teeth stain score were analysed separately as a change from baseline to compare between treatment regimens (Daily use and Weekly use).

Examiner repeatability

At each visit, for a random sample of participants, stain and plaque assessments were repeated by the examiner to check consistency in measuring plaque and stain levels on the denture surfaces. For each parameter of stain and plaque assessments, a Fleiss-Cohen weighted kappa coefficient (κ), along with the 95% CI was calculated for the repeatability analysis for each denture surface (tissue fitting surfaces, polished surfaces, denture teeth). Reliability was deemed excellent if $\kappa>0.75$; fair to good if $0.4\leq\kappa\leq0.75$; poor if $\kappa<0.4$

Results

Quantitative analysis of in vitro denture plaque biofilm

The *in vitro* analysis of different denture treatment regimens on multispecies denture plaque biofilms was carried out over the course of 5 days. Three groups were included: untreated biofilms as a positive control (UT), daily brushing followed by denture cleansing (DT) and intermittent denture cleansing (IT). Total aerobes, anaerobes and yeasts were initially quantified using CFU analysis over 5 days (Figure 4). For the DT group, no viable CFUs were detectable (ND) on any day for aerobes, anaerobes and yeasts, whereas for the IT group, viable bacteria were detected on Days 2, 3, 4 and 5 (approximately 10^3 to 10^5 CFU/mL), and on Days 2, 3 and 4 for yeasts (approximately 10^3 to 10^4 CFU/mL). No significant changes in overall microbial levels were observed in this time frame for the UT group, with consistent levels of 10^8 and 10^6 CFU/mL detected for bacteria and yeasts, respectively (data not shown). Both the DT and IT groups showed a statistically significant reduction in CFU's for aerobes, anaerobes and yeasts ($p < 0.001$) compared to the UT group, though DT was consistently and statistically significantly more effective than IT ($p < 0.001$). To visually

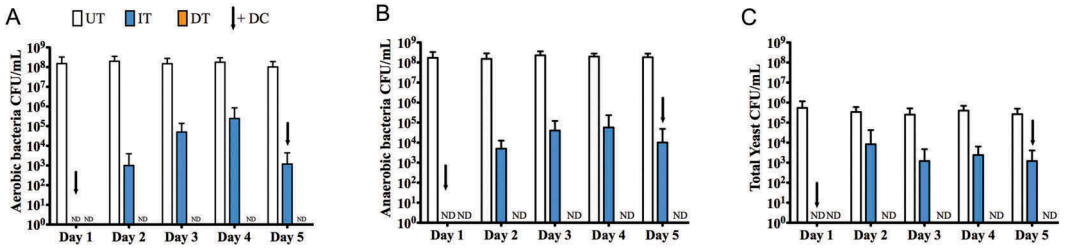

Figure 4. Daily CFU/mL counts of A) aerobic bacteria, B) anaerobic bacteria and C) total yeast count (±standard deviation) post treatment.

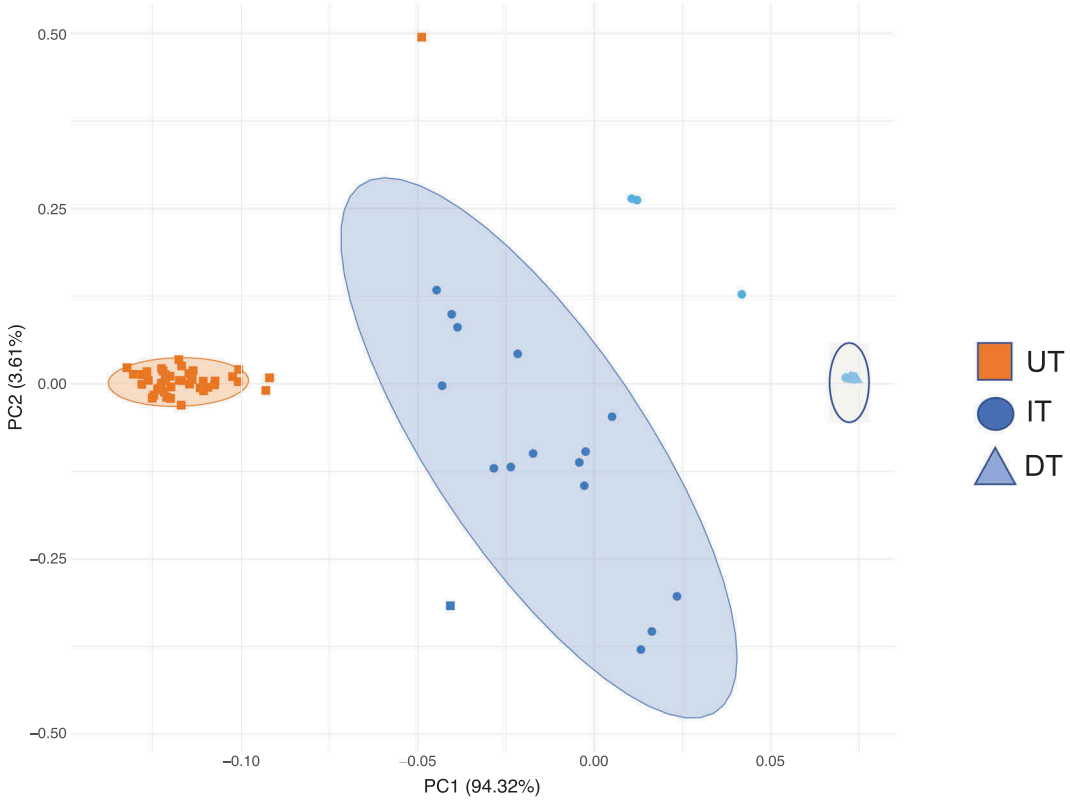

Figure 5. Principal component analysis showing different *in vitro* treatment outcomes associated with total and viable cell populations.

illustrate the overall effects of the treatment regimens, a PCA analyses was performed (Figure 5). Clustering demonstrated three independent clusters between the treatments as highlighted by the ellipses and colored clusters. PC1 and PC2 are displayed with PC1 displaying over 94% of the variation between samples, the variation along this component distinguishes between the treatment types.

Given that total viable cell counting is prone to inaccuracy (e.g. clumps of cells), and the possibility for the carry-over of actives, despite neutralisation, then a quantitative PCR was employed as an adjunctive assay to supplement these observations. For total bacteria retained on the PMMA surface following treatment (Figure 6), the number of bacteria was significantly lower in both the IT and DT groups compared to the untreated control by approximately 3 $\log_{10}$ ($p < 0.001$), though no discernible differences were observed in retained bacteria between these groups (data not shown). The number of yeasts isolated from the discs shows a similar pattern to that of the bacteria, in that by comparison with the UT group there were significantly fewer yeast cells in both the DT and IT groups, with an approximately 1 $\log_{10}$ reduction ($p < 0.001$), though again there were no differences between DT and IT groups. Live qPCR analysis was also performed to assess how many of the retained cells were viable, based on whether cell membranes were compromised or not. This showed that despite these treatments, approximately 1×10^4 and 1×10^3 CFE/mL of bacteria and yeasts, respectively, remained viable through Days 1 to 5, irrespective of treatment group (data not shown).

To assess whether any of the treatment regimens impacted the composition of the denture plaque biofilms, changes in the individual species contribution to

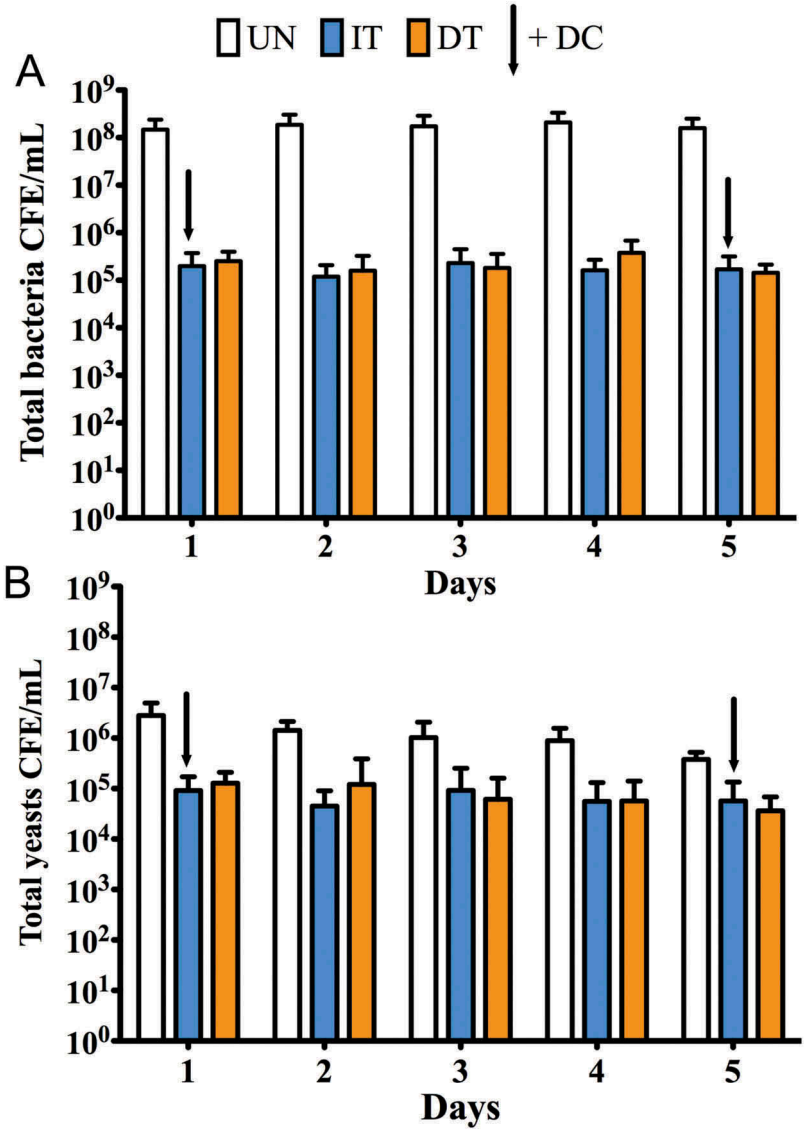

Figure 6. Daily CFE counts of (a) total bacteria and (b) total yeasts post treatment (±standard deviation).

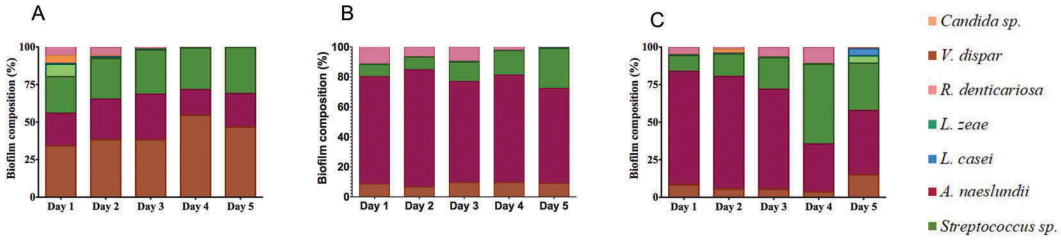

Figure 7. Microbial composition as assessed by qPCR from *in vitro* denture disc samples. (a) Untreated [UT], (b) Denture cleanser [DT], (c) Brushing [IT].

each biofilm were investigated over the 5-day time course. The total cell count was quantified and converted into a proportion of the overall biofilm to determine the contribution of each species. Interestingly, it was observed that the UT group showed a number of changes over the 5 days, with *V. dispar, A. naeslundii* and *Streptococcus* species dominating the biofilm, with a notable increase in *V. dispar* at Days 4 and 5 (Figure 7 (a)). Reciprocally, a reduction in *R. dentocariosa, C. albicans, L. zeae* and *L. casei*, was observed daily as the biofilms matured. The DT group showed a biofilm dominated by *A. naeslundii*, with increasing proportions of *Streptococcus* species (Figure 7(b)), though the IT group was initially dominated by *A. naeslundii*, followed by *Streptococcus* species (Figure 7(c)). Overall, these data showed that different interventions have the capacity to alter denture plaque composition, in a treatment dependent manner.

Quantitative analysis of in vivo denture plaque biofilm

Clinical

A total of 25 participants were screened, with a total of 19 participants randomized to a treatment sequence. There were no participant withdrawals with all 19 randomized participants completing the study. There was one protocol violation in the Daily use regimen and one in the Weekly use regimen treatment periods, which led to data exclusion. Of the 19 participants the majority were female (n = 12, 63.2%) and white (n = 19, 100%), with a mean age of 68.7 years (SD 5.10, range 60–75 years). At baseline (Day 0, pre-treatment), the transformed microbial counts [Log_{10} (Count+1)] were slightly higher in the Weekly use treatment at 2.27 Log_{10}(CFU/disc) compared to 2.04 for Daily use for aerobic bacteria. These values for anaerobic bacteria were 2.47 and 2.07, respectively.

Microbial counts

A reduction from baseline in both aerobic and anaerobic bacteria in the denture disc samples was observed on Day 7 for both treatments, resulting in values of adjusted mean values of 0.26 Log_{10}(CFU/disc) and 1.06 Log_{10}(CFU/disc) aerobic bacteria in the Daily Use and the Weekly use treatment respectively, and 0.50 Log_{10}(CFU/disc) and 0.93 Log_{10} (CFU/disc) anaerobic bacteria for each treatment, respectively (Figure 8). A statistically significant difference between treatments was observed for aerobic bacteria microbial count at Day 7, with a greater reduction observed for the Daily use treatment (Table 2) (−0.86 adjusted mean treatment difference, p-value 0.0144). For the anerobic bacteria, a treatment difference was observed in favour of the Daily use treatment; however, this difference was not statistically significant (−0.48 adjusted mean treatment difference, p-value 0.1879). *C. albicans* cultured from disc samples were mostly zero and were therefore not further analysed (data not shown).

At Day 3, a reduction from baseline in aerobic and anaerobic bacteria was observed for the Daily use treatment, resulting in adjusted mean values of 0.31 and 0.33 Log_{10}(CFU/disc) respectively. Subjects in the Weekly use treatment, who did not use the denture cleanser tablet but cleaned their denture with water instead, showed an increase in both microbial counts. Aerobic bacteria increased to 2.63 Log_{10}(CFU/disc) while anaerobic bacteria increased to 3.55 Log_{10} (CFU/disc). A statistically significant between treatment difference was observed for aerobic and anaerobic bacteria microbial count at Day 3 with the greater reduction observed for the Daily use treatment (−2.32 adjusted mean treatment difference, p-value 0.0002 for aerobic bacteria and −3.22, p-value <0.0001 for anaerobic bacteria). Following the Daily use treatment regimen, a high proportion of subjects had no microbial counts at Day 3 (17 out of 19 for both aerobic and anaerobic bacteria) while in the Weekly use treatment regimen no microbes were retrieved from fewer subjects (seven out of 19 subjects for aerobic bacteria and four out of 19 for anaerobic bacteria).

With regards to exploratory objective, denture sonicate microbial samples were collected only at Day −1 visit (Screening visit) before initial prophylaxis (pre-prophylaxis) and at Day 7 (post-treatment). No statistically significant between treatment difference was observed for aerobic or anaerobic bacteria microbial count at Day 7 in the denture sonicate samples (Table 2). *C. albicans* microbial count from both time points and treatments were retrieved at lower numbers than aerobic or anerobic bacteria. A between treatment difference was observed in favour of the Daily use treatment; however, this difference was not statistically significant (−0.60 adjusted mean treatment difference, p-value 0.325).

Microbial composition

Microbial composition by treatment and visit was assessed using specific primers targeting bacteria

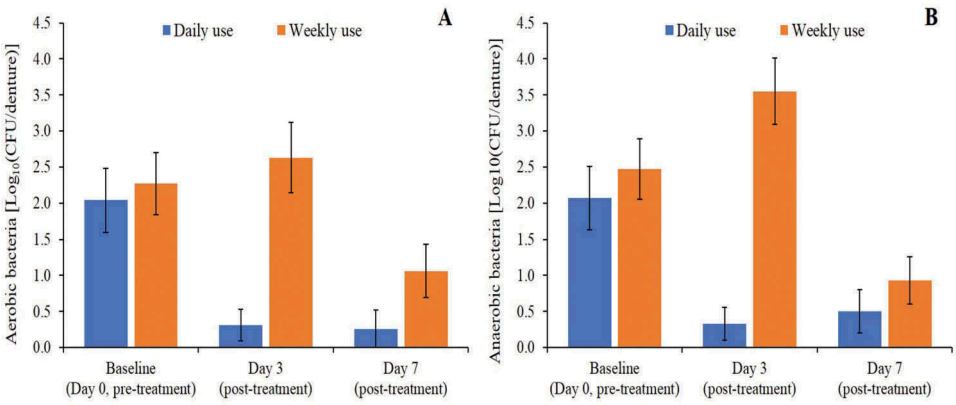

Figure 8. (a) Aerobic bacteria and (b) Anaerobic bacteria microbial count Log_{10} (CFU/disc) by visit and treatment from denture disc samples (±standard error) (ITT population).

Table 2. Statistical analysis of change from baseline in aerobic bacteria and anaerobic bacteria microbial counts (CFU/disc) from denture disc (DDisc) and denture sonicate (DSon) samples (ITT Population).

	Change from baseline		Treatment comparison		
	Daily use Mean (SE)	Weekly use Mean (SE)	Difference (95% CI)	p-value	Ratio (95% CI)
Aerobic bacteria (post treatment)					
DDisc Day 3	−1.85 (0.39)	0.47 (0.39)	−2.32 (−3.43, −1.20)	0.0002	0.005 (0.0004, 0.0628)
DDisc Day 7	−1.92 (0.32)	−1.06 (0.32)	−0.86 (−1.53, −0.19)	0.0144	0.137 (0.0295, 0.6369)
DSon Day 7	−0.36 (0.13)	−0.48 (0.13)	0.12 (−0.257, 0.505)	0.5118	1.33 (0.55, 3.20)
Anaerobic bacteria (post treatment)					
DDisc Day 3	−1.94 (0.38)	1.28 (0.38)	−3.22 (−4.24, −2.19)	<.0001	0.001 (0.0001, 0.0064)
DDisc Day 7	−1.80 (0.33)	−1.31 (0.32)	−0.48 (−1.23, 0.26)	0.1879	0.328 (0.0589, 1.8257)
DSon Day 7	−0.35 (0.13)	−0.42 (0.14)	0.07 (−0.33, 0.46)	0.7316	1.17 (0.47, 2.88)
***C. albicans* (post treatment)**					
DSon Day 7	−0.69 (0.44)	−0.08 (0.45)	−0.60 (−1.86, 0.66)	0.3250	0.25 (0.01, 4.53)

Difference is first named treatment minus second named treatment such that a negative difference favours the first named

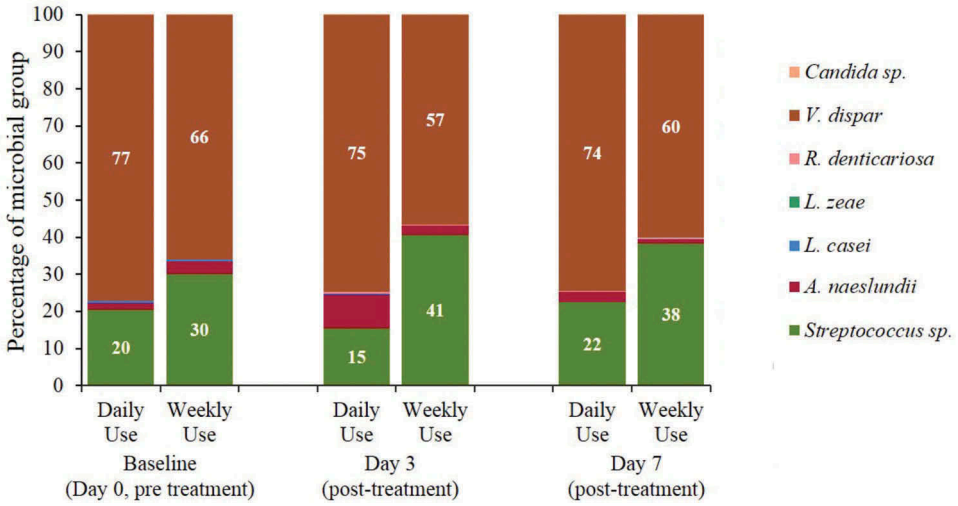

Figure 9. Microbial composition as assessed by qPCR from denture disc samples (ITT population).

known to be associated with dentures, as described above. A stacked bar chart is presented for their relative abundance, considering as a 100% the sum of the eight microbial groups targeted (Figure 9). There was no formal statistical analysis performed for the microbial composition data. A dominance of *V. dispar* and *Streptococcus* species was observed at the different time points and for both treatments (ranging between 57 and 77% and 15 and 41%, respectively). *A. naeslundii* was detected with both treatments and visits but in lower relative abundance (ranging between 1 and 9%). *R. dentocariosa, L. casei* and *Candida* species were only minor contributors to the microbial composition of the dentures (in the disc samples), detected at less than 1%. *L. zeae* was not detected in any of the samples. In the Weekly use treatment group, a slight increase in *Streptococcus* species was detected from Baseline (pre-treatment) to Day 3 and Day 7 (post-treatment) and a slight decrease in *V. dispar* and other minor groups. However, no overall evident changes in microbial composition were observed either for the Daily use or the Weekly use treatment, with both having a dominance of *V. dispar* and *Streptococcus* species.

Denture plaque and stain scores

Overall, low plaque scores were observed on all the denture surfaces, particularly on the polished surfaces (Table 3). Examiner repeatability for plaque was excellent with a weighted kappa of 0.968 [95% CI 0.922,1.00]. Despite the overall low plaque scores, a decrease following Daily use of the denture cleanser was observed at Days 3 and 7, while the Weekly use treatment regimen led to small changes depending on Day and denture area examined (Figure 10). At Day 3 there were differences observed in favor of the Daily use treatment for the three surfaces; however, these differences were not statistically significant. At Day 7, a statistically significant between treatment difference was observed for denture teeth and for tissue fitting surfaces with the greater reduction observed for the Daily use treatment in both surfaces (−0.54 adjusted mean treatment difference, p-value 0.0211 for denture teeth; −0.57, p-value 0.0320 for tissue fitting surface)

Stain levels were very low throughout the study, particularly at baseline where most of the surfaces had a 'no staining detectable' score. Overall, at Day 7 for the three surfaces there was a difference observed in favour of the Daily use treatment. However, these

Table 3. Statistical analysis of change from baseline in plaque score in denture teeth, tissue fitting surfaces and polished surfaces (ITT Population).

	Change from baseline			Treatment comparison
	Daily use Mean (SE)	Weekly use Mean (SE)	Difference (95% CI)	p-value
Denture teeth (post treatment)				
Day 3	−0.65 (0.20)	−0.30 (0.20)	−0.35 (−0.92, 0.23)	0.2307
Day 7	−0.79 (0.15)	−0.26 (0.15)	−0.54 (−1.99, −0.09)	0.0211
Tissue fitting surfaces (post treatment)				
Day 3	−0.58 (0.20)	−0.05 (0.20)	−0.52 (−1.11, 0.07)	0.0765
Day 7	0.52 (0.18)	0.05 (0.18)	−0.57 (−1.095, −0.05)	0.0320
Polished surfaces (post treatment)				
Day 3	−0.30 (0.05)	−0.28 (0.05)	−0.02 (−0.15, 0.12)	0.7875
Day 7	−0.30 (0.16)	0.09 (0.16)	−0.39 (−0.86, 0.07)	0.0953

Difference is first named treatment minus second named treatment such that a negative difference favours the first named

differences were very small and not statistically significant for any of the three surfaces (data not shown).

Safety results

Overall, 28 treatment emergent AEs (TEAEs) were reported by 13 (68.4%) participants, 14 in each of the treatment groups. There were 21 oral TEAEs reported by 12 participants (63.2%) (10 in the Daily Use and 11 in the Weekly Use groups). None of the TEAEs were considered related to treatment. All TEAEs were mild or moderate in nature and all but 'Lip injury' had resolved by study end. There were no serious AEs or incidents reported and no participants withdrew from the study due to AEs. The use of the denture cleanser tablet was shown to be generally well tolerated in the study.

Discussion

While there is limited evidence on how denture care should be implemented [6,7], the guidelines are clearer on what should not be done [8]. Cleaning in boiling water and storing the dentures dry should be avoided to minimize physical warping. The storage solution should be changed frequently to prevent microbial overgrowth within the water. Prolonged exposure to sodium hypochlorite/bleach containing products should also be avoided due to its detrimental impact on denture materials, particularly metals [33]. The use of microwave disinfection in combination with denture cleansers and brushing has also been shown to disinfect dentures *in vivo* [34], though microwaves may also physically distort denture acrylics [35]. A number of unconventional approaches to denture care, including soaking in vinegar, baking soda, sodium chloride (table salt) and liquid soaps, were identified in a recent study [36]. Many of these remedies are lacking in either efficacy and/or material compatibility [33]. Nevertheless, without proper professional advice many denture wearers may well continue these alternative practices.

Collectively the current evidence we have, which in many instances remains weak, highlights the need for improved denture cleansing techniques capable of dealing with a range of bacteria, in addition to highly tolerant candidal cells. Disruption of the denture biofilm is also critical to improving oral health. This may be achieved either through mechanical means such as a brush or using sonic cleaning devices which may be a more effective method of cleansing and decolonising dentures. Alternative strategies may include chemicals and enzymes capable of digesting and disaggregating biofilms, which could allow improved penetration and activity of agents in cracks, crevices and pores. On current evidence, mechanical disruption coupled with effective antimicrobial agents is likely to be the most desirable option.

PMMA is the primary denture material of choice, though this has an uneven surface that results in a heterogenous topography that yeasts and bacteria can co-colonise, forming biofilms and escaping from denture cleansing therapies [21,37,38]. Investigations to determine optimal methods for cleaning dentures have focussed on the various physical and chemical cleansing techniques, both individually and in combination. However, most of these investigations evaluate treatment over a short period of time and therefore do not accurately simulate an optimal denture routine clinically [8,39,40]. Daily denture cleansing treatment of *C. albicans* biofilms has been previously investigated, with results indicating that despite a significant reduction in viable *C. albicans* cells, a residual reservoir of yeast cells remained, indicative of ineffective cleansing [21,41,42]. A limitation of these studies was the use of a single species biofilm model, as this is not reflective of the polymicrobial denture environment. The present study aimed to address this by developing a more complex model that would allow assessment of repeated, longitudinal treatments as well as physical and chemical treatment modes.

Quantitative analysis of different denture treatment regimens on multispecies *in vitro* denture plaque biofilms was carried out over the course of 5 days. Results indicated that regular daily cleaning provided a significantly greater benefit than intermittent cleaning, even with the use of brushing

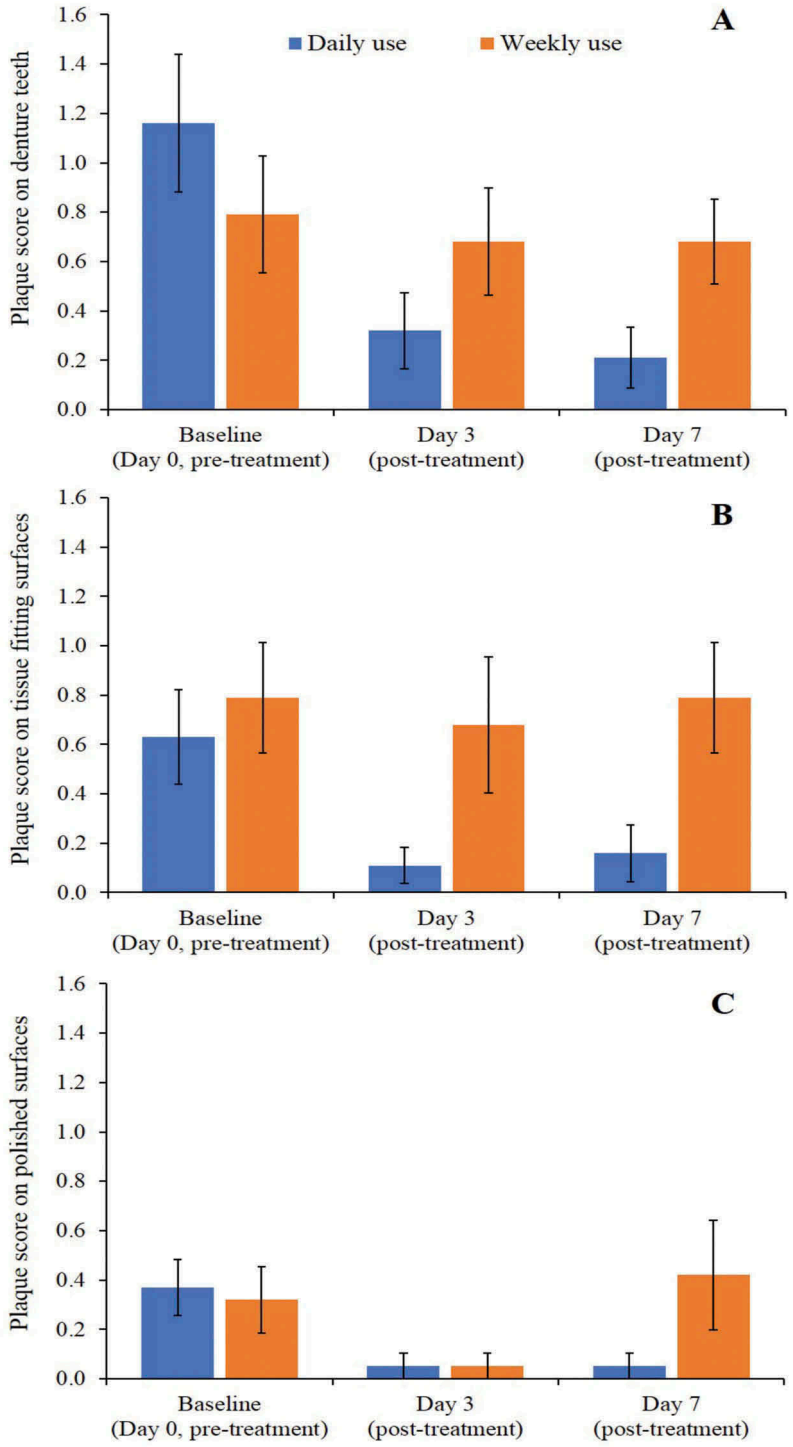

Figure 10. Raw means plaque score on (a) Denture teeth, (b) Tissue fitting surfaces and (c) Polished surfaces (±standard error) (ITT population).

together with the chemical disinfection. The results showed that despite active treatments of denture material, significant quantities of microorganisms were retained on the PMMA surface, that could only be released by sonication. Thus, although a considerable number of microbes remained on the discs post cleansing, the treatments employed were significantly effective given the extensive reduction in the overall microbial burden. Live qPCR results suggest that the denture plaque biofilm may contain the dormant persister cell phenotype that are unaffected by treatments, but cannot necessarily be cultured. Overall, these data suggest that denture biofilm composition is dependent on whether and how the biofilms are treated, which largely agrees with our previous studies [21,26]. However, this model benefits from its inception and design based on the first reported denture plaque microbiome [10]. This has facilitated us to use this as a robust first line screening tool capable of discerning quantitative differences in denture plaque biofilms in a reproducible manner.

The clinical study also followed a similar pattern with regard to the microbial count reduction between daily vs intermittent cleaning. Several studies have demonstrated the ability of alkaline peroxide based denture cleansers on reducing denture plaque biofilm [43,44]. The current study is first of its kind evaluating the impact of daily vs intermittent (once weekly cleaning) using alkaline peroxide based denture cleanser tablets.

A statistically significant greater reduction in aerobic bacteria microbial count was reported for the Daily use treatment regimen at Day 7 and in aerobic and anaerobic bacteria at Day 3 in comparison with the Weekly use treatment regimen. At Day 3 the aerobic and anaerobic microbial count in the Weekly use treatment was higher than at Day 0, indicating that the microbial biofilm grew from baseline when participants cleaned their dentures daily with water. At Day 7, where participants in both treatment regimens used the denture cleanser tablet, the aerobic and anaerobic bacterial count was reduced from baseline in both treatments. The result at Day 3 presumably reflects the antimicrobial activity delivered from the denture cleanser tablet, in comparison with water. However, the differences observed at Day 7 are intriguing since both groups had received identical treatments immediately prior to sampling. The data suggest that the biofilm developed in the weekly treatment group was more resistant to a single treatment with denture cleanser in comparison to the biofilms developed in the daily treatment group. This is in accordance with the results seen in the *in vitro* element of the present study, and in many other studies of mature biofilms from oral and other sources. It provides a microbial line of evidence to support previous clinical studies that have suggested regular cleaning of dentures to be beneficial to overall oral health [6,7]. Soaking alone in a denture cleanser may not be sufficient for adequate plaque removal [45], and is in line with the widely held belief around mechanical cleaning methods being important for physical plaque removal. Some studies have demonstrated an additional benefit linked to use of denture cleanser tablets on plaque removal compared to brushing alone. Sheen and colleagues demonstrated use of alkaline peroxide based cleanser resulted in 42% reduction ($p = 0.0014$) in plaque levels after 2 weeks of use compared to brushing with water [46].

Despite the relatively low denture plaque scores throughout this study, a statistically significantly greater reduction in denture teeth and tissue fitting surfaces plaque scores were found for the group using the Daily use treatment regimen compared to the Weekly use treatment at Day 7. Nishi et al (2012) collected denture plaque from denture wearers and analysed the effect of denture brush use, cleansing frequency and cleaning solution [47]. They concluded that the use of brush was associated with lower amounts of microbes and that, unsurprisingly, daily use was better than monthly use. In the general population they did not find a difference between daily and 3–4 times per week, but in those patients who were in nursing homes daily cleaning was the most effective. It is possible that these most vulnerable patients are unable to clean sufficiently themselves and our study would confirm that daily cleaning is advisable. Our results are also in agreement with the small clinical study carried out by Sheen et al (2000), which showed that denture plaque levels could be reduced using a daily brushing technique, but that the addition of an active cleanser reduced the rate of plaque formation and was more effective than water and brushing alone [46]. Moreover, results of the present study correlate with the conclusion of Kiesow et al (2016), who reported that specialist denture cleanser tablets provide a good combination of microbial efficacy, while also maintaining material compatibility [33]. The use of denture cleansers was also shown to lead to a significant reduction of microbial burden compared to a mouthwash [48]. This study, alongside the preceding evidence, has in part addressed the inadequacies in the literature that was concluded from previous systematic reviews [6,7]. Indeed, these data provide greater evidence that frequent use of denture cleansers is an effective strategy for supporting a low microbial bioburden that logically will maintain mucosal health. To further define and evolve our understanding of mucosal health we have developed techniques to investigate and evaluate microbial population dynamics. This approach may have translational benefits for improving existing denture cleansers developed to target specific groups of pathogenic denture plaque microorganisms.

The denture microbial composition was investigated from the disc samples by a qPCR targeted approach at Days 0, 3 and 7. Eight microbial groups were selected based on findings from a previous microbiome high throughput sequencing study of denture wearers [10]. In the present study a relative dominance of *V. dispar* and *Streptococcus* species was observed in both treatment groups and at all time points, with other microbial groups contributing in smaller proportions. No apparent difference between treatments were observed. *Streptococcus* and *Veillonella* have been documented as early colonizers and dominant microbes in healthy oral biofilms [49–51] and have been reported as major components of dentures in participants without stomatitis [10,52]. *Actinobacteria* spp. have been reported as abundant components of the denture's microbiome [10]; however, in this study they were present in a relative low abundance (<10%). This study would benefit from a full microbiome analyses, though whether these data would add value in terms of driving evidence for the best treatment regimens remains to be seen. Our qPCR approach provides an intermediate and more economical approach to assessing changes in microbial dynamics.

Conclusions

The present study shows how basic science understanding has enabled the development of an *in vitro* denture plaque model system that mimics the development of plaque on dentures in the mouth. Treatment with denture cleansers on a regular, daily basis in both an *in vitro* model and in a clinical study of denture wearers was more effective in reducing microbial numbers and plaque scores in comparison with intermittent treatments.

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