Treatment of Dental Plaque Biofilms using Photodynamic Therapy

By

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

BACKGROUND: Photodynamic therapy (PDT) is a treatment modality involving a dye that is activated by exposure to light of a specific wavelength in the presence of oxygen to form oxygen species causing localised damage to microorganisms.

AIM: To determine the most effective bactericidal incubation and irradiation times of erythrosine-based PDT, using a tungsten filament lamp, on *in vivo*-formed dental plaque biofilms.

MATERIALS AND METHODS: The study was a two-phase randomised controlled study consisting of *in-vitro* and *in-situ* phases. Phase-1 aimed to determine the most appropriate incubation-time using erythrosine(220μM) based-PDT on *lactobacillus* species grown *in-vitro*. Phase-2 was conducted on 18-healthy adult participants wearing intraoral appliances with human enamel slabs to collect dental plaque samples in two separate periods for use in arm-1 and arm-2.

For phase-2, accumulated dental plaque samples were tested under different experimental conditions; a) Control-1 (No erythrosine, no light); b) Control-2 (+Erythrosine, no light); c) Treatment-1 (+Erythrosine, +15min continuous light); d) Treatment-2 (+Erythrosine, +30sec light pulses for 5-times separated by 1min dark periods). Incubation-times of 15min and 2min were used in arm-1 and arm-2, respectively; as adapted from the previous pilot study and phase-1.

Following treatment, percentage reduction of total bacterial counts were compared between the different groups. Additionally, Confocal Laser Scanning Microscopy(CLSM) was used to investigate the effect of PDT on *in vivo*-formed plaque biofilms.

RESULTS: Significant reductions in the percentage of total bacterial counts (~93-95%) of *in vivo*-formed biofilms were found when using either 2min or 15min incubation-times and applying 15min continuous light. Whereas, when applying fractionated light, there was more cell death when 15min incubation-time was used (~91%) compared with the 2min incubation-time (~64%). CLSM results supported these findings.

CONCLUSION: Improving the clinical usefulness of PDT by reducing its overall treatment time seems to be promising and effective in killing *in vivo*-formed dental plaque biofilms.

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Introduction and Literature review

1.1 Dental plaque

The microbial communities in humans are highly regulated; structurally and functionally organised communities attached to surfaces as biofilms (Li and Tian, 2012). The composition of these microbial communities is significantly different between and within individuals; however, the overall functions are quite similar (Gillings *et al.*, 2015). The oral cavity has one of the most diverse bacterial populations as it offers several distinct habitats for microbial colonisation, such as teeth and the soft tissues of the oral mucosa (Dewhirst *et al.*, 2010). Teeth are the only natural non-shedding surfaces in the human body and it provides unique opportunities for biofilm formation and microbial persistence (Marsh and Devine, 2011). Dental plaque is a typical biofilm. It has been defined By Marsh and Martin (1992) as “the diverse microbial community found on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin”. Formation of this dental plaque is a multistep process; it involves adhesion of initial bacterial colonisers to the enamel salivary (acquired) pellicle, that is formed on tooth surface, followed by attachment of secondary colonisers to the already attached initial colonisers (co-aggregation) through molecular interactions, such as protein-protein interactions (Marsh and Bradshaw, 1995). As the plaque layers build up, provided that no disturbance of biofilm is achieved, new environmental conditions are established, favouring the colonisation and adaptation of different colonisers with different requirements, including oxygen level, to grow (Marsh and Bradshaw, 1995).

Oral microflora acts as part of the host’s defences by prevention of colonisation of exogenous and pathogenic microorganisms by, for instance, producing inhibitory compounds and developing an environment that is not favourable for the invading microorganisms (Marsh *et al.*, 2011). However, if

the balance of the composition and function of this oral microflora is disturbed by biological or physiological changes, such as hormonal changes, then disease can occur (Kilian *et al.*, 2016; Marsh *et al.*, 2011; Marsh and Bradshaw, 1995). Other factors that can contribute to unbalanced oral microflora include salivary gland dysfunction, poor oral hygiene, dietary habits, stress and smoking (Cho and Blaser, 2012). Therefore, it is essential to understand the relationship between the oral microflora and the host, and the importance of keeping the oral microflora at levels that is compatible with health (Marsh *et al.*, 2011).

1.1.1 Dental plaque structure

Dental plaque consists of dense, mushroom-like clumps of bacteria that arise from the enamel surface, scattered with bacteria-free channels filled with extracellular polysaccharide secreted by the bacteria that can serve as diffusion channels (Figure 1) (Nizet and Esko, 2009). It was examined under confocal laser scanning microscopy where it revealed a highly heterogeneous architecture in terms of distribution of cells, matrix and fluid filled spaces (Auschill *et al.*, 2001; Wood *et al.*, 2000). These fluid filled pores and channels found to extend through the whole thickness of the biofilm sample, providing a link between the oral environment and the tooth surface (Auschill *et al.*, 2001; Wood *et al.*, 2000). When using live/dead stains to visualise bacterial viability in a dental plaque sample, a high percentage of viable bacteria were found in the central part of the biofilm sample and surrounding the pores and channels (Auschill *et al.*, 2001). This structure of plaque biofilms is considered a significant factor in relation to the penetration and distribution of molecules, including antimicrobial agents. The biofilm matrix, which contains polysaccharides, proteins and other metabolites, is seen to protect the microorganisms from the environment and could prevent diffusion of these molecules in the biofilm (Marsh, 2005; Marcotte *et al.*, 2004; Robinson *et al.*, 1997).

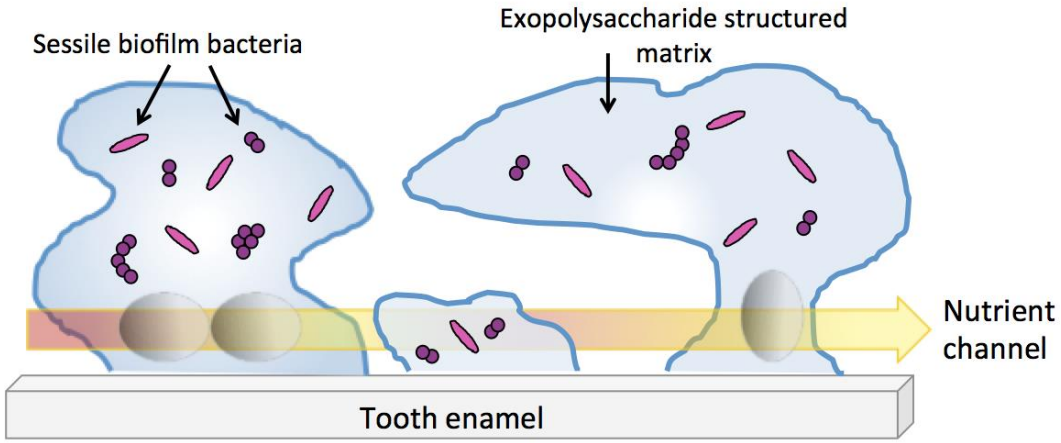


Figure 1 Structure of Dental plaque biofilm (Nizet and Esko, 2009)

1.1.2 Bacterial composition of dental plaque

There are over 700 bacterial species that have been discovered in the oral cavity, however, many of them cannot be cultivated in pure culture in the laboratory (Kilian *et al.*, 2016). Approximately 80-90% of the weight of plaque is water (Marsh and Bradshaw, 1995). While 70% of the dry weight is bacteria (Gram-positive and Gram-negative species), the remaining 30% consists of a matrix of polysaccharides and salivary proteins and glycoproteins (Marsh and Bradshaw, 1995). As stated earlier, the resident oral microflora contribute to the individual's health and the imbalance of its composition will lead to disease.

The composition of plaque differs at different sites of the tooth (Marsh and Bradshaw, 1995). This variation is due to the differences in the tooth-site biological properties (Marsh and Bradshaw, 1995). For instance, the plaque microflora at approximal surfaces of the tooth is more diverse, with high numbers of obligately anaerobic Gram-negative species, than that of occlusal fissures due to a lower redox potential (Marsh and Bradshaw, 1995; Marsh *et al.*, 1989b). Whereas the gingival crevice plaque consists of higher levels of obligately anaerobic species than that of former sites as their nutrients are provided by gingival crevicular fluid (Marsh and Bradshaw, 1995; Slots, 1977).

Furthermore, the bacterial composition in the dental plaque varies at different stages of its formation (Marsh and Bradshaw, 1995). The following are the types of bacterial species that can be observed under microscopy at different stages of dental plaque development:

- Early biofilm (after 2-4 hours of plaque formation): Gram-positive coccoid microorganisms (*Streptococci spp*) and few rods microorganisms (*Actinomyces spp*) (Marsh and Bradshaw, 1995).
- After 1-2 days: Gram-positive rods and filaments (Marsh and Bradshaw, 1995).
- Older biofilm (after several days): more diverse and complex microflora. High numbers of rod-shaped species (*Actinomyces spp*) and increased numbers of obligately anaerobic species (Marsh and Bradshaw, 1995; Nyvad, 1992).
- After 2-3 weeks (undisturbed): depth of biofilm is approximately 50-100μm on exposed surfaces and thicker on protected surfaces such as occlusal fissures (Nyvad, 1992).

1.1.3 Properties of Dental plaque

Microorganisms in a biofilm have different properties from when they are in isolation (Table 1) (Marsh *et al.*, 2011). These properties are not just the summation of the properties of the resident population (Marsh *et al.*, 2011). Bacteria within a plaque biofilm can communicate with each other by generating, perceiving, and reacting to small diffusible signal molecules (quorum sensing process). They act as a community, which facilitated their colonisation on the host, as well as resisting their opponents and adaptation with environment's changes (Li and Tian, 2012). Additionally, this communication has enhanced the virulence and pathogenic potential of these bacteria (Li and Tian, 2012). Therefore, it is important to understand the properties of bacteria grown in a biofilm, including physiological properties, susceptibility to antimicrobial agents and interaction with the host,

to be able to control dental plaque related diseases (Li and Tian, 2012; Marsh, 2005, 2004).

Table 1 General properties of biofilms and dental plaque

(Marsh *et al.*, 2009)

General property	Dental plaque example
Open architecture	Presence of channel and voids
Microbial protection	Production of extracellular polymers to form a functional matrix; physical protection from phagocytosis
Host protection	Colonization; resistance
Enhanced tolerance to antimicrobials	Reduced sensitivity to chlorhexidine and antibiotics; gene transfer
Neutralization of inhibitors	β lactamase production by neighboring cells to protect sensitive organisms
Novel gene expression	Synthesis of novel proteins on attachment or on binding to host molecules; up-regulation of *gtfBC* in mature biofilms
Coordinated gene responses	Production of bacterial cell-to-cell signaling molecules (e.g. CSP, AI-2)
Communication with host	Down-regulation of pro-inflammatory responses by resident oral bacteria; remodeling of the cytoskeleton of epithelial cells
Spatial and environment heterogeneity	pH and O_2 gradients; co-adhesion
Broader habitat range	Obligate anaerobes in an overtly aerobic environment
More efficient metabolism	Complete catabolism of complex host macromolecules (e.g. mucins) by microbial consortia (food chains and food webs)
Enhanced virulence	Pathogenic synergism in periodontal diseases

1.1.4 Dental plaque related diseases

The oral cavity provides environments for a wide range of microorganisms, including bacteria, yeasts and viruses (Allaker and Douglas, 2015). These microorganisms are associated with numerous oral infections as a consequence of imbalances in the normal oral microflora (Marsh *et al.*, 2011). Dental caries and periodontal disease are one of the most prevalent bacterial diseases occurring in man worldwide. According to the World Health Organization, dental caries continues to be a major oral health problem in most industrialised countries, affecting 60-90% of schoolchildren and the majority of adults (Petersen *et al.*, 2005). Whereas periodontal disease affects 30-50% of the adults, 10% of them have the severe form (Meisel and Kocher, 2005).

Dental caries is the demineralisation of dental hard tissues by acidogenic plaque bacteria colonizing the tooth surface, including *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus species* (Marsh *et al.*, 2011; Marsh *et al.*, 2009). It forms through a complex interaction over time between the acidogenic bacteria, fermentable carbohydrate and host factors such as teeth and saliva (Figure 2) (Marsh *et al.*, 2009). Individuals with high numbers of these cariogenic bacteria, low salivary flow, poor oral hygiene, inadequate fluoride exposure, high in amount and frequency of sugar consumption, low socioeconomic status, and medical or physical disability are all at risk of developing caries (Scottish Intercollegiate Guidelines Network, 2014).

Whereas in periodontal disease the host mounts an inappropriate and uncontrolled inflammatory response to an increased microbial load, due to plaque accumulation that is either confined to the gingiva (gingivitis) or extending to the deeper supporting tissues, resulting in damage to the periodontal ligament and alveolar bone that supports the teeth (periodontitis) (Allaker and Douglas, 2015). The predominant pathogens in advanced

periodontal disease are mainly *Porphyromonas gingivalis*, *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* (Marsh *et al.*, 2009; Marsh and Bradshaw, 1995). Similar to dental caries, periodontal disease is multifactorial; It results from interaction of environmental triggers, such as resident microflora, poor oral hygiene, exposure to antimicrobial drugs, and the host susceptibility (Figure 2) (Marsh *et al.*, 2009). Although plaque accumulation is the crucial aetiological factor in periodontal disease, the progression from gingivitis to periodontitis can only occur if the individual has impaired immunological defences (Kilian *et al.*, 2016). Systemic diseases, such as diabetes mellitus and Down syndrome, smoking and emotional stress can modify the host response and enhance the destructive effects of the microbiota (Clerehugh and Tugnait, 2001).

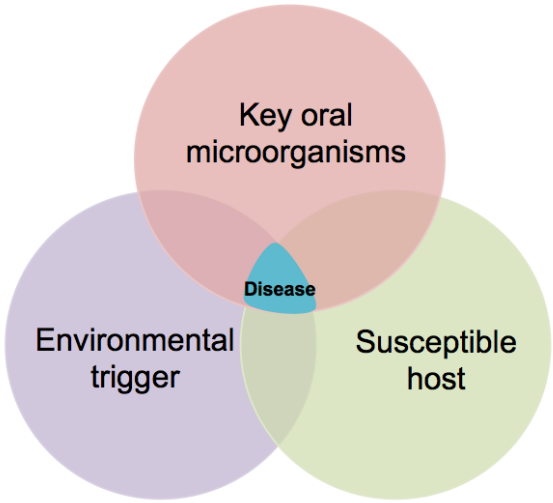


Figure 2 Interactions of factors involved in oral disease

(Marsh *et al.*, 2009)

These dental diseases and their sequelae can cause significant pain and are affecting the individuals' quality of life (Scottish Intercollegiate Guidelines Network, 2014). It has been reported that there is an association between periodontal disease and systemic conditions, such as cardiovascular disease and complications during pregnancy (Kim and Amar, 2006; Xiong *et al.*,

2006). It is also very expensive to treat dental disease; the NHS, for instance, spends approximately £2.25 billion per year on dental treatment (The British Dental Health Foundation, 2016). In addition, the burden of dental disease lasts a lifetime as once the tooth structure is destroyed it will require restorative treatment and regular maintenance throughout lifetime (Selwitz *et al.*, 2007). Therefore, It is essential to understand the relationship between the above-mentioned risk factors and also to develop strategies and approaches in oral disease prevention and health promotion.

1.1.5 Approaches to control dental plaque

The current most common practices for controlling dental plaque are mechanical removal, such as tooth brushing and flossing, and using antimicrobial mouth rinses (Allaker and Douglas, 2015). These approaches should ideally prevent dental plaque accumulation without disturbing the microflora's balance in the oral cavity (Allaker and Douglas, 2015). However, such practices are not always effective or practical (Tahmassebi *et al.*, 2015). Mechanical removal requires patients' compliance and, also, good manual dexterity which is usually absent in patients with, for example, physical or mental disabilities (Baker, 1992; Ciancio, 1988) and younger children (Tahmassebi *et al.*, 2015).

In addition, there is an increased tolerance to antimicrobial agents by bacteria grown in a biofilm; this is due to the fact that properties of bacteria grown in a biofilm are significantly different compared with planktonic growth (Table 1) (Marsh *et al.*, 2011). For instance, the concentration of Amine fluoride and chlorhexidine required to kill *Streptococcus sobrinus* growing in a biofilm is around 100 times greater than the concentration required to kill the same amount of planktonic bacterial cells (Shani *et al.*, 2000). One of the reasons that explains this increased tolerance of biofilms to antimicrobial agents compared to planktonic cells is that these biofilm bacteria are slow growing cells, as they divide slowly, which makes them less sensitive to antimicrobial agents (Marsh *et al.*, 2011). The age of the biofilm is also

considered as a significant factor in restricting the penetration of the antimicrobial agent as had been reported by Millward and Wilson (1988); Older biofilms of *Streptococcus sangius* were more resistant to chlorhexidine than younger biofilms (Millward and Wilson, 1988).

Other than the growth rate and biofilm's age, it is well established in the literature that biofilm-associated bacterial infections have several mechanisms in resisting antibiotics, as a consequence of a high antibiotic consumption that has contributed to the emergence of multidrug-resistant strains that has become a public health problem (Pozo and Patel, 2007; Sharma *et al.*, 2005; Wilson, 1996). One of these mechanisms is that antibiotics can be inactivated by enzymes within the biofilm matrix (Pozo and Patel, 2007). An example of this is the inactivation of penicillin and cephalosporin antibiotics by β-lactamase enzyme produced by gram-positive bacteria in oral biofilm (Soares *et al.*, 2012). Alteration of the channels located in the bacterial cell wall also will reduce the uptake of antibiotic into the bacterium (Soares *et al.*, 2012). The genetic information that encodes for these mechanisms may arise by a random spontaneous mutation or by interspecies gene transfer (Soares *et al.*, 2012; Barker, 1999).

Furthermore, there are other limitations associated with the use of these antimicrobial agents, such as disruption of the oral microflora and the difficulty of maintaining therapeutic concentrations in the oral cavity (Wilson, 2004). Therefore, alternative approaches to control dental plaque are needed. One such alternative is photodynamic therapy (Allaker and Douglas, 2015; Mang *et al.*, 2012).

1.2 Photodynamic therapy (PDT)

1.2.1 History

PDT basically involves three nontoxic components: visible light; a photosensitizer; and oxygen (Takasaki *et al.*, 2009). It is a treatment modality where the photosensitizer (photoactive dye) binds to the target cells and can

be activated by light of a wavelength that corresponds to an absorption maximum of the photosensitizer. Following activation of the photosensitizer by the application of light, in the presence of oxygen, reactive oxygen species (singlet oxygen and free radicals) are produced that are toxic to eukaryotic cells and microorganisms (Figure 3) (Soukos and Goodson, 2011).

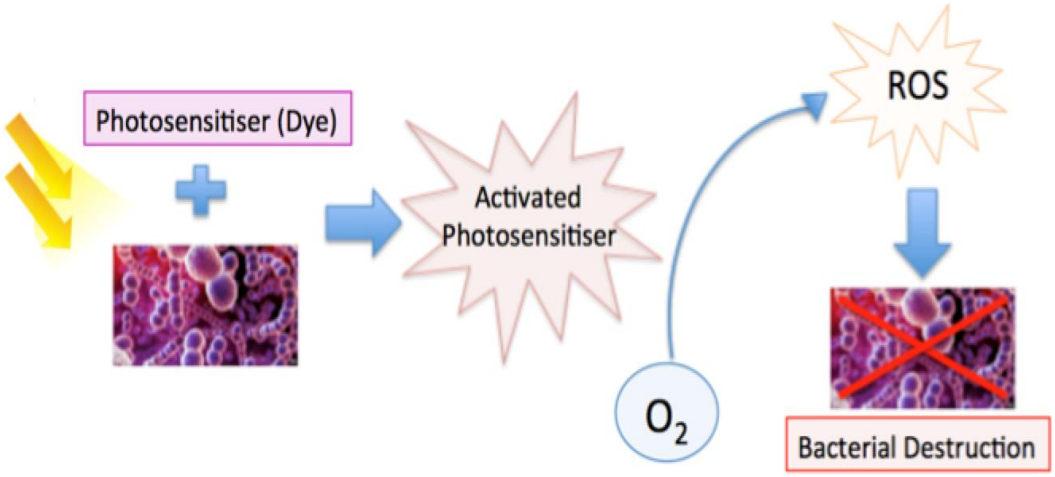


Figure 3 PDT mechanism of action

PDT has been widely applied in the medical field (Takasaki *et al.*, 2009). Historically, It has been more prominent in cancer treatment as an alternative method to traditional treatments such as chemotherapy, radiotherapy or surgery. It has been shown to kill cancerous cells by necrosis or apoptosis (Carrera *et al.*, 2016). It has been approved for clinical treatment of certain tumours in the Unites States (US), the European Union, Canada, Russia, and Japan (Konopka and Goslinski, 2007). The US Food and Drug Administration (FDA) has also recently approved PDT for the treatment of selected skin problems (Salva, 2002). Furthermore, PDT is being used either clinically or in experimental studies in different medical fields such as ophthalmology, gastroenterology, cardiology, and dermatology (Meisel and Kocher, 2005). However, It is only recently being considered as an antimicrobial treatment, even though it was discovered more than 100 years

ago that microorganisms can be killed by light in the presence of a photosensitizing agent by Oscar Raab in 1900 (Soukos and Goodson, 2011). This late consideration is due to the current increase in drug resistance bacteria (Meisel and Kocher, 2005; Wainwright and Crossley, 2004). The science behind antimicrobial PDT follows the same principles to PDT of tumour cells (Gursoy *et al.*, 2013).

PDT is also known as photo-chemotherapy, photo-radiation therapy, photodynamic antimicrobial chemotherapy, photodynamic inactivation and antimicrobial PDT (Gursoy *et al.*, 2013; Nagata *et al.*, 2012; Konopka and Goslinski, 2007).

1.2.2 Mechanism of action of PDT

Again, PDT is a treatment involving a photosensitizer, which absorbs light and can be taken up by microorganisms, that in the presence of oxygen is activated by exposure to light of a specific wavelength (Soukos and Goodson, 2011). The result of the irradiation is that the photosensitizer molecule moves from its low-energy ground state to an excited singlet state that receives the light energy (Soukos and Goodson, 2011). The lifetime of this excited singlet state is very short (nanosecond range) to permit significant interactions with the surrounding molecules (Konan *et al.*, 2002; Dougherty *et al.*, 1998), therefore, it may either return to its ground state by emitting light (fluorescence), or may move to a higher-energy triplet state (Soukos and Goodson, 2011; Konopka and Goslinski, 2007). The key role of the singlet state in the photosensitization process is to act as precursor of the triplet state (Konan *et al.*, 2002). This triplet state (photoactive state), with a lifetime in the microsecond-millisecond range, interacts with cell components to generate cytotoxic species that causes a rapid and selective destruction of the target bacterial cells by undergoing one or both of the following two reactions (Figure 4) (Soukos and Goodson, 2011; Konopka and Goslinski, 2007; Ochsner, 1997):

1. **Type 1 reaction:** involves electron/hydrogen transfer directly from the photosensitizer triplet state with the involvement of a substrate to produce radical ions, or electron/hydrogen removal from the substrate molecule to form free radicals. These radicals can react rapidly with oxygen to produce highly reactive oxygen species, such as superoxide and hydroxyl radicals.

2. **Type 2 reaction** (interaction between the photosensitizer triplet state and oxygen): involves energy transfer from the photosensitizer triplet state to oxygen to produce excited state singlet oxygen, which can oxidize many biological molecules, such as proteins, nucleic acids and lipids, and lead to bacterial cell death.

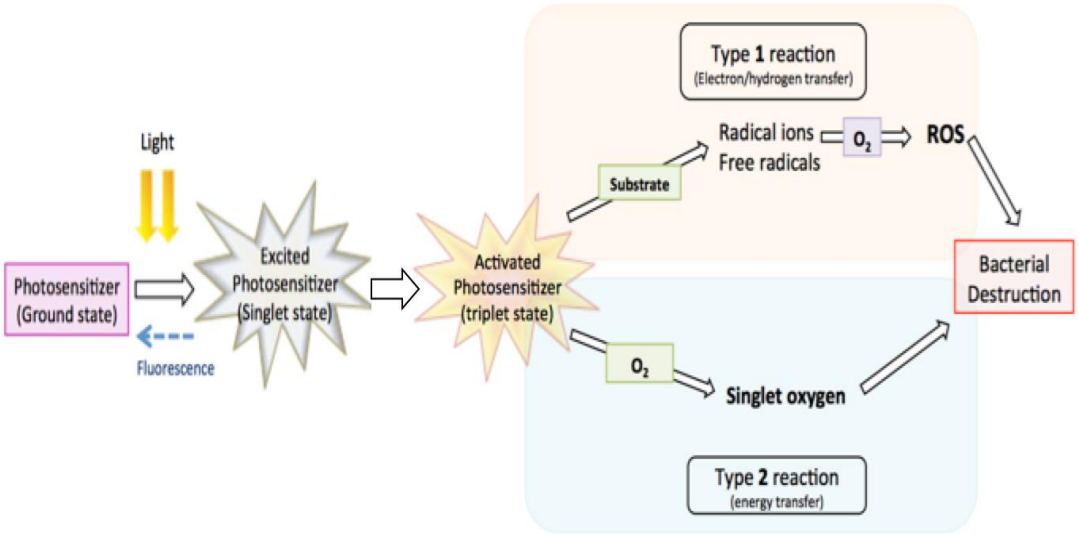


Figure 4 Type 1 and Type 2 reactions in PDT

(Soukos and Goodson, 2011)

These reactive oxygen species (ROS) are responsible for the photo-destruction to cell components such as cytoplasmic membrane of bacteria and DNA, causing cell death (Gursoy *et al.*, 2013; Konan *et al.*, 2002). Of

these ROS, singlet oxygen is thought to be the major damaging species in PDT, which has a half-life of <0.04μs (Soukos and Goodson, 2011; Moan and BERG, 1991). It has been reported that distinguishing between these two reactions is difficult in PDT, and if both Types 1 and 2 reactions were involved in PDT, this implies that the mechanism of photo-damage is dependent on both oxygen tension and photosensitizer concentration (Konopka and Goslinski, 2007). However, there are numerous factors influencing the photo-damage, including the type, dose, incubation time and localisation of the photosensitizer, the availability of oxygen, the wavelength of light (nm), the light power density (mW/cm^2) and the light energy fluence (J/cm^2) (Soukos and Goodson, 2011; Konan *et al.*, 2002).

1.2.3 Advantages of PDT

There are several advantages of PDT including topical application of the photosensitizing chemical agent on the target tissue and then followed by selective irradiation of the target tissue with visible light (dual selectivity) (Gursoy *et al.*, 2013; Hamblin and Hasan, 2004; Salva, 2002). This means that only cells with selective application of photosensitizer and also receiving light exposure are killed (Pfitzner *et al.*, 2004). Therefore, damage to the surrounding normal tissue is limited (Salva, 2002). In addition, it has been reported that bacterial resistance to the cytotoxic action of singlet oxygen or free radicals is unlikely to develop, since these reactive species interact with several microbial cell structures and different metabolic pathways (Konopka and Goslinski, 2007). The primary target of the photo-destruction in microbial cells is the cytoplasmic membrane, followed by DNA damage. However, this is dependent on the photosensitizer dose and the irradiation time (Bertoloni *et al.*, 2000). This disruption of biofilms may inhibit plasmid exchange involved in the transfer of antibiotic resistance, and disrupt colonization (Konopka and Goslinski, 2007).

Moreover, PDT has the advantage of being equally effective against antibiotic-resistant and antibiotic-sensitive bacteria and, in addition, repeated applications do not result in the selection of resistant organisms as with antimicrobial agents, where drug resistance involves drug inactivation or target alteration as mentioned earlier (Konopka and Goslinski, 2007; Wainwright and Crossley, 2004). But, singlet oxygen was not involved in these mechanisms of drug resistance. It has been reported that singlet oxygen is highly reactive and difficult to defend against as it can inactivate the antioxidant enzymes such as superoxide dismutase and catalase (Wainwright and Crossley, 2004; Kim *et al.*, 2001).

The clinical advantages of PDT are that it is non-invasive and it can be carried out in outpatient settings (Gursoy *et al.*, 2013; Konopka and Goslinski, 2007). Furthermore, studies of PDT for periodontal diseases have reported some advantages of PDT over conventional mechanical therapy (i.e. scaling and root planning), such as reducing treatment time and no need for anaesthesia, suggesting using it as an adjunct therapy to the conventional methods (Qin *et al.*, 2008; de Oliveira *et al.*, 2007).

1.2.4 Application of PDT in dentistry

Dental applications of PDT are rising rapidly (Gursoy *et al.*, 2013). It has been used to treat oral cancer, bacterial and fungal infections, and the photodynamic diagnosis of the malignant transformation of oral lesions (Konopka and Goslinski, 2007). Recently, it has been used to target dental plaque microorganisms in planktonic phase and in biofilms (Fontana *et al.*, 2009), periodontal diseases (Raghavendra *et al.*, 2009; Qin *et al.*, 2008; de Oliveira *et al.*, 2007; Meisel and Kocher, 2005), necrotic pulps (Pinheiro *et al.*, 2009; Garcez *et al.*, 2008), peri-implantitis (Dörtbudak *et al.*, 2001) and oral candidiasis (Dovigo *et al.*, 2011; Donnelly *et al.*, 2007) either *in vitro* or *in vivo*.

These PDT studies that target periodontal and endodontic diseases, for example, have suggested the potential of PDT as an adjunctive therapy to the conventional therapies, scaling and root planing in the case of periodontal disease, and endodontic chemo-mechanical debridement in the case of treatment of necrotic pulps (Soukos and Goodson, 2011). Lately, treatment kits for clinical PDT are available in some countries, such as Canada and the UK, for the treatment of periodontal diseases. This kit consists of a laser system with a custom-designed hand-piece and a photosensitizer (methylene blue or toluidine blue O) (Soukos and Goodson, 2011).

In terms of dental plaque microorganisms, several PDT studies have shown that oral bacteria in planktonic cultures (Rolim *et al.*, 2012; Chibebe Junior *et al.*, 2010; Bevilacqua *et al.*, 2007; Paulino *et al.*, 2005; Soukos *et al.*, 1998; Wilson *et al.*, 1992), plaque scrapings (Williams *et al.*, 2003; Sarkar and Wilson, 1993), growing *in vitro* as a biofilm (Pereira *et al.*, 2013; Teixeira *et al.*, 2012; Metcalf *et al.*, 2006; Wood *et al.*, 2006; Zanin *et al.*, 2006; Zanin *et al.*, 2005; Dobson and Wilson, 1992), and *in vivo* biofilm generation (Tahmassebi *et al.*, 2015; Teixeira *et al.*, 2012; Lima *et al.*, 2009; Wood *et al.*, 1999) are susceptible to PDT. However, it has been reported that oral bacteria in biofilms were less affected by PDT than the bacteria in the planktonic phase and this may be related to biofilms' distinct characteristics (Fontana *et al.*, 2009). Despite this reduced efficacy, it is thought that its antibacterial effect is greater than that which had been reported for treatment with antibiotics (Fontana *et al.*, 2009).

Therefore, PDT seems to be a promising therapeutic approach in the management of dental biofilms. However, the FDA has not yet approved it for dentistry use. In clinical studies, the treatment procedure of the patients should be conducted according to FDA and local institutional review board approval (Gursoy *et al.*, 2013; Konopka and Goslinski, 2007).

1.2.5 Safety of PDT

As stated earlier, PDT is a two-step procedure that involves the topical application of a photosensitizing chemical agent and selective illumination of the target lesion with visible light (Salva, 2002). It combines two non-toxic compounds, drug and light, to destroy the target cells (Salva, 2002). Due to the topical application of the photosensitizer on the target lesion and then followed by selective illumination of the lesion with visible light, the damage to the surrounding normal tissue is unlikely (Salva, 2002).

However, it is important to determine the safety of PDT and the therapeutic window where bacteria would be affected but not the host tissues (Xu *et al.*, 2009; Soukos *et al.*, 1996). Therefore, a team of researchers has investigated the PDT effects on human gingival keratinocytes and fibroblasts and they have revealed no evidence of cytotoxic effect on these cells *in vitro* when using a low dose of PDT (Toluidine blue O and HeNe laser) that still resulted in sufficient killing of *Streptococcus sanguis*, which is one of the most common species found in dental plaque (Soukos *et al.*, 1996). Similarly, Zeina *et al.* (2002) have demonstrated minimal cytotoxic effect on keratinocytes *in vitro* when using a dose of PDT (Methylene blue and white light) that resulted in sufficient killing of cutaneous bacterial species by seven log cycles. The kill rates of the keratinocytes were up to 200-fold lower and slower than the kill rates of cutaneous bacterial cells using the same dosage of PDT (Zeina *et al.*, 2002; Zeina *et al.*, 2001). The reasons for this killing difference have been thought to be due to the existence of a nuclear membrane in keratinocytes that acted as an additional barrier to the photosensitizer or it might be due to the difference in the size of the bacterial cell and keratinocytes, where the keratinocytes are about 25-50 times larger than bacterial cells and may thus enclose a higher number of targets per cell (Zeina *et al.*, 2002). Therefore, the cytotoxic effects of PDT to host tissues is considered to be dose-dependent, where a higher dose is required to cause damage to mammalian cells compared to bacterial calls (Soukos *et al.*, 1996).

Furthermore, a study by Xu *et al.* (2009) have compared the cytotoxic effects of PDT and sodium hypochlorite on human gingival fibroblasts and osteoblasts *in vitro* and they have found that sodium hypochlorite has greater cytotoxic effects on these cells, suggesting PDT as a safe adjunctive therapy for endodontic disinfection.

The genotoxic effects of PDT have also been investigated on keratinocytes *in vitro* using comet assay to detect any DNA damage to these cells following PDT (Zeina *et al.*, 2003). The assay was not able to detect any DNA damage, indicating that PDT has a wide safety margin between bacterial killing and keratinocyte damage (Zeina *et al.*, 2003).

Therefore, PDT seems to provide a rapid, noninvasive, confined bacterial killing without harming host tissues (Soukos and Goodson, 2011).

1.2.6 Light Sources

A light source is required to activate the photosensitizer (Konopka and Goslinski, 2007). It should have a specific wavelength that corresponds to the activation absorption spectrum of the photosensitizer and also it should provide an adequate dose of energy to the photosensitizer to be enable transition to the higher-energy triplet state (Wilson and Patterson, 2008).

Visible light, which is a form of electromagnetic radiation (Figure 5), is most applicable to photodynamic therapy (Soukos and Goodson, 2011). It is defined as the wavelengths that are detected by the human eyes. It falls in the range of the electromagnetic radiation spectrum between infrared and ultraviolet and it has wavelengths of about 400-700nm (Figure 5). These waves are seen as the colours of the rainbow. Each colour has a different wavelength. Violet has the shortest wavelength and red has the longest wavelength. White light is seen when all the colours are combined, whereas,

black is a total absence of light (National Aeronautics and Space Adminstration, 2014).

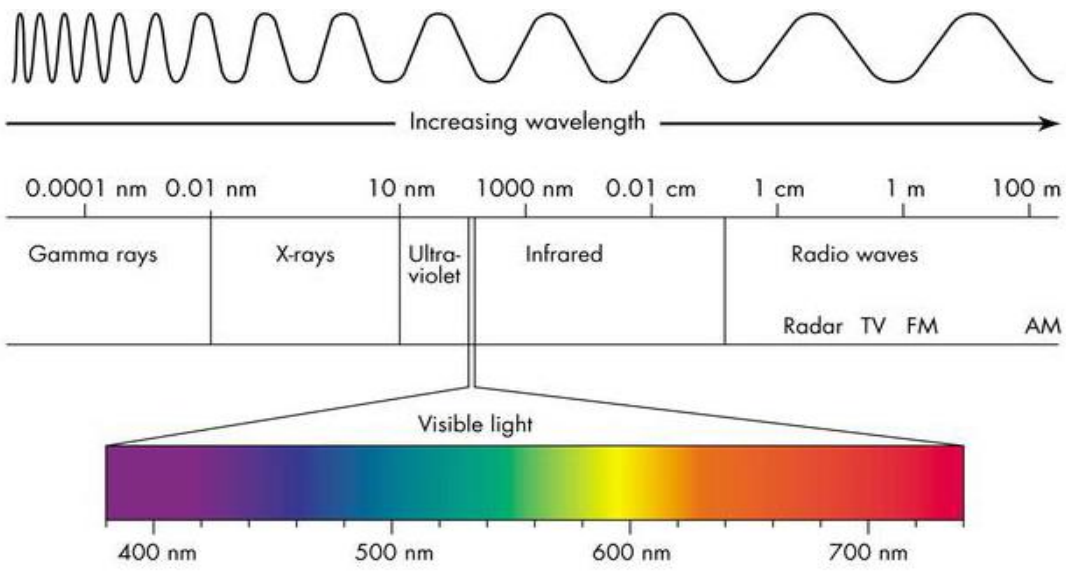


Figure 5 Electromagnetic radiation spectrums: Visible light wavelength (http://www.cyberphysics.co.uk/topics/radioact/Radio/EMSpectrumcolor.jpg)

With regards to PDT, red light at wavelengths of 630nm penetrate the superficial layers of human tissues up to 5mm (Salva, 2002), while deeper penetration is reached at longer wavelengths (Konopka and Goslinski, 2007). The benefits of using a visible light source is to visualize the target area and localisation of the PDT effect without harming adjacent host tissue (Wilson, 1994).

Several light factors need to be considered in order to activate the photosensitizer, including the total light dose required, the dose rates, the light penetration depth and the localisation of target area (Raghavendra *et al.*, 2009).

Different sources of light delivery are available depending on the location and morphology of the target lesion (Raghavendra *et al.*, 2009). The main light sources used in PDT are lasers, light-emitting diodes (LEDs) and filtered lamps (Wilson and Patterson, 2008). The superiority of one light source over the other has not been established; therefore the use of either one depends on the specific application (Brancaleon and Moseley, 2002).

1.2.6.1 Lasers- Coherent light sources

The laser (light amplification by stimulated emission of radiation) is defined as “a device capable of producing a beam of light that consists of parallel waves, all of which have the same frequency and are coherent” (Wilson, 1993). The power output of the laser light beam is greater than that produced by a non-laser light source (Wilson, 1993). Lasers with specific wavelengths are being used recently in PDT, such as helium–neon lasers (633nm), gallium–aluminum–arsenide diode lasers (630–690, 830, or 906nm), and argon lasers (488–514nm) (Gursoy *et al.*, 2013).

High efficiency (greater than 90%), precise application and monochromaticity that gives maximum efficiency of photo-activation, are the main advantages of lasers (Wilson and Patterson, 2008; Salva, 2002). However, lasers are single-wavelength devices, this means that each photosensitizer with different absorption spectrum will require a different laser unit, and they are also expensive (Wilson and Patterson, 2008).

1.2.6.2 Incoherent light sources

Most natural and artificial light sources are incoherent, which means that the waves of the light beam are not in phase, polychromatic and divergent (Wilson, 1993). Lately, these non-laser sources, such as LEDs (440-636nm) (Bevilacqua *et al.*, 2007; Zanin *et al.*, 2005), tungsten filament lamp (400-1100nm) (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006;

Wood *et al.*, 1999), quartz halogen lamps (620-640nm) (Lee *et al.*, 2013) and xenon arc lamps (300-1200nm) have been used widely in PDT of oral biofilms, since these devices are lighter, inexpensive, and portable compared with the traditional lasers (Gursoy *et al.*, 2013; Takasaki *et al.*, 2009; Wainwright and Crossley, 2004). In addition, they are flexible in configuring arrays into different irradiation geometries to compensate for difficult anatomic areas; for example, a U-shaped tube arrays that is used for irradiation of the entire face and scalp for dermatological problems (Wilson and Patterson, 2008; Brancaleon and Moseley, 2002). They also can be spectrally filtered to match any photosensitizer (Wilson and Patterson, 2008). Lamps can be used direct or coupled to liquid light guides of between 5-10mm in diameter, therefore, they are suitable for accessible lesions (Brancaleon and Moseley, 2002).

Furthermore, the energy required from the light source is determined by the molecular structure of the photosensitizer; for instance, acridines have a chromophoric structure with a light excitation range of 400-450nm, which is different than phenothiazinium (600-690nm) (Wainwright and Crossley, 2004). Therefore, it is important to understand the photo-properties of the photosensitizer and the target tissue (Wainwright and Crossley, 2004).

The light source used in this study was a 400W tungsten filament lamp (white light). These lamps can deliver light over a wide spectrum (400-1100nm) and can be spectrally filtered to match any photosensitizer (Wilson and Patterson, 2008). This light source was used to assess killing of *in vitro* and *in vivo* dental plaque biofilms in combination with erythrosine dye in the wavelength range 500-550nm, which corresponds to the region of maximal absorption by erythrosine (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006). It was chosen due to its wide availability and low cost.

1.2.7 Photosensitizers

It is important to carefully choose a suitable and effective photosensitising agent that is able to highly absorb the light wavelength used (Gursoy *et al.*, 2013). These are usually coloured substances that absorb visible light (Wainwright and Crossley, 2004). Many natural and synthetic compounds, such as dyes, cosmetics, chemicals and natural elements, possess photosensitising properties (Meisel and Kocher, 2005). It has been suggested that the photosensitizer should have certain favourable properties to be used in antimicrobial PDT. These properties include: a broad spectrum of action; high absorption in the spectral region of the excitation light; high binding affinity for microorganisms; low binding affinity for mammalian cells to avoid the risk of photo-destruction of host tissues; lack of selection of resistant strains after multiple treatments; a minimal risk of promoting mutagenic processes; low chemical toxicity; a high quantum yield of triplet state and singlet oxygen to obtain large concentrations of the activated drug (Gursoy *et al.*, 2013; Soukos and Goodson, 2011; Wilson and Patterson, 2008; Jori *et al.*, 2006).

The main photosensitizers that have been used in dentistry are listed in Table 2.

Table 2 Examples of the main photosensitizers used in dentistry (Carrera *et al.*, 2016; Soukos and Goodson, 2011; Wainwright and Crossley, 2004)

Photosensitizers (Dyes)	Absorption wavelength	Antimicrobial activity by PDT	Examples of Studies
Phenothiazine - Methylene blue - Toluidine blue	600-660nm	Against dental biofilm and planktonic cells	(Fontana *et al.*, 2009; Bevilacqua *et al.*, 2007)
Phthalocyanines	660-700nm	Against *in vivo* biofilm	(Wood *et al.*, 1999)
Porphyrins	600-690nm		
Chlorines	652nm		(Pfitzner *et al.*, 2004)
Curcumin	300-500nm	Against biofilms and planktonic forms *Candida* spp.	(Andrade *et al.*, 2013; Dovigo *et al.*, 2011)
Xanthene dyes - Rose Bengal - Erythrosine	450-600nm 500-550nm	Against *in vitro* or *in vivo* biofilms	(Tahmassebi *et al.*, 2015; Shrestha *et al.*, 2014; Metcalf *et al.*, 2006; Wood *et al.*, 2006)

The susceptibility of bacteria to destruction by PDT seems to be associated to the charge of the photosensitizer (Konopka and Goslinski, 2007). Some photosensitizers, for example, toluidine blue O and methylene blue, carry a positive charge, and it is reported that these cationic photosensitizers can target both Gram-positive and Gram-negative bacteria. In contrast, neutral or

anionic (negatively charged) photosensitizers bind efficiently to Gram-positive bacteria but to some extent to Gram-negative bacteria (Soukos and Goodson, 2011; Wainwright and Crossley, 2004). This is due to the structural difference in their cell wall (Figure 6). Gram-positive bacteria have a relatively porous layer of peptidoglycan and lipoteichoic acid outside the cytoplasmic membrane, which allows the negatively charged photosensitizer to bind efficiently and diffuse into sensitive sites (Konopka and Goslinski, 2007). Whereas in Gram-negative bacteria the outer membrane structure is more complex, forming a physical and functional barrier between the bacterial cell and its environment (Raghavendra *et al.*, 2009; Konopka and Goslinski, 2007). However, the permeability of the Gram-negative bacteria to photosensitizers can be modified by several mechanisms, such as linking the photosensitizer to a cationic molecule (poly-L-lysine-chlorine e6) or conjugating the photosensitizer with a monoclonal antibody that binds to cell-surface-specific antigens (Raghavendra *et al.*, 2009; Hamblin and Hasan, 2004).

Therefore, in terms of bacteria-photosensitizer interaction, the effectiveness of PDT is mostly associated with three points: the photosensitizer ability to bind and interact with the bacterial membrane; photosensitizer ability to diffuse into the cell, and reactive oxygen species formation around bacterial cell by irradiation of the photosensitizer (Nagata *et al.*, 2012). In addition, it is essential to put in mind that the effect of PDT on dental biofilms is different than on planktonic cells due to these structural variation in the bacterial cell membranes, in addition to the presence of other components, such as extracellular matrix and quorum-sensing factors, rendering the photosensitizer-microorganism interaction (Huang *et al.*, 2012).

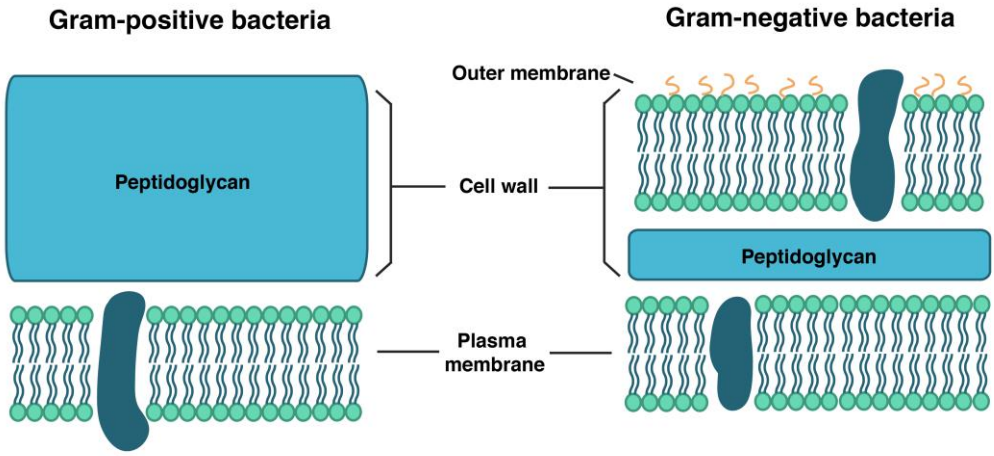


Figure 6 Morphological structure of Gram-positive and Gram-negative bacteria (https://www.khanacademy.org/science/biology/bacteria-archaea/prokaryote-structure/a/prokaryote-structure)

As we investigated the PDT effect using erythrosine as the photosensitizer in this study, it is discussed in more detail in the following section.

1.2.7.1 Erythrosine

Erythrosine is a cherry-pink synthetic food colouring dye with a poly-iodinated xanthene structure (Figure 7) (Chequer *et al.*, 2012). It is also known as Erythrosine B, FD & C Red No. 3, C.I. Acid Red 51, E127, and tetraiodofluorescein (Ganesan *et al.*, 2011). It belongs to a group of cyclic compounds called xanthenes, which has been reported to have the ability to absorb light in the visible region (500-550nm) and initiate photochemical reactions (Wood *et al.*, 2006). In addition, Its antimicrobial activity against Gram-positive and Gram-negative oral bacteria is well documented in the literature (Marsh *et al.*, 1989a; Baab *et al.*, 1983; Caldwell and Hunt, 1969; Begue *et al.*, 1966). Its main application in dentistry is staining and visualising dental plaque to facilitate oral hygiene practice (Wood *et al.*, 2006). In plaque disclosing solutions, it is used at concentrations of 9-25mM

(0.72-2% weight/volume) (Marsh *et al.*, 1989a), which is much higher than that used in several PDT studies (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006).

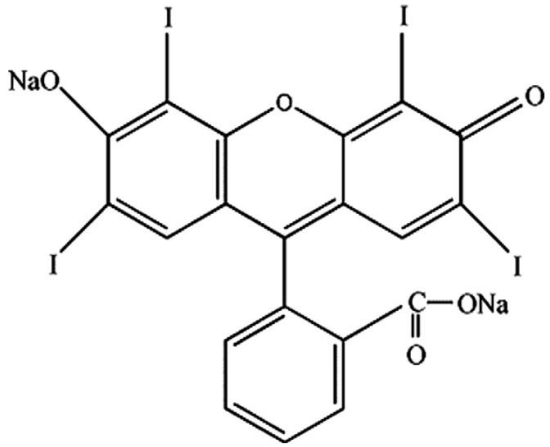


Figure 7 Chemical structure of Erythrosine

Erythrosine presents advantages over other dyes for use in PDT, because it is already been approved for use in dentistry by the US Food and Drug Administration (FDA) to visualise dental plaque and, in addition, does not show direct toxicity to the host tissue (Carrera *et al.*, 2016; Ganesan *et al.*, 2011; Allaker and Douglas, 2009; Wood *et al.*, 2006; Arnim, 1963). However, safety concerns of erythrosine were raised by the FDA in 1990 following publication of a report showing that, under experimental conditions, erythrosine at high dose levels (4% in the diet) can induce thyroid carcinogenicity in rats (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2011). Therefore, their response to this was to withdraw erythrosine permission to use in cosmetics and externally applied drugs, but it can be used in coloured foods and ingested drugs without any restrictions (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2011). In the EU, erythrosine is commonly used in pharmaceuticals and cosmetics and, in addition, it is permitted as a food colouring substance for certain uses such as cocktail cherries (EFSA Panel on Food Additives and Nutrient Sources Added to Food, 2011). The established Acceptable Daily Intake

(ADI) of erythrosine by the Joint FAO/WHO Expert committee on Food Additives (JECFA) in 1990 and the EU Scientific Committee for Food (SCF) in 1989 is 0-0.1mg/kg bodyweight per day. The EFSA Panel on Food Additives and Nutrient Sources Added to Food (2011) has re-evaluated the evidence of erythrosine from available studies and concluded that it is neither genotoxic nor mutagenic, which is consistent with previous evaluation of erythrosine by JECFA and SCF. Therefore, erythrosine is still being used in dentistry as a measure of dental plaque in either a disclosing solution form or as tablets.

As stated earlier, several studies have demonstrated erythrosine's antimicrobial property as well as its light absorbing property and the ability to initiate photochemical reactions. This is discussed in more details in the following section.

Erythrosine antimicrobial property

Numerous *in vitro* studies had been conducted to demonstrate the antimicrobial activity of erythrosine. Baab *et al.* (1983) found that erythrosine has a bactericidal effect and it inhibits the growth of both Gram-positive and Gram-negative oral bacteria when using different standard laboratory tests such as disc-diffusion susceptibility, agar-dilution susceptibility, and bactericidal testing. Similarly, Marsh *et al.* (1989a) also reported that erythrosine has a marked antimicrobial activity against a number of Gram-positive and Gram-negative oral bacteria. Whereas, Begue *et al.* (1966) and Caldwell and Hunt (1969) found that erythrosine inhibits only the Gram-positive bacteria and, additionally, Begue *et al.* (1966) suggested that the effect was bacteriostatic rather than bactericidal, which means that erythrosine inhibits the growth of bacteria rather than killing them. However, these studies determined the antimicrobial activity of erythrosine using only the disc-diffusion susceptibility test, and many of the bacteria tested were not representative of dental plaque.

These studies demonstrated only the antimicrobial property of erythrosine. However, erythrosine also has the property of absorbing light, and the ability to initiate photochemical reactions.

Erythrosine light absorbing property and PDT

Wood *et al.* (2006) compared the cell killing efficacy of three photosensitizers- erythrosine, photofrin, and methylene blue (MB) in *S.mutans* biofilms formed *in vitro* with application of 15min continuous light using a white light source. The study found that erythrosine was more effective at killing biofilm bacteria than the other two photosensitizers. Similarly, Lee *et al.* (2013) have evaluated PDT effect on *in vitro* formed *S.mutans* biofilms as well, when using the combination of erythrosine (20μM) and a standard dental halogen curing light. The results revealed a significant cell death of the bacteria.

In de Carvalho Goulart *et al.* (2010) PDT study, methylene blue and erythrosine's cell-killing efficacy were compared on planktonic and biofilm cultures of *Aggregatibacter actinomycetemcomitans* (A.a) *in vitro* using a resin photopolymeriser as a light source. Their results showed that erythrosine is more efficient at killing these bacteria in both cultures compared to methylene blue.

Moreover, a recent review of 18 articles (Nagata *et al.*, 2012) that were published between 1992 and 2010, related to PDT and dental caries concluded that erythrosine is the most suitable photosensitizer in killing Gram-positive bacteria (*S.mutans* group), since it has a hydrophilic property that makes it permeable to the bacterial cell wall and also it has a photodynamic effect even at very low concentrations.

a) Erythrosine concentration

As previously stated, there are several factors that influence the photo-damage, including the type, dose, incubation time and localisation of the photosensitizer, the availability of oxygen, the wavelength of light (nm), the light power density (mW/cm^2) and the light energy fluence (J/cm^2) (Soukos and Goodson, 2011; Konan *et al.*, 2002). Therefore, in terms of the photosensitizer properties, it is important to test and select a dose/concentration that is effective in bacterial killing and at the same time non-toxic to host cells.

For plaque disclosure, erythrosine is used at concentrations of 9-25mM (0.72-2%), which is much higher than that used in several PDT studies (Metcalf *et al.*, 2006; Wood *et al.*, 2006; Marsh *et al.*, 1989a). Different studies have used different concentrations of erythrosine ranging from 2-220μM (Table 3). Tahmassebi *et al.* (2015) found that the most effective erythrosine concentration, among 11, 22, 88, 220μM, was 220μM, resulting in a 98% reduction of the total bacterial counts on a 14-days *in vivo* formed biofilms. This confirmed the fact that PDT's cytotoxic effect is dose-dependent, where the higher the concentration of photosensitizer used, the more the damage to target tissues is expected (Tahmassebi *et al.*, 2015; Konopka and Goslinski, 2007). Therefore, in the present study, 220μM erythrosine concentration was used.

b) Incubation time with photosensitizer prior to irradiation (pre-irradiation time)

Various PDT studies have used different protocols including varying the pre-irradiation time with the photosensitizer. Some protocols that have used erythrosine as a photosensitizer, have used either 5min (Pereira *et al.*, 2013; Rolim *et al.*, 2012; Chibebe Junior *et al.*, 2010) or 15min incubation times

(Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006) where the bacterial solutions were left in the dark.

Pereira *et al.* (2013) and Chibebe Junior *et al.* (2010) evaluated the effects of PDT, using erythrosine and LED, on the viability of *S.mutans* and *S.sanguinis* biofilms, in the former study, and planktonic cultures of *S.mutans,* in the latter study. Both studies found significant reduction in bacterial count. Similarly, Rolim *et al.* (2012) noted a significant reduction in planktonic cultures of *S.mutans* count using erythrosine and photopolymerizer light.

Likewise, the studies that have used 15min incubation time have also reported a significant reduction in bacterial counts that are either formed *in vivo* or in *vitro* using erythrosine and a tungsten filament lamp (Table 3) (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006).

c) Continuous light *vs.* fractionated light

Metcalf *et al.* (2006) revealed that application of fractionated light to *S.mutans* biofilms grown *in vitro* has an increased cell-killing efficacy compared with continuous irradiation at the same overall light dose, when using erythrosine as a photosensitizer . The authors demonstrated that most of the cell killing occurs in the first 5min of irradiation, and for time periods longer than 5min the amount of killing is slowly increased, which they thought was probably due to photo-bleaching of erythrosine. Therefore, they fractionated the 5min irradiation period by two regimes. The first regime was five times 1-minute light pulses with 5min recovery periods between pulses, giving a total treatment time of 30min. The second regime was ten times 30-seconds light pulses with 2min recovery periods, giving a total treatment time of 25min. Both regimes were superior, in bacterial count reduction, to continuous irradiation times of up to 30min. The authors thought that the reason for this was the re-oxygenation for the excited photosensitizer or due

to the general replenishment and rearrangement of the erythrosine during the dark periods.

Additionally, Tahmassebi *et al.* (2015) have compared various irradiation regimes on erythrosine treated biofilms formed *in vivo*. In their study, continuous light of 2, 5 and 15min and fractionated light of five times 1-min pulses separated by dark periods of 2min (total treatment time=15min) were applied. Among these, both the 15min continuous light and the fractionated regime were most effective in bacterial killing.

Table 3 PDT studies on dental bacteria using erythrosine

Author/s	Microorganism	Photosensitizer	Light source
Metcalf *et al.* (2006)	*In vitro* formed *S.mutans* biofilms	Erythrosine (22μM)	Tungsten filament lamp (white light) 500-550nm
Wood *et al.* (2006)	*In vitro* formed *S.mutans* biofilms	Erythrosine, Photofrin, MB (22μM)	Tungsten filament lamp (white light) 500-550/600-650nm
de Carvalho Goulart *et al.* (2010)	*In vitro A.a* (planktonic and biofilm cultures)	Erythrosine, MB (0.5 & 1μM)	Photopolymeriser
Chibebe Junior *et al.* (2010)	*In vitro S.mutans* planktonic cultures	Erythrosine, Rose Bengal (2μM)	LED 440-460nm
Rolim *et al.* (2012)	*In vitro S.mutans* Planktonic cultures	Erythrosine, eosin Rose Bengal, Malachite green, MB, TBO (163.5μM)	Photopolymeriser 570nm LED 636nm
Lee *et al.* (2013)	*In vitro* formed *S. mutans* biofilms	Erythrosine (20μmol/L)	Dental halogen curing light
Pereira *et al.* (2013)	*In vitro* formed *S.mutans* and *S.sanguinis* biofilms	Erythrosine Rose Bengal(5μM)	LED (455±20nm)
Tahmassebi *et al.* (2015)	*In vivo* formed dental plaque biofilm	Erythrosine (11, 22, 88 & 220μM)	Tungsten filament lamp (white light) 500-550nm

1.2.8 Investigating the effect of PDT on dental plaque biofilms

A number of research techniques have been used to study the effect of PDT on dental plaque biofilms, such as:

1.2.8.1 Microbiological culture analysis

As stated earlier, several studies have investigated the effect of PDT on either *in vitro* growing oral bacteria in planktonic and/or biofilm cultures or on natural undisturbed oral plaque biofilms formed *in vivo.* In order to do so, oral bacteria require specific growth media, defined atmospheric conditions and incubation times (Kilian *et al.*, 2016). Therefore, bacterial viability was investigated by plating the serially diluted biofilm samples on different types of media for aerobic and anaerobic bacteria, such as Colombia blood agars (CBA), following incubation of these bacterial samples with various concentrations of the photosensitizer and then irradiated with light. After this, the agar plates are incubated at 37^0C for 48 hours to count the surviving cells.

These cultural analysis have been the gold standard for microbiology as it provided an understanding on the properties and ability of an organism, however, the knowledge of the diversity and architecture of the oral microbiome has been provided by culture-independent approaches, such as microscopy techniques (Kilian *et al.*, 2016).

1.2.8.2 Microscopy techniques

Various microscopy techniques have been used to investigate the architecture, composition, physiology and viability of dental biofilms (Surman *et al.*, 1996). Transmission electron microscopy (TEM) and Scanning electron microscopy (SEM) have been widely used to image biofilms as they provide high resolution and magnification images (Lawrence *et al.*, 2003). In

addition, TEM provides detailed information in bacterial structure in terms of its cell wall and intracellular material (Dige *et al.*, 2007). However, these techniques require dehydration and fixation of samples prior to observation, leading to sample distortion and artefacts (Wood *et al.*, 2000). On the other hand, when using CLSM, samples can be visualised in their natural intact hydrated state, which means that samples are free from preparation artefact (Marsh *et al.*, 2011; Wood *et al.*, 1999). CLSM provides detailed compositional information such as differentiating between different morph-types of bacteria, like cocci, rods and filaments, especially when used in combination with fluorescent probes (Dige *et al.*, 2007; Lawrence *et al.*, 2003). Moreover, CLSM has the advantage of scanning very thin sections throughout the depth of the plaque biofilm without out-of-focus blurring and, also, the digitalized data can be reconstructed to provide three-dimensional images of the biofilm sample (Wood *et al.*, 2000). For example, when using CLSM to visualise dental plaque formed *in vivo* on enamel slabs (Leeds *in situ* device- see section1.3.1) over a 4-day period it had showed that plaque samples exist with heterogeneous architecture in terms of cells, matrix, and fluid-filled pores and channels that extended throughout the entire thickness of the biofilm sample (Wood *et al.*, 2000). Thus, CLSM has improved our insight into the architecture of the oral biofilms (Marsh *et al.*, 2011).

Moreover, CLSM has been used to determine the effect of antimicrobial agents on bacterial viability in dental biofilms by using fluorescent dyes to distinguish live and dead bacteria depending on their cytoplasmic membrane permeability. In addition, CLSM provides an insight on the spatial distribution of bacterial populations in a dental biofilm (Netuschil *et al.*, 1998).

It has been shown that no single microscopic technique has comprehensive application, but used in combination they can supplement each other to achieve a realistic representation of biofilm structure and dynamics (Surman *et al.*, 1996). Wood *et al.* (1999) used both TEM and CLSM to visualise the effect of PDT on *in vivo* formed plaque biofilms for 7-days. TEM showed marked damage to bacterial cells following PDT and the most evident

damage was the vacuolation and condensation of the cytoplasm and membrane damage. While CLSM showed that biofilm samples were around half the thickness of the controls and this has been reported to be due to damage of bacterial membranes, as evident in TEM, which lead to reduced cell-to-cell or cell-to-matrix binding and subsequent loss of bulk of biofilm.

1.3 Dental plaque collection techniques

The great knowledge about the dental plaque biofilm structure and characteristics was obtained by development of *in vitro* biofilm models and by collection and examination of biofilm samples. There are several plaque-sampling techniques that have been used in different studies, including simple plaque scraping off the tooth surfaces using sterile dental instruments (Fontana *et al.*, 2009; Arweiler *et al.*, 2006; Petersson *et al.*, 2002) and paper points (Charles *et al.*, 1999; Sjögren *et al.*, 1996). However, these mechanical techniques disturb the biofilm structure, as these biofilms are very delicate and fragile (Wood *et al.*, 2000). Similarly, *in vitro* formed biofilms also have limitations, such as having a limited number of species and having a composition as well as a structure that is not comparable to those *in vivo* (Watson *et al.*, 2005). Therefore, there was a need to develop models to collect dental plaque with no distortion to overcome these limitations (Wood *et al.*, 2000), as this analysis of undisturbed human dental plaque biofilms has been considered as the best method for studying the architecture and physiology of biofilm formation on dental materials and, also the effect of antimicrobial therapies on the biofilm structure (Tomás *et al.*, 2010). This can be achieved through the use of *in situ* plaque generator devices.

Several designs of *in situ* plaque generator devices have been used in different studies on dental biofilm, such as buccal (Tahmassebi *et al.*, 2015; Arweiler *et al.*, 2004; Wood *et al.*, 2000), lingual (Re *et al.*, 2010) or palatal

(Teixeira *et al.*, 2012; Lima *et al.*, 2009; Auschill *et al.*, 2005) devices (Table 4). Among these, buccal devices were the most commonly used for the study of *in situ* biofilm, since other devices had some design drawbacks (Prada-López *et al.*, 2016); The collected biofilm on these devices was disturbed as it was exposed to contact with the tongue (Prada-López *et al.*, 2016), even though some authors have designed protections, such as applying a plastic mesh in the palatal devices (Cury *et al.*, 2001).

In general, buccal devices can be classified to either fixed or removable devices.

1.3.1 Fixed devices

Fixed devices such as the Leeds *in situ* device, which was developed at the University of Leeds, has been used in many studies (Robinson *et al.*, 2006; Wood *et al.*, 2000; Wood *et al.*, 1999; Robinson *et al.*, 1997). It consists of a nylon ring attached to human enamel slab and then bonded to the tooth surface by composite resin, allowing the accumulation of plaque *in vivo* (Table 4) (Wood *et al.*, 1999; Robinson *et al.*, 1997). The device was placed at buccal surfaces of posterior teeth and left *in situ*, and then removed after a specified period of time without disturbing the bacterial plaque and also without the need to stop oral hygiene (Robinson *et al.*, 1997). However, care must be taken when brushing to ensure that the accumulated biofilm remains undisturbed (Wood *et al.*, 2000). In addition, careful de-bonding of the appliance and removal of the composite resin from the tooth surface was required to avoid damaging the enamel of the tooth (Prada-López *et al.*, 2016). Another example of a fixed device is the sub-gingival plaque carrier, which was developed by Wecke *et al.* (2000). It consists of a gold foil or polytetrafluoroethylene (e-PTFE) membranes that were attached to a plastic carrier for placement at the bottom of the periodontal pocket. The carrier was fixed sub-gingivally to the tooth surface by using cyanoacrylic glue (Table 4).

The carrier was left *in situ* for 3-6 days and then was removed for the purpose of sub-gingival plaque biofilm sampling.

1.3.2 Removable devices/appliances

Various designs and materials of removable appliances have been used in different studies of oral biofilms (Table 4). For example, acrylic appliances (Nascimento *et al.*, 2014; Diaz *et al.*, 2006), acrylic and metal appliances (Tahmassebi *et al.*, 2015; Arweiler *et al.*, 2014; Auschill *et al.*, 2005), metal appliances (Simion *et al.*, 1997), thermoplastic appliances (Hannig *et al.*, 2013), and disk-holding splints (Prada-López *et al.*, 2016; Quintas *et al.*, 2015). In the literature, specific limitations of the above mentioned appliances have been demonstrated, such as bulky acrylic appliances that may affect the phonetics and aesthetics of the volunteers, costly and complex laboratory fabrication of metal appliances, and inability to chew with the thermoplastic and disc-holding appliances as they cover the occlusal surfaces of the teeth (Table 4) (Prada-López *et al.*, 2016). However, removable appliances, in contrast to fixed appliances, has the advantage of allowing the volunteers' to perform their daily oral hygiene measure without disturbing the growth of plaque biofilms (Prada-López *et al.*, 2016).

Furthermore, different substrates, which are attached to the removable appliances, for the accumulation of *in situ* oral biofilm have been used in different studies, including human enamel slabs (Tahmassebi *et al.*, 2015; Teixeira *et al.*, 2012; Diaz *et al.*, 2006; Cury *et al.*, 2001; Palmer *et al.*, 2001), bovine enamel slabs (Arweiler *et al.*, 2014; Hannig *et al.*, 2013) and glass slabs (Tomás *et al.*, 2010; Auschill *et al.*, 2005; Arweiler *et al.*, 2004). From these, the most commonly used substrate was the human enamel slabs, as it is similar to the natural tooth surface (Prada-López *et al.*, 2016). This is probably be due to the fact that artificial slabs compromises bacterial adhesion and colonisation, which is associated to surface roughness and free energy (Robinson *et al.*, 1997). However, Netuschil *et al.* (1998) found

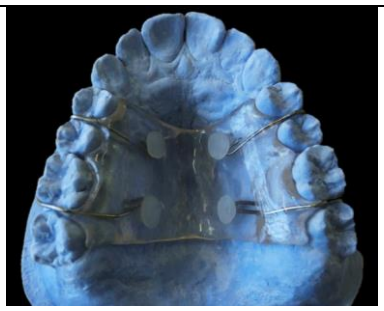
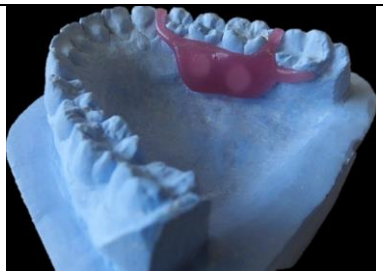
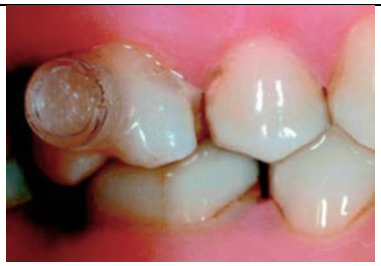
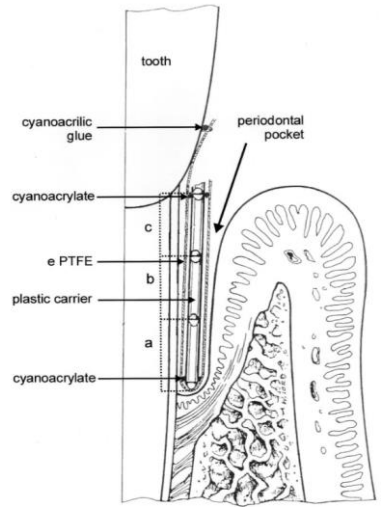
no great difference, when using enamel or glass slabs, in the 48-hour biofilm thickness.

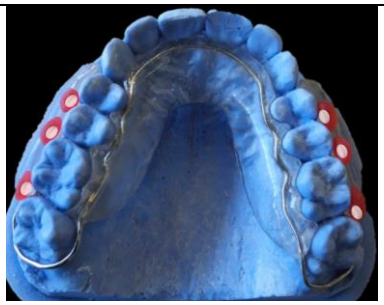
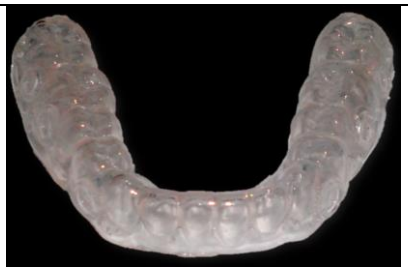
These slabs are placed internally of each buccal flange of the appliance, in such a way that biofilm growth is protected and not disturbed by the tongue and the cheeks, depending on the design of a specific framework (Prada-López *et al.*, 2016).

In terms of the location of the appliance in the oral cavity (maxillary buccal region versus mandibular buccal region) or the position of the slabs in the appliance (right versus left; distal versus mesial), researchers have reported that there was no significant difference in the mean thickness of the accumulated biofilm nor the mean bacterial viability values or their pattern in 48-hour biofilm (Arweiler *et al.*, 2004; Auschill *et al.*, 2004) and 14-days biofilm (unpublished data-Tahmassebi *et al.* (2015)).

An example of the removable appliance is the one developed by Koulourides and Volker (1964), which was introduced to study dental caries on enamel slabs *in situ*. It consists of a labial arch wire, two acrylic buccal flanges extending from first premolar to first permanent molar area, and two U metal clasps attached to the first permanent molars. The slabs are placed in wells of the buccal acrylic flanges at the same level with that of the acrylic, and are covered with gauze to encourage plaque formation (Koulourides and Chien, 1992). Later studies have modified this appliance by eliminating the need to use gauze to allow more comprehensive study of plaque architecture (Robinson *et al.*, 1997).

Table 4 Examples of *in situ* devices

• **Palatal acrylic and metal device** (Prada-López *et al.*, 2016)	
• **Lingual acrylic device** (Prada-López *et al.*, 2016)	
• **Buccal devices:** - **Fixed Leeds *in situ* device** (Pessan *et al.*, 2008)	
- **Fixed sub-gingival plaque carrier** (Wecke *et al.*, 2000)	
- **Removable acrylic device** (Prada-López *et al.*, 2016)	

- **Removable acrylic and metal device** (Prada-López *et al.*, 2016)	
- **Removable metal device** (Simion *et al.*, 1997)	
- **Removable thermoplastic and polysiloxane splint** (Prada-López *et al.*, 2016)	
- **Removable Disc-holding splint** (Prada-López *et al.*, 2016)	

1.4 Conclusion of literature review

From the preceding literature, the *in vitro* use of erythrosine-based PDT in the management of oral plaque bacteria, growing in planktonic and/or biofilm cultures *in vitro*, as well as on *in vivo* formed biofilms seems to be encouraging (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006). However, to our knowledge there were no reports in the literature that have compared the efficacy of different incubation times with photosensitizer prior to irradiation. Therefore, in order to further study the bactericidal effect of erythrosine-based PDT, this study was conducted aiming to reduce the overall treatment time for PDT by investigating the efficacy of different incubation times and irradiation times in bacteria killing to enhance its clinical

usefulness. In addition, CLSM was used to visualise the PDT effect on *in vivo* formed biofilms.

1.5 Aims and objectives

The aim of the present study was two-fold. Firstly, to investigate, *in vitro*, the PDT effect on *Lactobacilli casei* spp. in planktonic form using different incubation times, including 2, 5 and 15min, and determine, among these, the most effective bactericidal incubation time . Secondly, to investigate the PDT effect on *in vivo* formed biofilms using different incubation and irradiation times. In addition, to investigate and visualise the bacterial viability (Live/dead cells) of *in vivo* formed biofilms using CLSM for both control and PDT treated groups.

The objective of the *in vitro* study is:

- Determine the most effective bactericidal incubation time of erythrosine-based PDT (220μM), using a tungsten filament lamp (white light), on *L.casei* spp. in planktonic form.

The objectives of the *in situ* study are:

- Determine the most effective *in vitro* bactericidal incubation time and irradiation time of erythrosine-based PDT (220μM), using tungsten filament lamp (white light), on *in vivo* formed dental plaque biofilms.

- Investigate and visualise the bacterial viability (Live/dead cells) of *in vivo* formed dental plaque biofilms using CLSM for both control and PDT treated groups.

1.6 Null hypothesis

- There is no significant difference in the total viable bacterial counts among different incubation and irradiation times.

Chapter 2
Materials and methods

2.1 Study design

This study has two-phases, an *in vitro* phase and *in situ* phase (Figure 8).

2.1.1 *In vitro* phase:

The aim of this phase was to determine the most effective incubation time (2, 5 or 15min) for erythrosine-based PDT (220μM), using a 400W tungsten filament lamp, that would significantly reduce *L.casei.* viable counts. Different incubation times were compared to investigate whether there was any difference in the bacterial viability among them; attempting to reduce the overall treatment time for PDT. The amount of bacterial killing obtained would then allow us to identify the best incubation time to use for the 2nd arm of the following *in situ* phase of the study.

2.1.2 *In situ* phase:

The *in situ* phase was a single centre, randomised with two arms design (Figure 8). The aim of this phase was to determine the most effective incubation time between a 15min time (Arm-1) that was based on the pilot study by Tahmassebi *et al.* (2015) and an incubation time (Arm-2) that was based on the initial *in vitro* part, for erythrosine-based PDT, using tungsten filament lamp, that would significantly reduce total viable bacterial counts of *in vivo* formed dental plaque biofilms among control and treatment groups. To determine also the most effective irradiation time, among 15min continuous light and fractionated light of 30sec light pulses for 5 times separated by 1min dark periods, for erythrosine-based PDT (220μM), using the tungsten filament lamp, that would significantly reduce total viable bacterial counts of *in vivo* formed dental plaque biofilms among control and

treatment groups. In addition, to investigate bacterial viability (Live/dead cells) of *in vivo* formed dental plaque biofilms using CLSM for both control and PDT treated groups.

2.2 Ethical approval

This study was based on a previous pilot study entitled “A pilot *in situ* study of a new method for the control of plaque biofilms via a photoactive solution” by Drogkari, E. as a part of her MDentSci dissertation, University of Leeds, 2011 (Tahmassebi *et al.*, 2015).

Ethical approval was sought and obtained from the National Research Ethics Service (NRES) committee of South Central – Berkshire B (REC reference number: 14/SC/1226 – Appendix A). Following this, the study received approval from the Leeds Research and Innovation (R&I) committee in order for it to be conducted at the Leeds Teaching Hospitals NHS Trust (LTHT R&I number: DT14/11310 (149271/WY)- Appendix B).

Summary of this study was registered online in a publicly accessible database (NHS Health Research Authority) before subject recruitment. The study investigator (AA) ensured that this study was conducted in full conformance with the laws and regulations of the country in which the research was conducted and as per the World Medical Association Declaration of Helsinki.

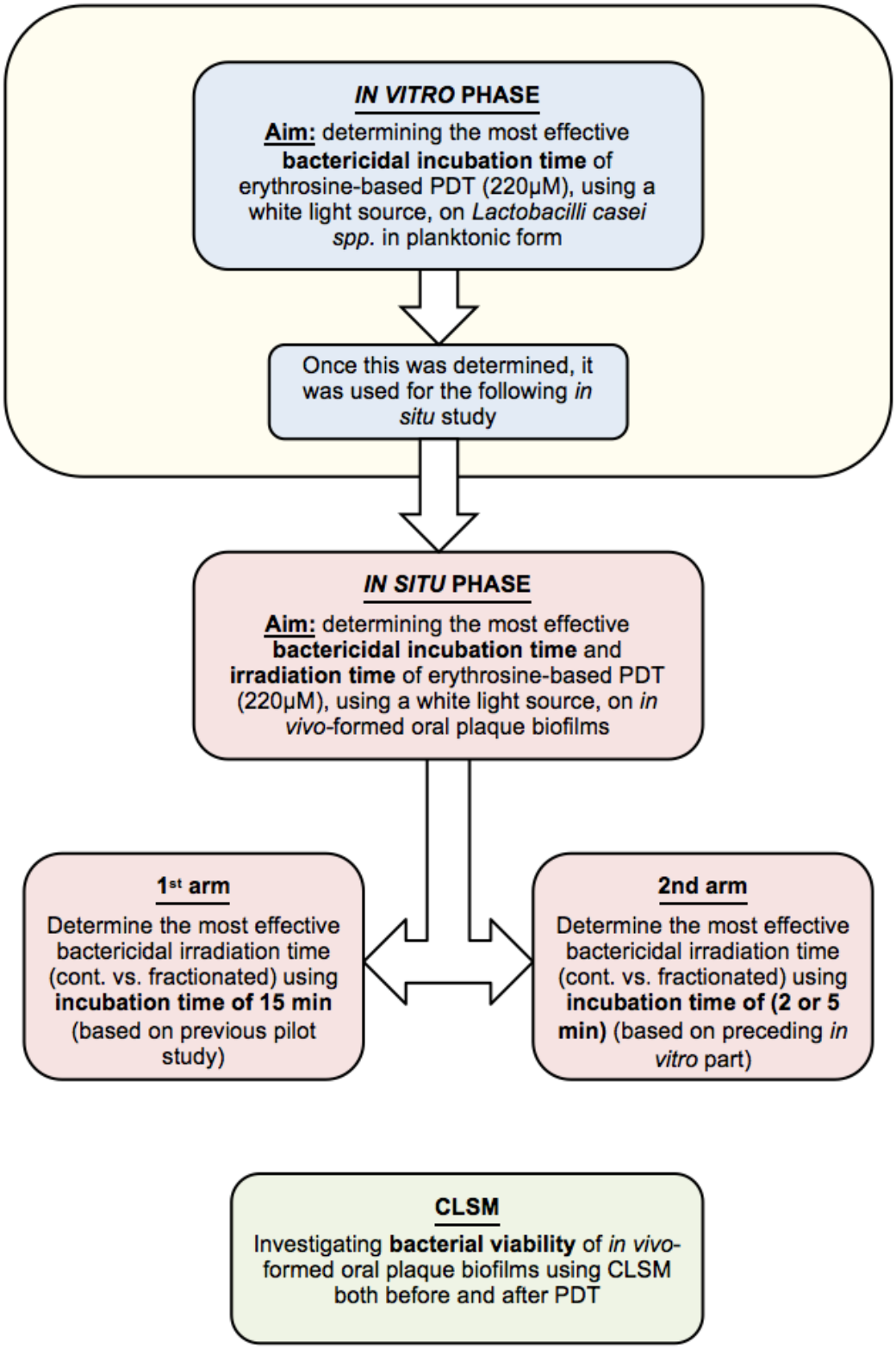


Figure 8 Study design

2.3 Training and calibration

The study investigator (AA) was trained and calibrated for the use of aseptic microbiological procedures in microorganisms' cultures, preparation of study solutions and dilution series, making CBA plate, counting bacterial colonies, using microscopy and CLSM, preparation of enamel slabs and making *in situ* appliances prior to commencement of the study and prior the usage of these facilities or materials.

2.4 Materials

2.4.1 Materials used in the clinic

- Dental examination kit
- High and low-speed hand-pieces
- Straight hand-piece
- Diamond, Acrylic, and Polishing burs
- Rubber cups
- Air-scaler and tips
- Prophy-paste (3M ESPE Clinpro™, USA)
- Graduated saliva sample tubes (Corning, Sigma-Aldrich, Germany)
- Digital Timer (TruLab®, Philippines)
- Alginate impression (Xantalgin®- Heraeus Kulzer, Germany)
- Impression trays (Ash polytrays - DENTSPLY, UK)
- Pressure indicator paste (Mizzy paste- Keystone, USA)
- Fix-tray adhesive (DENTSPLY, UK)
- Sterile gauze
- Ortho retainer box (Henry Schein, USA)
- Sticky wax (Bredent, Germany)

2.4.2 Materials used in the laboratory

- Enamel slabs from intact human premolars and permanent molars, inserted in experimental acrylic and metal removable appliances
- *Lactobacillus casei* (2104A)
- Sterile Pipette tips (Star lab, UK)
- Sterile loops (5µl**)**, spreaders (Star lab, UK)
- Sterile Stripette (Costar, Sigma-Aldrich, Germany)
- Sterile Glass beads (3mm) (Sigma-Aldrich, Germany)
- Sterile Universal 30 ml (Thermo Scientific, USA)
- Sterile plastic vials, Bejou tubes (Sterilin, UK)
- Universal racks
- Cuvettes (Sarstedt, Germany)
- 6-well plates (Costar, Sigma-Aldrich, Germany)
- 12-well plates (Costar, Sigma-Aldrich, Germany)
- 48-well plates (Corning, Sigma-Aldrich, Germany)
- Gilson & electric pipettes
- Petri Dish 90mm (Thermo Scientific, USA)
- Brain Heart Infusion (BHI)- (Oxoid, UK)
- Columbia Blood Agar (CBA)- (Oxoid, Basingstoke, UK)
- Horse Blood oxalated (Oxoid, UK)
- Reduced Transport Fluid (RTF)
- Erythrosine B (Sigma-Aldrich, Germany)
- Frosted glass slides (VWR, UK)
- Gram staining kit (Sigma-Aldrich, Germany)
- Petroleum jelly (Vaseline)
- Foil
- Paraffin film (Parafilm®, Sigma-Aldrich, Germany)
- Sterile wax knife
- Sterile excavator
- Sterile tweezers
- Bunsen burner

- Vortex Genie-2 (scientific industries, Inc., USA)
- 400W Tungsten filament lamp (Aurora, USA)
- Diamond Wire Saw cutting machine (Well® Walter EBNER, CH-2400 Le Loche, Germany)
- Autoclave (Prestige medical, UK)
- Water bath (Stuart, SBS40, UK)
- CO_2 incubator (Forma Direct Heat, Thermo Scientific, USA)
- Colony counter (Stuart R, SC6, UK)
- Balance (Mettler™, Thermo Scientific, USA)
- Spectrophotometer (Jenway 6305, UK)
- LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) (Molecular Probes, USA)
- CLSM (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany)

2.4.3 Microorganism

Lactobacillus casei strain (2104A) was obtained from the Microbiology laboratory stock collection, School of Dentistry, University of Leeds. It was maintained in a freezer at -80°C and it was used for the *in vitro* phase of the study. It is one of the most commonly isolated species from oral sample and it belongs to the *Lactobacillus* genus (Bagg *et al.*, 2006). These species are highly acidogenic and acid tolerant, and are associated with advanced caries lesions and carious dentine (Marsh *et al.*, 2009). It is a mesophilic bacterium (grows in moderate temperature 20-45°C) that is Gram-positive (Figure 9), rod shaped, non-sporing, non-motile, facultative anaerobe (Holzapfel *et al.*, 2001).

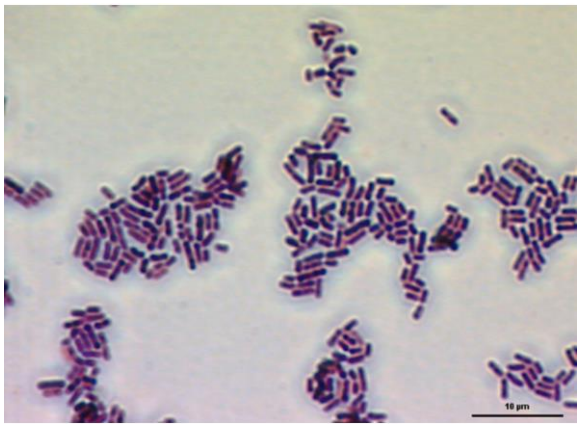


Figure 9 Gram stain of *Lactobacillus spp.*
(http://www.chsr.mmu.ac.uk/microbiology/education_and_communication/resources/images.asp#/microbiology/images/free/rev/gram_stain.jpg)

2.4.4 Erythrosine solution and Reduced Transport Fluid

Erythrosine (Molecular weight- 879.86g/mol) was prepared as a filter-sterilised 1mg/ml stock solution in Reduced Transport fluid (RTF) and stored in the dark at 4°C. This solution was then diluted to the required 220µM concentration (the concentration that was found, by the previous pilot study (Tahmassebi *et al.*, 2015), to be the most effective bactericidal concentration for PDT on *in vivo* formed oral biofilms). This diluted solution was used for all treatment groups, with PDT, in this study.

RTF consisted of 0.45gm K_2HPO_4, 0.45gm KH_2PO_4, 0.9gm NaCl, 0.1875gm $(NH_4)_2SO_4$, 0.4gm Na_2CO_3, 0.2gm dithiothreitol, 10ml of 0.1M EDTA and 1000ml of distilled water. This solution was adjusted to pH 8, filter sterilised into sterile containers and kept in the fridge until required. Ms Shabnum Rashid, Oral biology laboratory technician, University of Leeds, was responsible for its preparation. This solution was used for all the control groups in this study.

2.4.5 Light source

The tungsten filament lamp, white light source, was used to activate erythrosine (Figure 10). The lamp emitted 22.7mW/cm^2 of light intensity, in the presence of a heat-dissipating water bath, in the wavelength range 500-550nm (corresponding to the region of maximal absorption by erythrosine) (see section 1.2.6.2). Bacterial samples were placed at 30cm from the lamp and the heat-dissipating water bath was positioned between the bacterial samples and the lamp to prevent hyper-thermic effects.

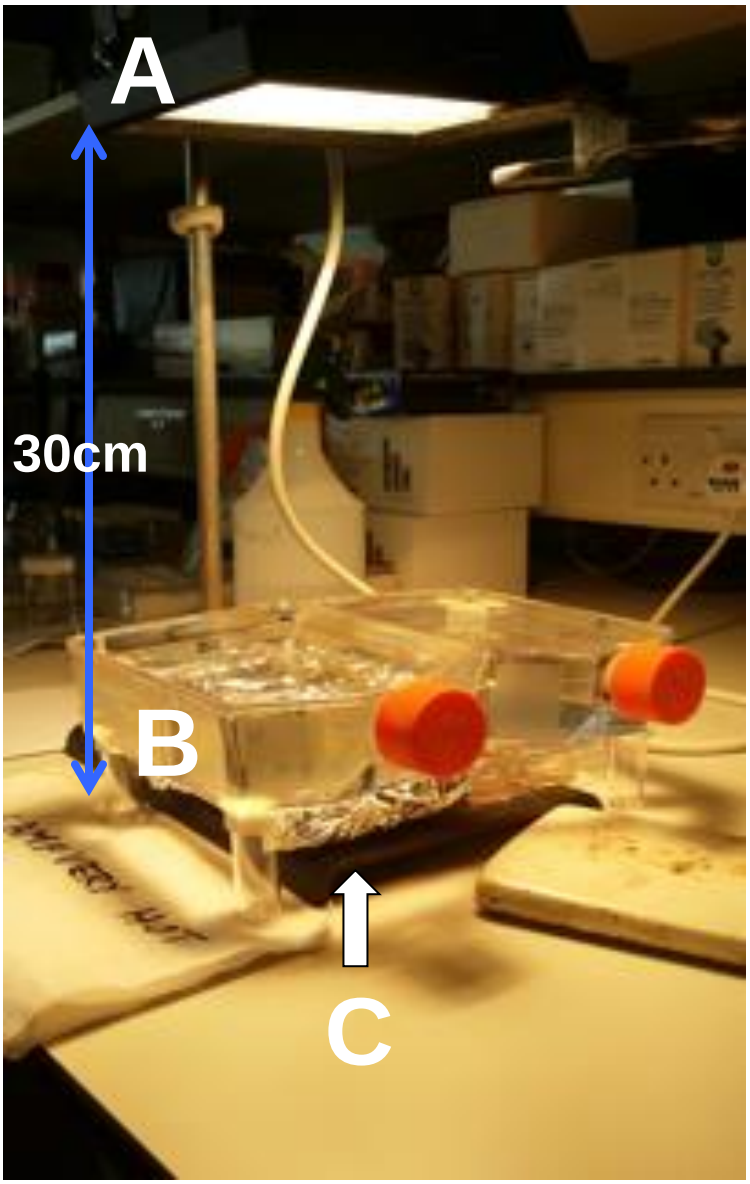


Figure 10 Light source: (A) Tungsten filament lamp (B) Heat-dissipating water bath (C) Bacterial samples in 6-well plates

2.5 *In vitro* phase

2.5.1 PDT on planktonic cultures of *L.casei*

The aim of this phase of the study was to determine the most effective bactericidal incubation time of erythrosine-based PDT by comparing the reduction of *L.casei* viable counts (CFU) among four different control and treatment groups. These four groups were as follows; a) Control 1: No erythrosine, no light (-E-L); b) control 2: +Erythrosine, no light (+E-L); c) control 3: No erythrosine, +light (-E+L); d) treatment group: +Erythrosine, +light (+E+L). The procedure of the microbiological analysis is explained in the following section.

Planktonic *L.casei* were spread on Petri dishes containing Colombia blood agar (CBA) and were incubated overnight at 37°C in a 10% CO_2 incubator. After this, gram staining was performed to check the strain purity. Following this, a loopful of the bacteria was inoculated into 20ml of Brain Heart Infusion (BHI) growth media and grown overnight at 37°C, in a 10% CO_2 incubator. Later, this overnight bacterial suspension was spectrophotometrically standardised, using a wavelength of 600nm and an optical density (OD) of ~0.5. Spectrophotometer measures the turbidity (OD), which is the measure of the amount of light absorbed by a bacterial suspension (Figure 11). The degree of turbidity is directly related to the number of microorganisms present, which indicates the cell growth rate of the organism. *L casei* growth rate at OD ~0.5 indicated that cells are in a rapidly growing and dividing state (mid log phase), therefore, all bacterial suspensions, used in following experiments, were treated with PDT once this was reached.

Next, 1ml aliquots of the bacterial standardised suspension were individually transferred to separate wells of two 6-well plates. Then, 1ml of diluted erythrosine solution (220μM) was added to the appropriate wells (+E-L and +E+L). While in the control wells (-E-L and -E+L), 1ml of RTF solution was added. Both 6-well plates were covered with foil and incubated at room

temperature for 2, 5, or 15min. After the dark incubation, the samples were irradiated under the tungsten filament lamp for 15min (+E+L) (Figure 13). To determine the effect of erythrosine alone on bacterial cells viability, some samples were covered with foil (+E-L). Additionally, to determine whether irradiation alone had any effect on bacterial cell viability, some samples were irradiated without erythrosine (-E+L). The samples that were not exposed to both light (covered with foil) and erythrosine acted as control (-E-L). To emphasize, the samples with no irradiation (-E-L and +E-L) were kept in dark by covering them with foil in the same duration as the light exposure time (15min). Ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were then produced from the bacterial suspensions of the control and treatment groups. To determine the number of bacteria in each sample, each dilution (100µl) was plated in triplicate on CBA growth medium. The plates were then incubated for 48hr at 37°C in a humidified 10% CO_2 incubator. After incubation, bacterial counts of each plate were performed, using a colony counter (Figure 12), and then the number of colony-forming units per millilitre (CFU/ml) was calculated from the mean count of each triplicate. Percentage reduction of bacterial counts was obtained from the different groups with different incubation times (Figure 13).

All experiments, with 2, 5, or 15min incubation times, were performed three times at different time points and, in addition, in triplicate samples. This is to minimize the bias related to microbiological procedures and also to validate and increase the reliability of the results.

Figure 11 Spectrophotometer (Jenway 6305, UK)

Figure 12 Colony Counter (Stuart R, SC6, UK)

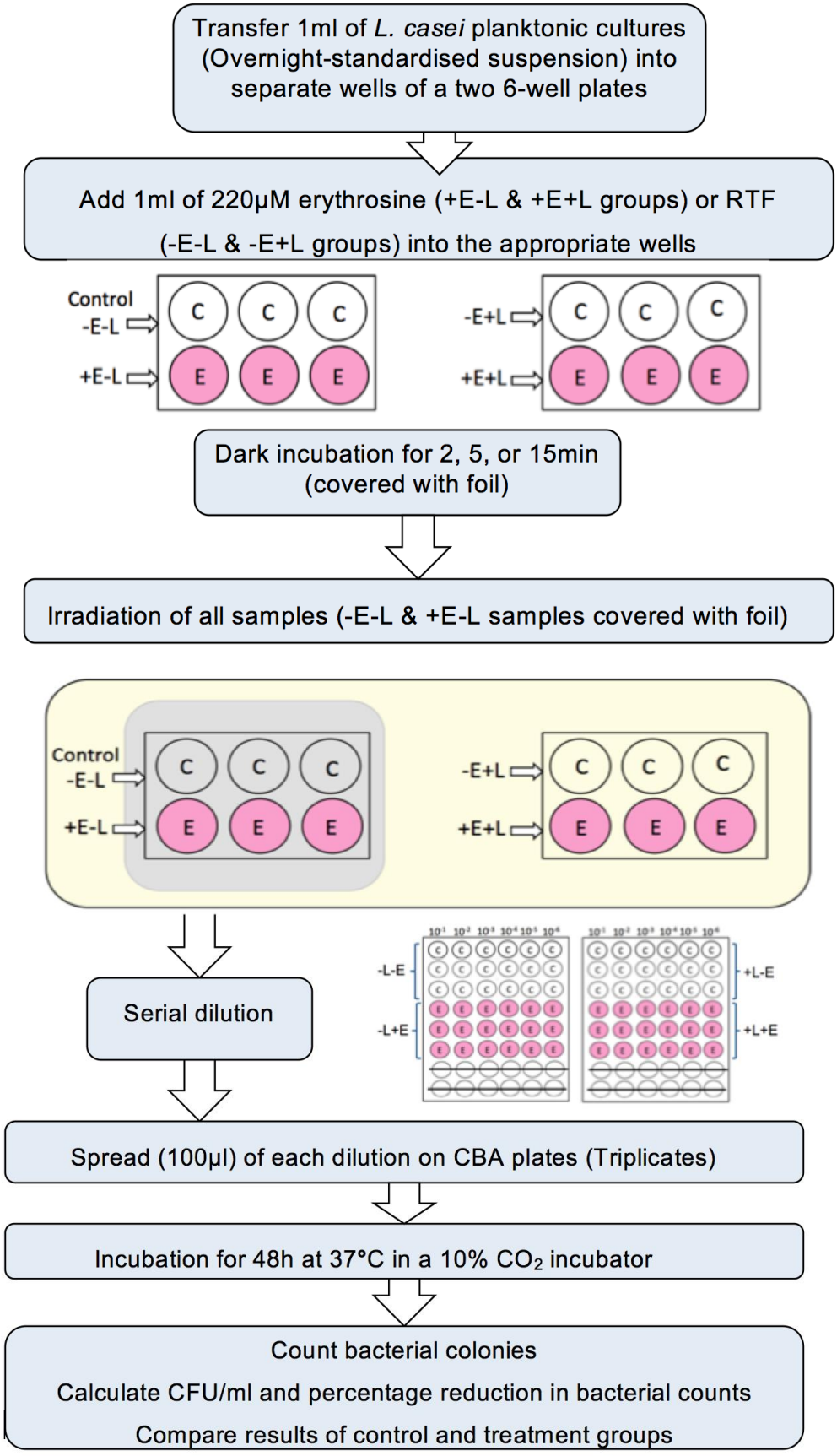


Figure 13 Protocol of *in vitro* PDT on planktonic cultures of *L. casei* bacteria

2.6 *In situ* phase

The *in situ* phase was a single centre, randomised with two arms design. The study was designed, conducted and reported according to GDC guidelines for good clinical practice.

2.6.1 Recruitment and selection of participants

In total, 18 healthy participants were recruited at the Leeds School of Dentistry. The sample size was determined with the statistician's help with performing the power calculation (see section 2.6.4). Recruitment was through poster advertisement at the University of Leeds (Appendix D). The chief investigator (AA) was the person responsible for the recruitment of the participants. An informed consent (Appendix E) was obtained from each participant prior to the commencement of the study. An explanatory information sheet (Appendix F) was also provided to each participant. Participants recruited in the study had to fulfil the following inclusion and exclusion criteria:

2.6.1.1 Inclusion criteria

- 18 years old and older.
- Both males and females.
- Medically fit and healthy.
- Dental examination:
    - Presence of at least 20 natural teeth.
    - Free from visual signs of untreated caries, periodontal disease, or any other dental/oral health conditions that could be exacerbated by the study interventions.
    - DMFT ≥ 1.
    - Salivary flow rate ≥ 0.25ml/min.

- Able to fully understand the procedures and restrictions and willing and likely to comply, as evidenced by voluntary written informed consent.

2.6.1.2 Exclusion criteria

- Disease:
  - Disease or recurrent disease that could affect the oral cavity or interfere with the dental examination and/or wearing of oral appliance.
  - Severe psychiatric, physical and medical disorders requiring treatment or making the participant unlikely to give informed consent or to cope with the procedures required by the study protocol.
- Medication:
  - Antimicrobial therapy within 14-days prior to screening or during the study.
  - Antibiotic treatment within 28-days prior to screening or during the study.
  - Use of medication affecting salivary flow.
- Dental history and examination:
  - Dental disease requiring treatment in the short or long term.
  - Oral surgery or extraction 6-weeks prior to screening of the study or during the study.
  - Wearing of prostheses or orthodontic appliances that could affect the study procedures.
- Others:
  - Pregnant women or intending to become pregnant or lactating
  - Smokers.
  - Known or suspected intolerance/ hypersensitivity to study materials closely related to compounds or ingredients that will be used in the study.

- Participation in another clinical study within 30-days of screening and during the study.

2.6.2 Pre-operative screening and visits record

After participants were consented to take part in the study, the chief investigator (AA) screened the participants for fulfilment of the inclusion and exclusion criteria. The screening included medical and dental history, dental examination, and measurement of the salivary flow rate. A referenced salivary flow rate (a whole un-stimulated saliva ≥ 0.25ml/min) was measured, by passive drooling into graduated test tube for 5min, to ensure that a standard remineralisation effect of the saliva of all participants was achieved. After that, dental scaling and polishing was performed for the participants, when required, by the chief investigator, prior to taking impressions to construct a lower removable appliance. The participants were instructed to wear their appliances at all times for two separate periods of two weeks each (total of four weeks), except when eating, drinking, and tooth brushing. At these times, participants had to place the appliance in damp gauze inside a plastic case that was provided to them, to prevent any drying of the accumulated plaque. Additionally, instructions and restrictions form was provided to all participants at appliance fitting visit (Appendix G). A case record form (Appendix H) was used to record the visits, clinical findings and side effects of each participant.

2.6.3 Participants' withdrawal criteria

Participants had the right to withdraw from the study at any time and for any reason. The investigator also had the right to withdraw subjects from the study in the event of inter-current illness, adverse events after a prescribed procedure, protocol deviations, administrative reasons or other reasons. It was understood by all concerned that an excessive rate of withdrawal of

subjects could render the study underpowered; therefore, unnecessary withdrawal of subjects was avoided.

2.6.4 Sample size determination

Statistical advice was sought from Leeds university statistician (Dr Jing Kang) and the sample size was calculated by using data from the previous pilot study (Tahmassebi *et al.*, 2015). Assuming a standard deviation of 1.11, effect size 1.8, significance level 0.05 and power 80%, it was found that the study requires at least 3-slabs per group. After adjusting for multiple comparisons by lowering the significance level (alpha=0.003), the power calculation resulted in a sample size of at least 5-slabs per group.

However, 18 participants were screened to ensure a sufficient sample size that would successfully complete the study. Among these, samples from 3 subjects were used for CLSM analysis and the rest for the microbiological analysis. Each participant were provided with a lower removable appliance containing 6 enamel slabs. The study consisted of two arms and each arm lasted 2-weeks.

2.6.5 Laboratory methods

2.6.5.1 Teeth selection

Approval from the Leeds Dental Institute Tissue Bank was obtained (reference number 270314/AA/128 – Appendix C) for collection of teeth fulfilling the following inclusion and exclusion criteria:

Inclusion criteria

- Sound (i.e. free from signs of decay and restorations) upper and lower premolars and permanent molars.

Exclusion criteria

- Carious upper and lower premolars and permanent molars.
- Upper and lower premolars and permanent molars with signs of trauma, erosion, restorations and malformations.

2.6.5.2 Enamel slabs

Enamel slabs were prepared from teeth fulfilling the inclusion and exclusion criteria mentioned above.

2.6.5.2.1 Preparation of enamel slabs

Enamel slabs were cut from human premolars and permanent molars extracted for orthodontic or periodontal reasons at school of Dentistry and were stored in a solution of distilled water and 0.1% thymol (Sigma Aldrich, Germany) at room temperature. Before their sectioning, teeth were cleaned using a spoon excavator and a toothbrush with pumice powder and stone to remove any soft tissue. Additionally, they were visually checked for cracks, caries or other malformations.

Following this, each tooth was mounted in “greenstick” impression compound (Kerr, UK) on cutting discs (Figure 14) and sectioned using a water cooled, Diamond Wire Saw, cutting machine (Well® Walter EBNER, CH-2400 Le Loche, Germany- Figure 15). The crowns and the roots of each tooth were separated first, followed by the buccal and lingual surfaces of each crown. Three enamel slabs of about 3mmx 2mmx 2mm were produced from the buccal section, and another three slabs of exactly the same dimensions were produced from the lingual section of each tooth. A total of 18 teeth and 108 slabs were used in this study.

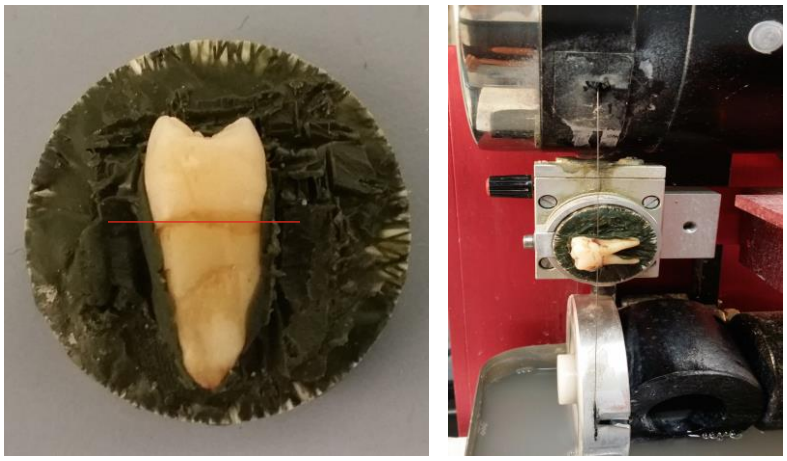


Figure 14 Illustration of tooth sectioning

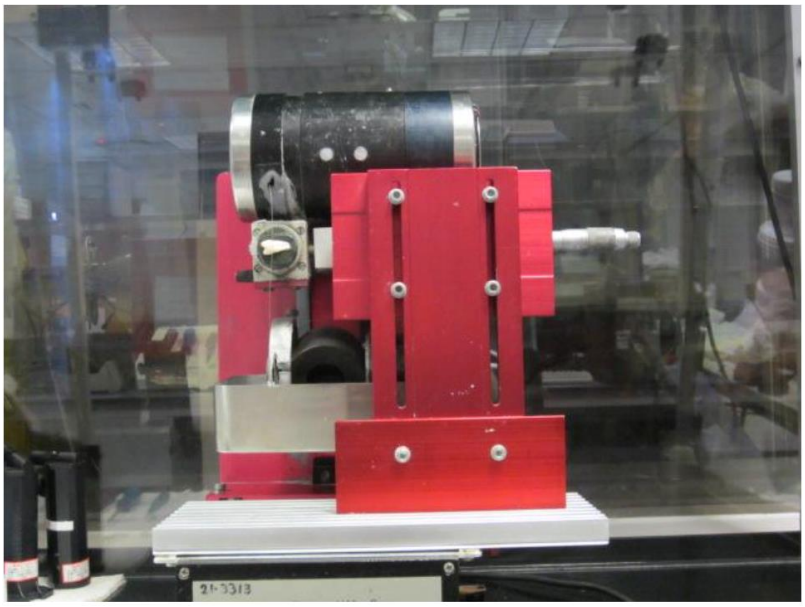


Figure 15 Well® Diamond wire Saw cutting machine

2.6.5.2.2 Sterilisation of enamel slabs

Once the enamel slabs were prepared, they were kept moist in distilled water with thymol, in micro-centrifuge tubes sealed with sealing film (Parafilm®, Sigma-Aldrich, Germany) to prevent leakage of the thymol solution and dehydration of the enamel. Later, they were immersed overnight in sodium

hypochlorite (12%), followed by thorough rinsing with deionized water and, again, immersed overnight in phosphate buffered saline (pH 7.4), then sent to the Department of Immunology of the University of Liverpool, where they were exposed to gamma radiation (4080Gy). This level of exposure provides sterilisation without altering the structural integrity of the enamel slabs (Amaechi *et al.*, 1999) and, in addition, the use of hypochlorite to treat enamel does not affect biofilm development (Watson *et al.*, 2004). This sterilisation procedure was according to University of Leeds sterilisation protocol of human slabs used *in situ* appliances.

Enamel slabs were sterilised twice prior to each arm of the *in situ* study.

2.6.5.3 *In situ* appliance framework

The appliance used in the study was the mandibular removable appliance, introduced by Koulourides and Volker (1964). It consisted of a labial arch wire, acrylic flanges buccally on premolars and first permanent molars, and a U clasp attached to each of the first permanent molars (Figure 16). Three enamel slabs were inserted in the right buccal flange, and another three in the left buccal flange of each appliance (Figure 17).

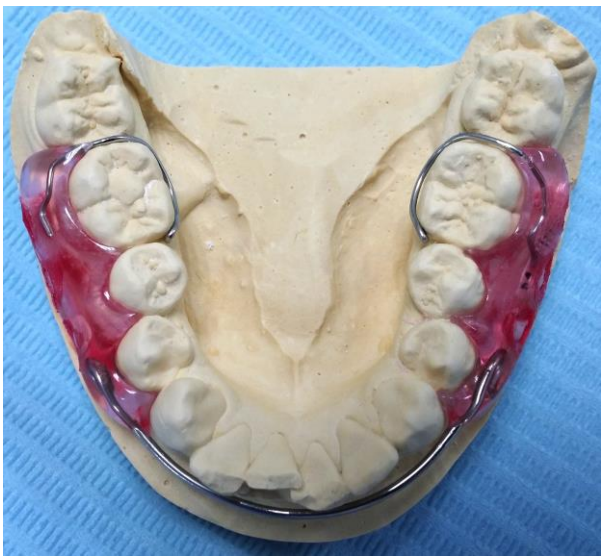


Figure 16 Occlusal view of *in situ* appliance

Figure 17 Right and left lateral views of *in situ* appliance

The following modifications to this original appliance were made in order to place the enamel slabs and collect the undisturbed layer of plaque (Figure 18):

- A 3mm deep space was made for each enamel slab to be placed in the appliance, leaving a 1mm depth for plaque accumulation on the surface of the slab. In the original appliance, the surfaces of the enamel slabs were placed at the same level with that of the acrylic without leaving a 1mm depression.
- No gauze was placed on the surface of the enamel slabs, which was placed for plaque accumulation in the original device.
- A 1mm space was left on the sides of each slab and filled with sticky wax to accommodate the removal of the slab with a dental wax knife without disturbing the accumulated plaque after collection of the appliance.

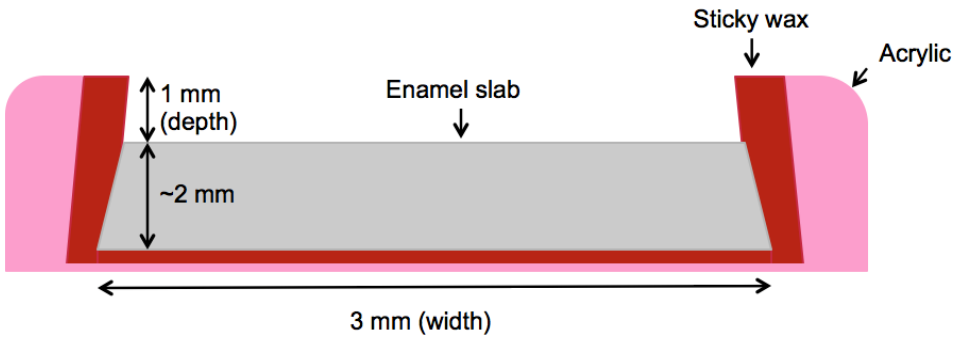


Figure 18 Enamel slab in *in situ* appliance

All slabs were secured in position with sticky wax (Bredent, Germany). Care was taken to not cover the exposed surface of enamel. The distance between every two slabs in sequence varied from 2 to 5mm, as it was dependent on the different thickness of the appliance at different areas and the presence of the metallic wire extending partially to the right and left flanges of the appliance.

2.6.5.4 Randomisation of enamel slabs

Randomisation of enamel slabs was applied in order to allocate them to:

- The *in situ* appliance
- The control and treatment groups

2.6.5.4.1 Randomisation of enamel slabs in the *in situ* appliance

All enamel slabs were coded based on random table of numbers, according to a computer programme of random allocation (http://stattrek.com/statistics/random-number-generator) and every 6-slabs were randomly assigned to each appliance for all the participants taking part in the study. All participants were allocated the same appliance with the same slabs for the second arm of the study.

2.6.5.4.2 Randomisation of enamel slabs in the control and treatment groups

Following removal of the enamel slabs from the appliance, enamel slabs were coded again, based on random table of numbers as mentioned above, and randomly allocated to the control and treatment groups by using sealed envelope. The list of the codes were kept with a member of staff (GD) and the chief investigator (AA) were given an envelope at each visit of appliance

collection. The same method of random allocation was performed in the second arm of the study.

2.6.5.5 Removal of enamel slabs from appliances

All six enamel slabs were removed in the laboratory using sterile instruments after collection of the appliance from the participant. Each one of the six slabs with its accumulated plaque was transferred to a separate well of a 12-well tissue culture plate. Petroleum jelly was used to prevent the slabs floating in the control and treatment solutions. Care was taken that the area of the slab with the accumulated plaque was uppermost in the well.

Figure 19 shows a summary of the laboratory protocol of the *in situ* phase following removal of enamel slabs from appliances.

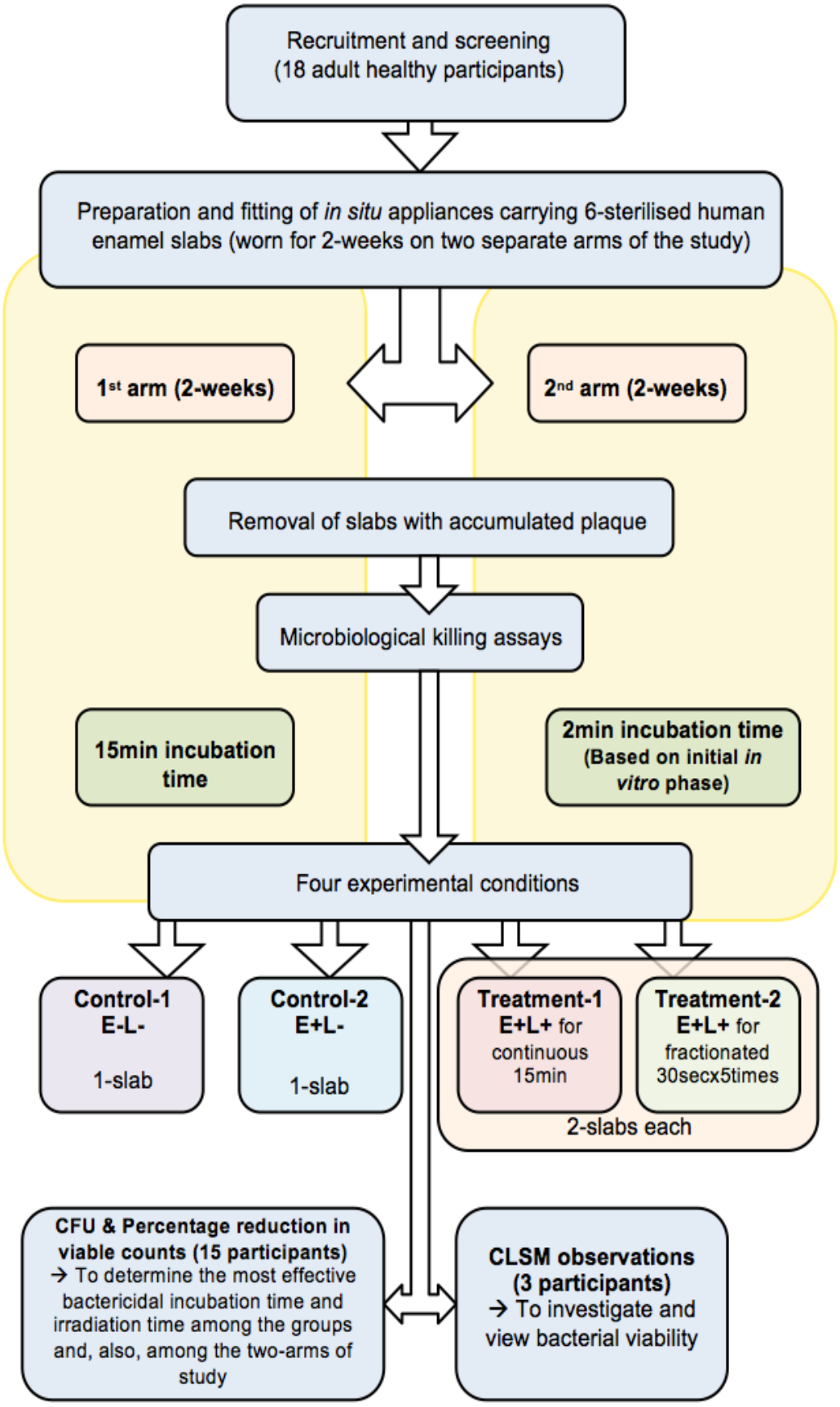


Figure 19 Protocol of the *in situ* phase of the study

2.6.5.6 Microbiological killing assays

2.6.5.6.1 Investigation of variation in one participant

Prior to the investigation of PDT effect on *in vivo* formed biofilm, an investigation on the variation of bacterial viability values among six enamel slabs in an appliance was undertaken. This was to ensure that the baseline measurement of accumulated plaque in all six slabs was comparable. Despite the fact that the previous pilot study (Tahmassebi *et al.*, 2015)- unpublished data- had already investigated this and found that there was no significant difference in the bacterial viability values among the six slabs.

Therefore, following removal of enamel slabs from the appliance of the first participant, the six enamel slabs were individually transferred to separate wells of a 12-well tissue culture plate. Petroleum jelly was used to prevent the slabs floating in the solution. Care was taken that the area of the slab with accumulated plaque was the uppermost in the well. Then, slabs were covered with 1.5ml of RTF and incubated at room temperature for 15min. No light was applied, as these were the control groups. Ten-fold serial dilutions were produced and plated on CBA plates in triplicates (total= 18 plates). The plates were then incubated for 37°C in a 10% CO_2 incubator for 48h. After incubation, bacterial counts of each plate were quantified, using colony counter, and then CFU/ml was calculated from the mean count of each triplicate.

2.6.5.6.2 PDT on *in vivo* formed plaque biofilms

As stated earlier, this *in situ* phase of the study consisted of two arms. Participants were requested to wear the appliances for a period of two weeks, on these two separate arms of the study, and at the end of each arm the slabs with the accumulated plaque were removed from the appliance and treated under the different experimental conditions. The experimental conditions were the same for the two arms of the study, except that the

incubation time of the plaque biofilm was different, attempting to reduce the overall PDT time. In the 1st arm, the incubation time was 15min, a duration of time that was used in the previous pilot study (Tahmassebi *et al.*, 2015), whereas, in the 2nd arm, the incubation time that was used was determined from the findings of the *in vitro* phase of the study. This was carried out in order to test whether this finding was also applicable on the *in vivo* formed biofilms and not only in planktonic cultures, attempting to reduce the overall PDT time. Figure 19 shows the protocol of the *in situ* phase.

As 18 participants were recruited, samples from15-participants were used for the microbiological killing assay to determine the bacterial viability values between the control and treatment groups. While the remainder of the samples of 3 participants were used for viewing under CLSM.

Arm-1

This part of the *in situ* phase involved 15 participants, where they had to wear the appliances for 2-weeks as already stated. At the end of this arm, the six slabs with accumulated plaque were removed from the appliance and treated under four different conditions, as follows; control one group-C1 (No erythrosine, no light) represented by 1 slab; control two group-C2 (Erythrosine, no light) represented by 1 slab; treatment one group-T1 (Erythrosine, +15min continuous light) represented by 2 slabs (T1A and T1B); treatment two group-T2 (Erythrosine, +30sec light pulses for 5 times, separated by dark periods of 1min) represented by 2 slabs (T2A and T2B). The reason for treating duplicate slabs, in treatment 1 and 2 groups, was to assess the variation of the outcome measurement, bacterial viability values, between the duplicates of the same group with a view to validate and increase the reliability of the results.

To enumerate the procedure, following collection of the appliance from the participant, randomisation of the slabs to control and treatment groups have had occur at this stage by opening a sealed envelope included the randomisation numbers (see section2.6.5.4.2). Next, the six slabs, with its accumulated plaque, were removed from the appliance and individually transferred to separate wells of 12-well tissue culture plates (Figure 20) and, then, were completely covered with either 1.5ml of RTF (C1) or 1.5ml (220μM) erythrosine (C2, T1A, T1B, T2A and T2B). Later, the 12-well plates were covered with foil and incubated for 15min at room temperature. After dark incubation, foil was removed from the treatment groups (T1A, T1B, T2A, and T2B) wells and all plates were placed under the tungsten filament lamp. Treatment groups (T1A and T1B) were irradiated for continuous 15min, while, treatment groups (T2A and T2B) were irradiated for 30sec light pulses for 5 times, separated by dark periods of 1min, using foil to cover these wells during the dark periods (total treatment time= 7.5min). To determine the effect of erythrosine alone on bacterial cells viability, control 2 group was covered with foil (Erythrosine, no light). Although the control groups (C1 and C2) were placed under the lamp, they were kept in dark by covering them with foil, attempting to treat all groups under same conditions as much as possible. Following completion of the irradiation regime, the bacterial solution from each well, with the enamel slab and the accumulated plaque, were individually transferred to plastic vials, using sterile instruments, and then mixed for ~30-60sec, using vortex mixer, with 5-6 sterile glass beads of 3mm diameter each, to disaggregate the plaque biofilms. Ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}), 50μl on 450μl of RTF using 48-well plates, were produced from the bacterial suspensions of the control and treatment groups, to be able to determine the number of bacteria in a sample, and each dilution (100μl) was plated on CBA in triplicates (Total=108 plates for each appliance). The plates were then incubated at 37°C in a 10% CO_2 incubator for 48h. After incubation, bacterial counts of each plate were quantified, using colony counter, and then colony-forming unit per millilitre (CFU/ml) was calculated from the mean count of each triplicate. Percentage reduction of bacterial counts was obtained from

the different groups with the aim to determine any significant difference in the bacterial viability counts among the different control and treatment groups.

Arm-2

The protocol regime for the 2nd arm was exactly the same as the 1st arm, except; the incubation time of samples was different (Figure 20). The incubation time was determined from the initial *in vitro* study on planktonic cultures of *L.casei* bacteria (see section 2.5.1) where different incubation times of 2, 5 and 15min were used and, then, the bacterial viability were compared between the different control and treatment groups. As it was found, from the *in vitro* study, no significant difference in the bacterial viability counts using either incubation time, it was decided to use 2min incubation time in this part, to investigate whether it has any effect on the bacterial viability of the *in vivo* formed plaque biofilms and, also, to compare this arm's results with the 1st arm, attempting to reduce the overall PDT time.

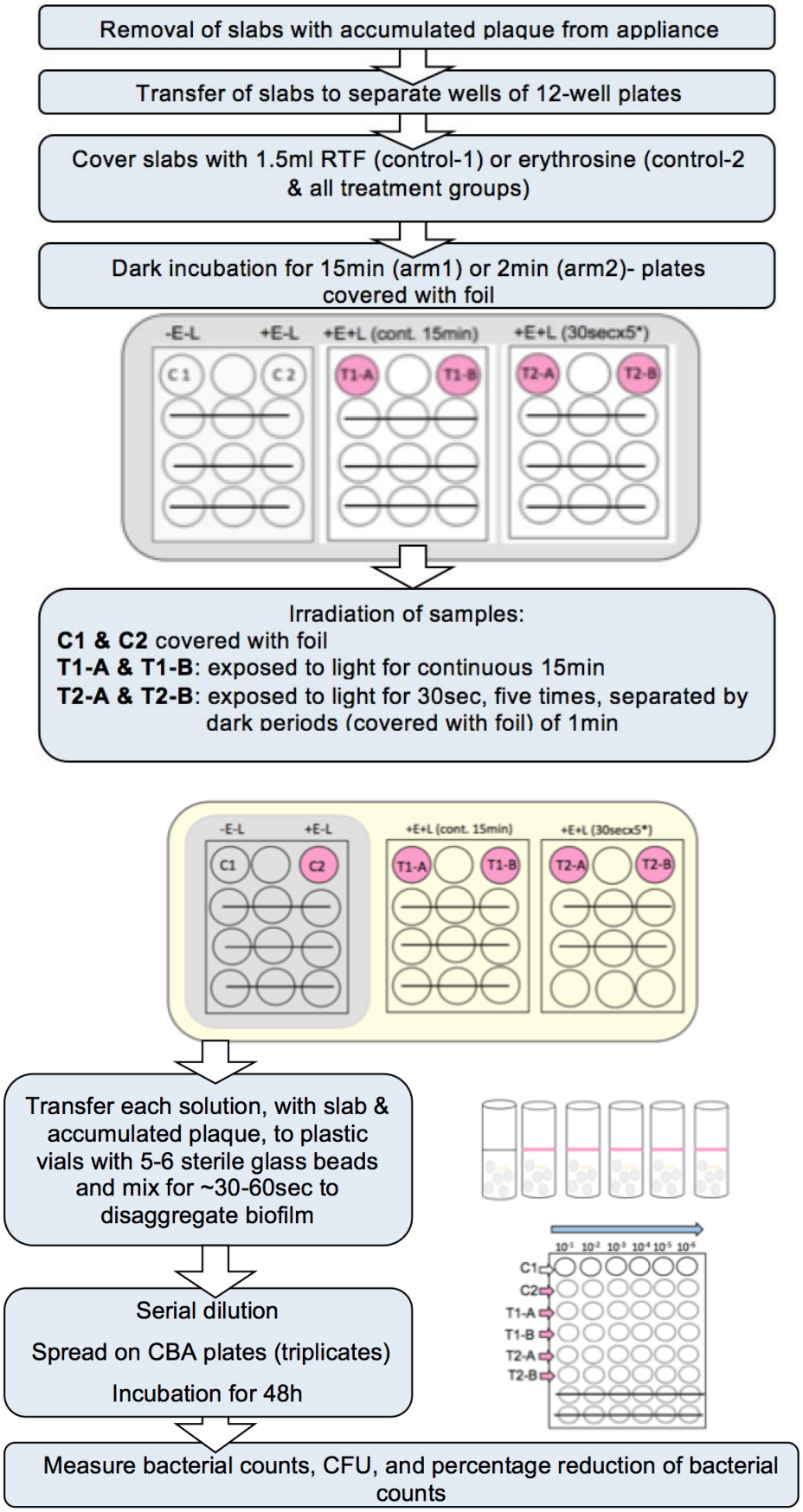


Figure 20 Laboratory protocol for the *in situ* phase of the study

Intra-examiner reproducibility

To determine the intra-examiner reproducibility, the study investigator (AA) randomly re-counted the total bacterial counts of 10% of the total number of CBA plates used in both arms of the *in situ* phase of the study. They were re-counted 5-7days following the initial counting. The plates were kept in microbiology fridge during this time. This was performed to establish and quantify reproducibility, and thus provide an indication of the reliability of the measurements, which was assessed using the Bland Altman plot.

2.6.5.7 CLSM observations

Plaque samples from 3 participants were treated under different conditions including; a) control (No erythrosine, no light); b) treatment-1 (Erythrosine, +15min continuous light); c) treatment-2 (Erythrosine, +30sec light pulses for 5-times, separated by dark periods of 1min). Following treatment, these samples were viewed immediately under CLSM in order to investigate PDT effect on the *in vivo* formed plaque biofilms.

The LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) (Molecular Probes, Eugene, OR, USA) was used in this study to determine the viability of bacteria within the biofilm, before and after PDT (Figure 22). This kit consists of two fluorescent nucleic acid-binding stains: SYTO 9 and Propidium iodide. SYTO 9 stains all viable bacteria in green, while Propidium iodide stains in red the bacteria whose membranes were damaged (non-viable bacteria).

Therefore, following treatment, samples were placed into a small Petri dish and then RTF was carefully added, covering the sample, to gently wash off the samples. Next, LIVE/DEAD® stains were mixed according to the

manufacturer's instructions and applied directly to the surface of each sample. The Petri dish was mixed gently and, then, covered with foil and incubated for 15min at room temperature. After the dark incubation, the slab, with accumulated plaque, was transferred to another Petri dish, with RTF, for wash off and then observed using a Leica SP2 CLSM (Figure 21). It was examined with an argon (488nm) and HeNe (543nm) laser with x10 and x63 water-dipping objective lenses. For confocal optical sectioning, sequences of images were taken along the horizontal x-y plane at 5μm (x10 lens) and 0.5μm (x63) increments from the bottom of the biofilm that was contact with the enamel slab to the top (z-axis). Images were collected at x1 magnification, x2 and x4 electronic zoom. Live bacteria with intact membranes were stained fluorescent green, while dead bacteria with damaged membranes were stained fluorescent red.

Figure 21 CLSM (Leica TCS SP2, Leica Microsystems GmbH, Wetzlar, Germany)

Figure 22 LIVE/DEAD® BacLight™ Bacterial Viability Kit (L7012) (Molecular Probes, Eugene, OR, USA)

2.7 Data analysis

2.7.1 Variables collected

Microbial counts were expressed as the number of colony forming units (CFU) per ml. Each CFU value represented the mean count from triplicate samples. Logarithmic transformation (log_{10}) was applied to the original observations (CFU/ml) to make the distribution more symmetric. Percentage reduction in bacterial counts in each group was calculated by dividing the difference in CFU between control and treatment groups with the number of CFU from the control group from the same subject (see the following calculation- Equation 1):

$$\boldsymbol{Percentage}\ (\%)\ \boldsymbol{reduction} = \frac{\boldsymbol{CFU}\ (\boldsymbol{control}) - \boldsymbol{CFU}\ (\boldsymbol{treatment})}{\boldsymbol{CFU}\ (\boldsymbol{control})} \times \mathbf{100}$$

Equation 1 Calculation of percentage reduction in viable counts

2.7.2 Statistical analysis

All the collected data were entered into Microsoft office Excel 2010 spread sheets. Differences between controlled and treated samples were statistically tested. All tests were performed using IBM® SPSS® Statistics Version 23. A significance level of $\alpha < 0.05$ was implemented.

All continuous data were checked for normality by comparing means and medians. Descriptive statistics such as mean, median, and standard deviation were used to summarise the data. The parametric ANOVA test was used for the normally distributed continuous data and the non-

parametric Friedman test was used for all the skewed continuous data to compare means, for the former test, and medians, for the latter test, between the control and treatment groups. Additionally, a series of Wilcoxon tests were conducted to identify where the specific difference lay, but with adjustments for multiple comparisons to control for inflation of type 1 error. In order to avoid this error, the statistical significance level (α) was adjusted according the following equation: α = 0.05/number of comparisons.

Intra-examiner reproducibility was evaluated in both arms of the *in situ* phase of the study by using the Bland Altman plot. Total bacterial counts of 10% of the total number of CBA plates used in both arms were re-counted.

Chapter 3 Results

3.1 *In vitro* phase

3.1.1 PDT on planktonic cultures of *L.casei*

L.casei bacterial suspensions used in all experiments were spectrophotometrically standardised, using a wavelength of 600nm and an optical density (OD) of ~0.5 (see section 2.5.1). Figure 23 shows an *L.casei* growth curve, which indicates that cells were in log phase at OD ~0.5. All bacterial suspensions used in all experiments were treated with PDT once this phase was reached.

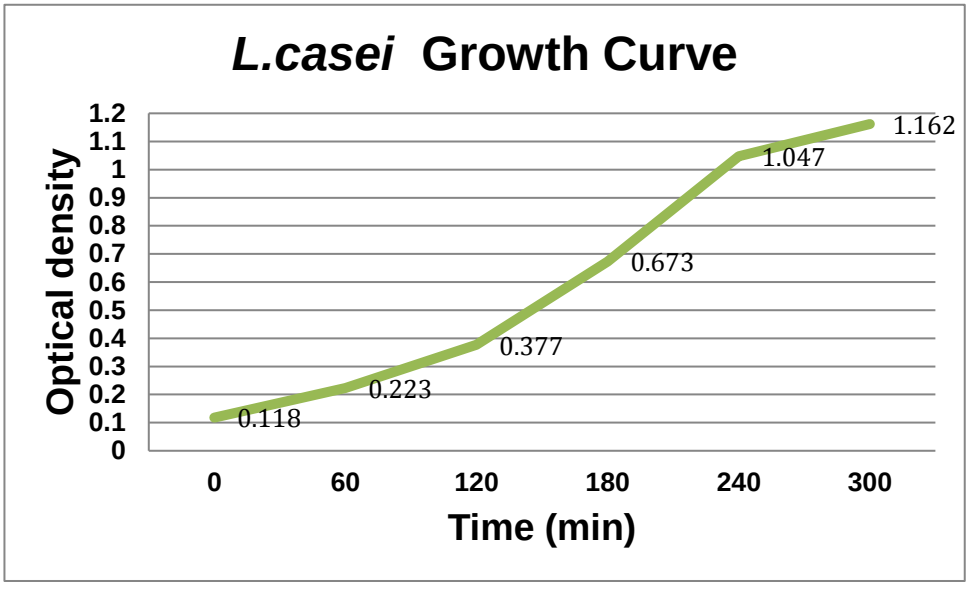


Figure 23 *L.casei* growth curve

L.casei bacteria were treated with PDT using different incubation times with photosensitizer to determine the most bactericidal incubation time. The times that were used were 2min, 5min and 15min, where the bacteria were incubated with erythrosine (220µM) in the dark. For each incubation time, three experiments were performed to increase the reliability of the results. In

addition to this, in each of these experiments, the bacteria were experimented under different conditions in triplicates to increase the reliability of the results. The experimental conditions were: a) Control-1 (No erythrosine, no light irradiation); b) Control-2 (+Erythrosine, no light irradiation); c) Control-3 (No erythrosine, +Light irradiation); d) Treatment group (+Erythrosine, +Light). Following this, total bacterial counts (CFU/ml) were measured, and the percentage reduction of bacterial counts were calculated and compared between the different experimental conditions and different incubation times to assess the bactericidal effect of PDT.

3.1.1.1 Total bacterial counts (CFU/ml) measurements

The CFU values were slightly decreased in samples exposed to erythrosine alone (Control-2) compared with groups of Control-1 (No erythrosine, no light irradiation) and Control-3 (No erythrosine, +Light irradiation). However, the combined treatment of light irradiation in the presence of erythrosine (E+L+) resulted in significant reduction in CFU counts at all incubation times (Table 5) (Figure 24).

Table 5 Mean count CFU/ml, mean log_{10} CFU/ml and standard deviation (SD) for the different experimental conditions using different incubation times.

Treatment conditions	2min incubation-time	5min incubation-time	15min incubation-time
Control-1:	2.18E+08	1.59E+08	3.17E+09
E-L-	8.26 (±0.32)	8.20 (±0.06)	8.81 (±1.01)
Control-2:	1.02E+08	8.63E+07	9.37E+07
E+L-	7.98 (±0.22)	7.93 (±0.10)	7.97 (±0.09)
Control-3:	2.41E+08	1.80E+08	2.74E+09
E-L+	8.35 (±0.20)	8.25 (±0.07)	8.68 (±1.08)
Treatment:	0.00	0.00	0.00
E+L+	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)

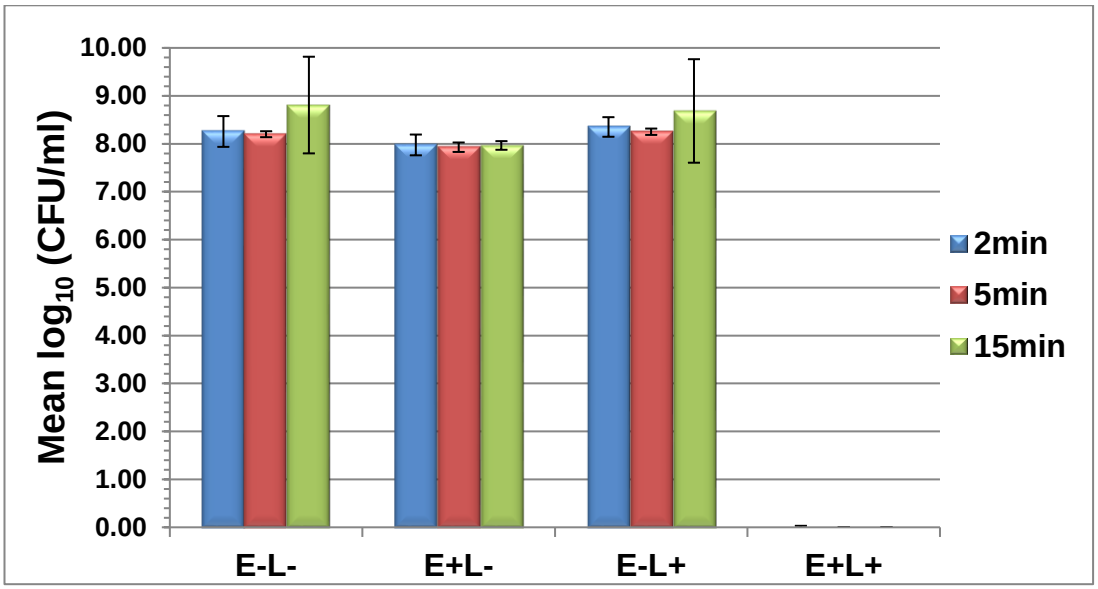


Figure 24 Total *L.casei* viable counts (mean log_{10} CFU/ml ± SD) obtained for the different experimental conditions when using incubation times of 2, 5 and 15min.

3.1.1.2 Percentage reduction in total bacterial counts (cell death)

Figure 25 shows that there was a variation in percentage reduction of bacterial counts between the different control groups, (E+L-) and (E-L+), when using 2, 5, and 15min incubation times, whereas in the treatment group (E+L+), there was a 100% reduction in bacterial counts among the three incubation times. This indicated that there was no difference in percentage reduction in bacterial counts of the treatment group (E+L+) in the three different incubation times. Additionally, as data were normally distributed, Univariate analysis of variance test (ANOVA) was carried out. It showed that there was a statistically significant difference in the percentage reduction of the bacterial counts between the different control and treatment groups (p=0.000, <0.05). However, there was no significant difference in the percentage reduction of bacterial counts between the three different incubation times in all the experimental conditions (p=0.266, >0.05). Therefore, two minutes incubation time was used in the 2nd arm of the following *in situ* study.

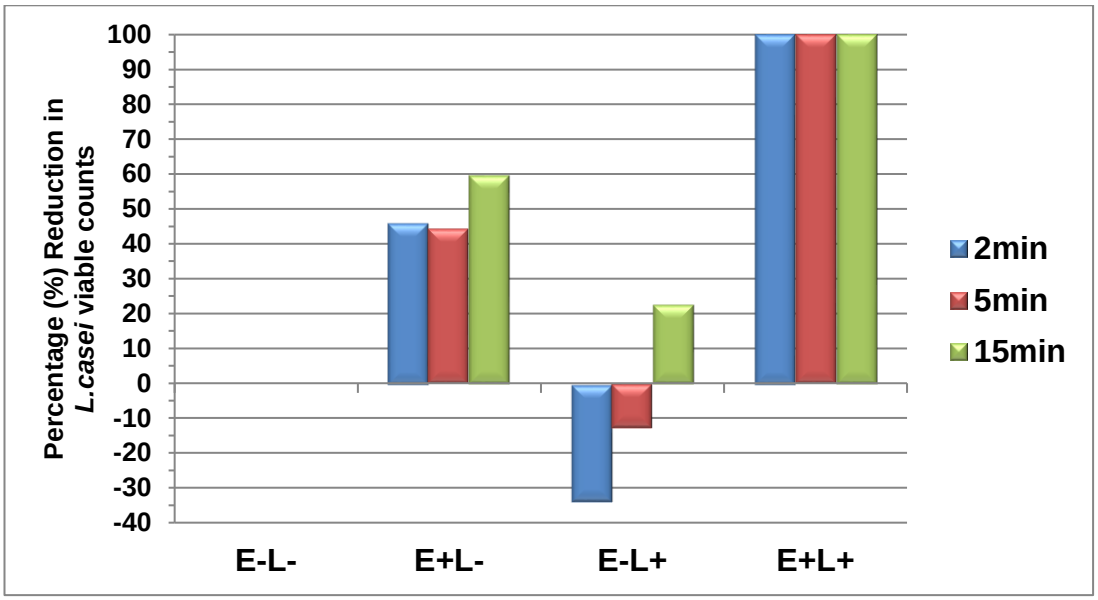


Figure 25 Percentage (%) reduction in *L.casei* viable count for the different experimental conditions using different incubation times.

3.2 *In situ* phase

3.2.1 Demographic data

In total 18 healthy adults participants who had to fulfil the inclusion and exclusion criteria for this study (see section 2.6.1.1 and 2.6.1.2), were recruited from the NHS Trust and University of Leeds. Their mean age was 34 years old, mean DMFT 5.94, and mean salivary flow rate 0.95 ml/min (Table 6).

Table 6 Description of the study sample

	N	Range	Minimum	Maximum	Mean	SD
Age	18	37	21	58	34.00	10.27
DMFT	18	16	1	17	5.94	4.33
Salivary flow rate (ml/min)	18	0.90	0.60	1.50	0.95	0.22

The accumulated plaque samples from 15 participants were used to investigate PDT effect by microbiological cultures and bacterial killing assay. While the accumulated plaque samples from the remaining three participants were used to view the PDT effect under CLSM.

3.2.2 PDT on *in vivo* formed biofilms

3.2.2.1 Baseline measurement- Investigation of variation in one appliance

Assessment of the degree of variation in the total bacterial counts (log_{10} CFU/ml) among six control groups (six enamel slabs with the accumulated plaque) was carried out by using a Coefficient of variation (CV) test. It represents the ratio of the standard deviation (SD) to the mean. CV value was 0.01 which is close to zero. This means there was no variation between the six samples. In addition, the small variation in the confidence interval (CI) indicated a significant and precise result (Table 7).

Table 7 Descriptive statistics of log_{10} CFU/ml among six control groups

Mean of log_{10} CFU/ml	SD	95% CI for mean
6.17	0.04	6.13, 6.22

3.2.2.2 Arm-1

The aim of this arm was to determine the most effective irradiation time that would significantly reduce the total viable bacterial counts among the control and treatment groups when using 15min incubation time in 220µM erythrosine. Two irradiation regimes were used: a) 15min continuous light; b) 30sec light pulses for 5-times separated by 1min dark periods (fractionated irradiation). Duplicate slabs were used for each of the previous light regimes,

aiming to increase the reliability of the results. Following PDT, total bacterial counts (CFU/ml) were measured and percentage reduction of total bacterial counts (cell death) were calculated from the different experimental groups with the aim to assess the bactericidal effect of PDT and to determine any significant difference in the bacterial viability counts among the different treatment and control groups.

3.2.2.2.1 Total bacterial counts (CFU/ml) measurements

Table 8 shows a direct comparison of the mean $\log_{10}$ of the total bacterial counts (CFU/ml) of the different control and treatment groups when 15min incubation time with 220µM erythrosine was used.

The bacterial reduction observed in the positive control group (C2), treated with erythrosine alone, was 0.26 $\log_{10}$ compared with the negative control group (C1). Whereas, the groups treated with erythrosine and irradiated with 15min continuous light (T1A and T1B) resulted in a bacterial reduction by 1.33-1.36 $\log_{10}$ when compared with C1. When looking at the bacterial reduction in the groups treated with erythrosine and irradiated with fractionated light (T2A and T2B), there was a 1.02-1.13 $\log_{10}$ reduction compared with C1.

Table 8 Mean $\log_{10}$ CFU/ml and standard deviation (SD) obtained for the different experimental conditions when using incubation time of 15min (Arm-1)

Treatment conditions	Mean $\log_{10}$ CFU/ml (SD)
(C1): E-L-	6.08 (±0.79)
(C2): E+L-	5.82 (±0.76)
(T1A): E+(15min)L+	4.72 (±0.80)
(T1B): E+(15min)L+	4.75 (±0.81)
(T2A): E+(5*30s)L+	5.06 (±0.86)
(T2B): E+(5*30s)L+	4.95 (±0.90)

3.2.2.2.2 Percentage reduction in total bacterial counts (cell death)

Table 9 shows the descriptive statistics for the percentage reduction in total bacterial counts (cell death) in the six control and treatment groups when compared with the control 1 (No erythrosine, no light) in 13 subjects. Analysis of the accumulated plaque samples from one participant were excluded as there were spillage of samples during microbiological testing.

Table 9 Descriptive statistics- Percentage reduction in total bacterial counts (cell death) in the six groups with duplicate slabs (A) & (B).

Treatment conditions	N	Mean (%)	SD	Min. (%)	Max. (%)	Percentiles (%)		
						25th	50th Median	75th
(C1): E-L-	13	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(C2): E+L-	13	42.14	18.76	17.08	74.66	26.45	32.46	57.68
(T1A): E+(15min)L+	13	89.13	13.35	64.03	99.82	78.14	94.27	98.88
(T1B): E+(15min)L+	13	87.45	16.33	55.40	99.68	74.81	95.73	98.79
(T2A): E+(5*30s)L+	13	83.15	17.50	46.39	99.47	78.30	88.15	96.01
(T2B): E+(5*30s)L+	13	85.10	16.92	47.48	99.71	79.84	91.37	95.86

As can be seen from Figure 26 a small percentage reduction was seen in the positive control group E+L- (C2) compared with the negative control group (C1). On the other hand, the percentage reductions of the total bacterial counts following both 15min continuous light (T1A and T1B) and 30sec*5 fractionated light separated by 1min dark periods (T2A and T2B) were much higher than the control groups.

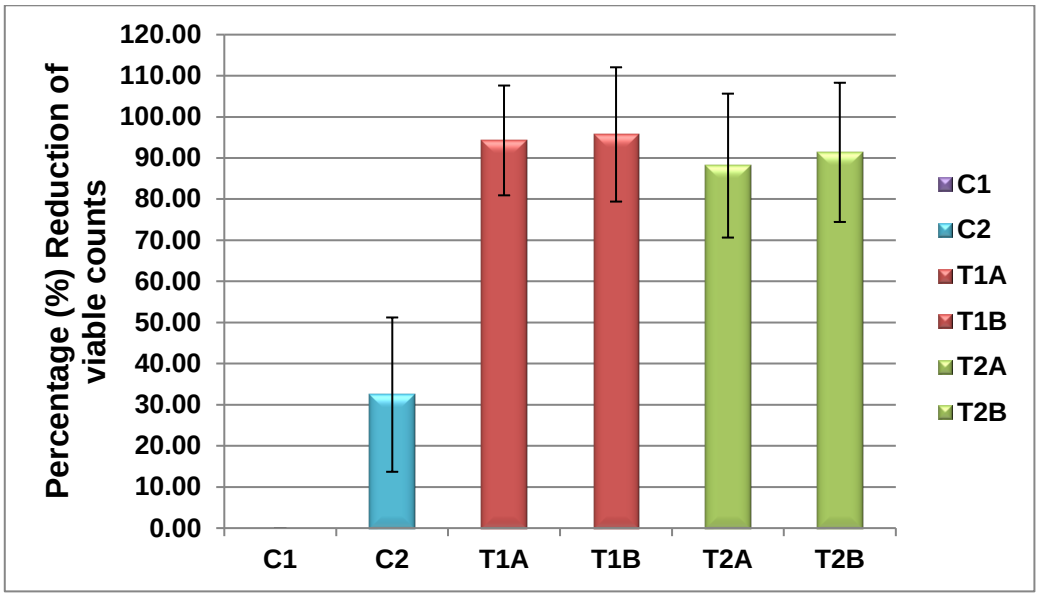


Figure 26 Arm-1: Percentage (%) reduction in viable counts in *in vivo* formed biofilm between the 6 groups.

As the data were not normally distributed (mean and median were not equal) in the six groups (Table 9), the non-parametric Friedman test was used. The results of the Friedman test indicated that there was a statistically significant difference in percentage of total bacterial counts between the six different groups in all subjects (p=0.000; <0.05). Inspection of median (Md) values showed an increase in percentage reduction of bacterial counts from C1 group (Md= 0.000) to C2 (Md=32.46) and a further increase in the four treatment groups (Md= 88.15 to 95.73) (Table 9).

As overall the Friedman test was significant, a series of Wilcoxon tests were conducted to identify where the specific differences lay, but with corrections to control for inflation of type 1 error. Bonferroni correction α= 0.05/15 = 0.003 (where 0.05 was the previous alpha (α) and 15 the number of comparisons). The test revealed statistically significant differences of the percentage reduction of bacterial counts between the negative E-L- (C1) and the positive E+L- (C2) control groups with a p value of 0.001, and, also between the control groups (C1 and C2) with the four treatment groups in all

subjects (n=13), p=0.001 (<0.003). All the four treatment groups had significantly higher percentage reduction in their bacterial counts than C1 and C2 groups. In addition, the test revealed non-statistically significant differences of the percentage reduction of bacterial counts between the four treatment groups (p>0.003). Therefore, both irradiation regimes, 15min continuous light and 30sec*5 fractionated light separated by 1min dark periods, were found to be effective for bacterial killing when 15min incubation time with 220μM erythrosine was used.

3.2.2.3 Arm-2

The aim of this arm was to determine the most effective irradiation time that would significantly reduce the total viable bacterial counts among the control and treatment groups when using 2min incubation time in 220μM erythrosine. Two irradiation regimes were used, similar to arm-1: a) 15min continuous light; b) 30sec light pulses for 5-times separated by 1min dark periods (fractionated irradiation). Duplicate slabs were used for each of the previous light regimes, aiming to increase the reliability of the results. Following PDT, total bacterial counts (CFU/ml) were measured and percentage reduction of bacterial counts (cell death) were calculated from the different experimental groups with the aim to assess the bactericidal effect of PDT and to determine any significant difference in the bacterial viability values among the different treatment and control groups.

3.2.2.3.1 Total bacterial counts (CFU/ml) measurements

Table 10 shows a direct comparison of the mean $\log_{10}$ of the total bacterial counts (CFU/ml) of the different control and treatment groups when 2min incubation time with 220μM erythrosine was used.

The bacterial reduction observed in the positive control group (C2), treated with erythrosine alone, was 0.1 $\log_{10}$ compared with the negative control

group (C1). Whereas, the groups treated with erythrosine and irradiated with 15min continuous light (T1A and T1B) resulted in a bacterial reduction by 1.14-1.24 log_{10} when compared to C1. When looking at the bacterial reduction in the groups treated with erythrosine and irradiated with fractionated light (T2A and T2B), there was a 0.63-0.81 log_{10} reduction compared with C1.

Table 10 Mean log_{10} CFU/ml and standard deviation (SD) obtained for the different experimental conditions when using incubation time of 2min (Arm-2)

Treatment conditions	Mean log_{10} CFU/ml (SD)
(C1): E-L-	5.76 (±0.72)
(C2): E+L-	5.66 (±0.70)
(T1A): E+(15min)L+	4.52 (±0.72)
(T1B): E+(15min)L+	4.62 (±0.65)
(T2A): E+(5*30s)L+	5.13 (±0.64)
(T2B): E+(5*30s)L+	4.95 (±0.83)

3.2.2.3.2 Percentage reduction in total bacterial counts (cell death)

Table 11 shows the descriptive statistics for the percentage reduction in total bacterial counts (cell death) in the six control and treatment groups when compared to control 1 (No erythrosine, no light) in 15 subjects.

Table 11 Descriptive statistics- Percentage reduction in total bacterial counts (cell death) in the six groups with duplicate slabs (A) & (B).

Treatment conditions	N	Mean (%)	SD	Min. (%)	Max. (%)	Percentiles (%)		
						25th	50th Median	75th
(C1): E-L-	15	0.00	0.00	0.00	0.00	0.00	0.00	0.00
(C2): E+L-	15	20.21	13.99	3.31	46.13	9.18	19.26	28.47
(T1A): E+(15min)L+	15	85.05	17.25	52.31	99.30	70.66	93.22	99.04
(T1B): E+(15min)L+	15	85.75	14.92	56.52	99.35	80.03	89.92	97.45
(T2A): E+(5*30s)L+	15	63.50	27.10	25.41	98.89	36.65	62.34	90.23
(T2B): E+(5*30s)L+	15	68.77	26.32	18.86	99.20	45.65	64.09	95.94

As can be seen from Figure 27 a small percentage reduction was seen in the positive control group E+L- (C2) compared with the negative control group E-L- (C1). On the other hand, the percentage reductions of the total bacterial counts following both 15min continuous light (T1A and T1B) or 30sec*5 fractionated light separated by 1min dark periods (T2A and T2B) were much higher than the control groups.

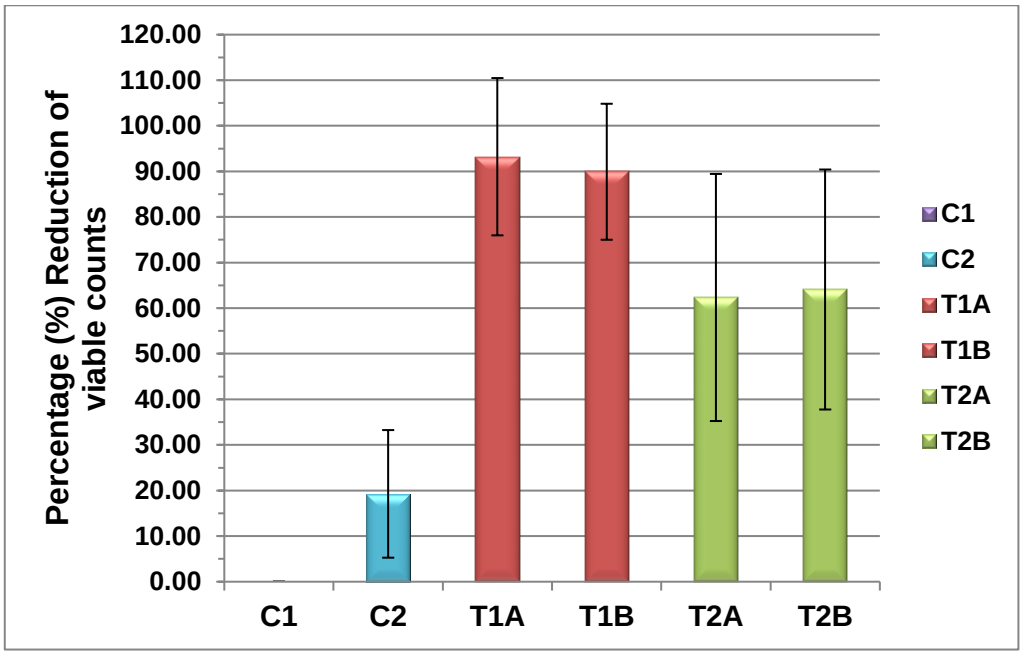


Figure 27 Arm-2: Percentage (%) reduction in viable counts in *in vivo* formed biofilm between the 6 groups.

As the data were not normally distributed (mean and median were not equal) in the six groups (Table 11), the non-parametric Friedman test was used. The results of the Friedman test indicated that there was a statistically significant difference in percentage of total bacterial counts between the six different groups in all subjects (p=0.000; <0.05). Inspection of median (Md) values showed an increase in percentage reduction of bacterial counts from C1 group (Md= 0.000) to C2 (Md= 19.26) and a further increase in the four treatment groups (Md= 62.34 to 93.22) (Table 11).

As overall the Friedman test was significant, a series of Wilcoxon tests were conducted to identify where the specific differences lay, but with corrections to control for inflation of type 1 error. Bonferroni correction α= 0.05/15 = 0.003 (where 0.05 was the previous alpha (α) and 15 the number of comparisons). The test revealed statistically significant differences of the percentage reduction of total bacterial counts between the negative E-L- (C1) and the positive E+L- (C2) control groups with a p value of 0.001, and, also between the control groups (C1 and C2) with the four treatment groups

in all subjects (n=15), p=0.001 (<0.003). All the four treatment groups had significantly higher percentage reduction in their bacterial counts than C1 and C2 groups. Surprisingly, there was statistically significant differences of the percentage reduction of bacterial counts between the treatment groups irradiated with 15min continuous light (T1A and T1B) and the treatment groups with 30sec*5 fractionated light separated by 1min dark periods with a p value of 0.001 (T2A vs.T1A and T1B) and 0.002 (T2B vs. T1A and T1B) (p<0.003) with higher percentage reduction of bacterial counts (up to ≈93%) seen in the 15min continuous light groups (T1A and T1B). This finding was not observed in arm-1 of the study where 15min incubation time was used. Therefore, 15min continuous light was found to be the most effective for bacterial killing when 2min incubation time with 220μM erythrosine was used.

3.2.2.4 Comparison of cell death percentage in *in vivo* formed biofilms between Arm-1 (15min incubation time) and Arm-2 (2min incubation time)

As can be seen from Figure 28 the combination of light irradiation, continuous or fractionated, in the presence of erythrosine (T1 or T2) resulted in a significant effect on the viability of the bacteria when using either 15min or 2min incubation times when compared to the control groups (Arm-1 & Arm-2).

However, when comparing the effect of 15min continuous light (T1) and 30sec*5 fractionated light separated by 1min dark periods (T2) regimes on the viability of bacteria, there was no significant difference in the percentage reduction of bacterial counts when they were incubated for 15min with erythrosine (Arm-1) with a percentage of cell death up to 95.73 with continuous light and up to 91.37 with the fractionated light. On the other hand, there was a significant difference in the percentage reduction of bacterial counts when the samples were incubated for 2min with erythrosine (Arm-2) and exposed to 15min continuous light (T1) and fractionated light

(T2) with a higher percentage of cell death (up to 93.22) noted when samples were irradiated with continuous light (T1).

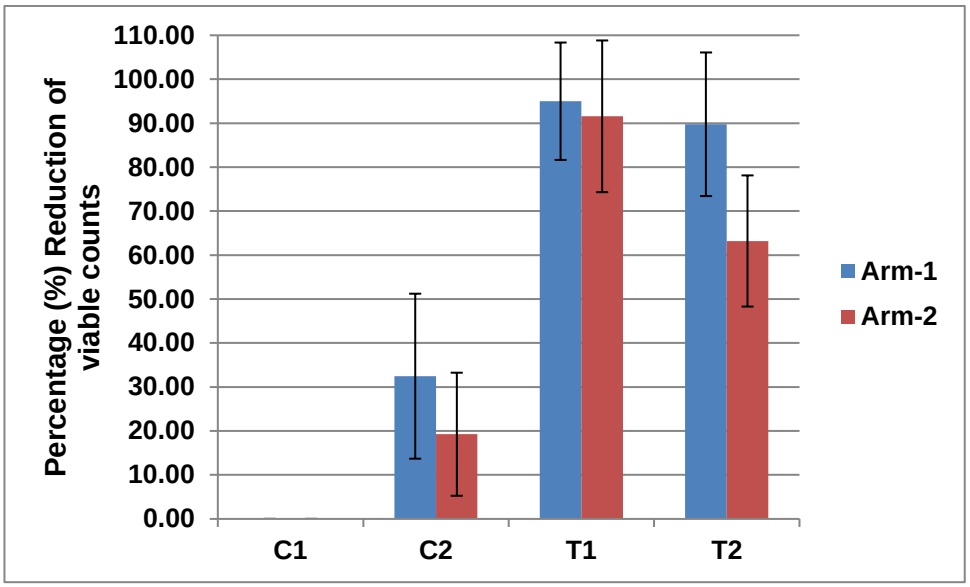


Figure 28 Comparison of percentage (%) reduction of viable counts in *in vivo* formed biofilms between Arm-1 (15min incubation time) and Arm-2 (2min incubation time).

3.2.3 Intra-examiner reproducibility

A comparison of reliability measures from one examiner (AA) were performed on two occasions to establish and quantify reproducibility, and thus provide an indication of the reliability of measurement. The investigator (AA) re-counted the total viable counts of 10% of the total number of blood agar plates used in both arms of the *in situ* phase. This means a total of ≈300 randomly selected blood agar plates were recounted. They were re-counted 5-7 days following the initial counting. The plates were kept in the microbiology fridge during this time.

One sample t-test was performed to investigate if there was a statistically significant difference between the two measurements. The test showed a

non-statistically significant difference between them (p=0.220, >0.05). As a result, the Bland Altman plot (Figure 29) was produced to assess the level of agreement between the two measurements. It shows that most of the values were close to the zero line (red line) and within the 95% confidence interval limits (-1.24, 1.34; green lines), suggesting a high level of agreement in the measurements. In addition, a Regression test was performed to assess if there was any potential proportional bias from the bland Altman plot. As the coefficient significant =0.282 (>0.05), this meant that there was no proportional bias.

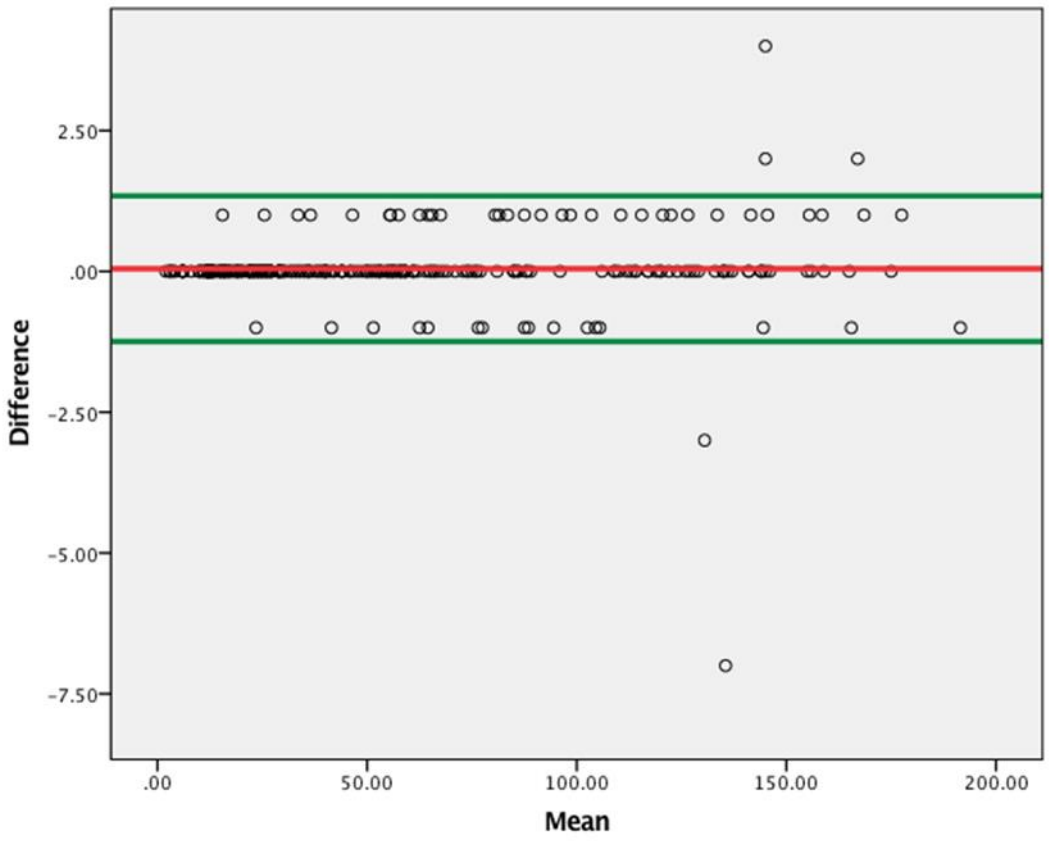


Figure 29 Bland Altman plot

3.2.4 CLSM observations

The effect of PDT on *in vivo* formed biofilms were visualised with CLSM. Images of the control and treated biofilms were captured following staining with LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, USA).

Figure 30 shows a CLSM image of a 14-days *in vivo* formed biofilm samples (untreated samples). It demonstrates a biofilm structure with a heterogeneous architecture in terms of types of cells present, such as cocci, rods and filaments, and also in terms of the overall structure where clumps of bacteria were surrounded by extracellular matrix and separated by fluid filled voids (black holes-red arrow). In addition, the majority of the cells showed green fluorescence in the absence of light irradiation and erythrosine (control), indicating a high level of cell viability (intact membranes). However, biofilm samples incubated with 220μM erythrosine for either 15min (Figure 31 and Figure 32) or 2min (Figure 33 and Figure 34) and then irradiated with either continuous 15min light (Figure 31 and Figure 33) or 30sec*5 fractionated light (Figure 32 and Figure 34), showed an increase in red/yellow fluorescence, indicating increased numbers of dead cells (damaged membranes).

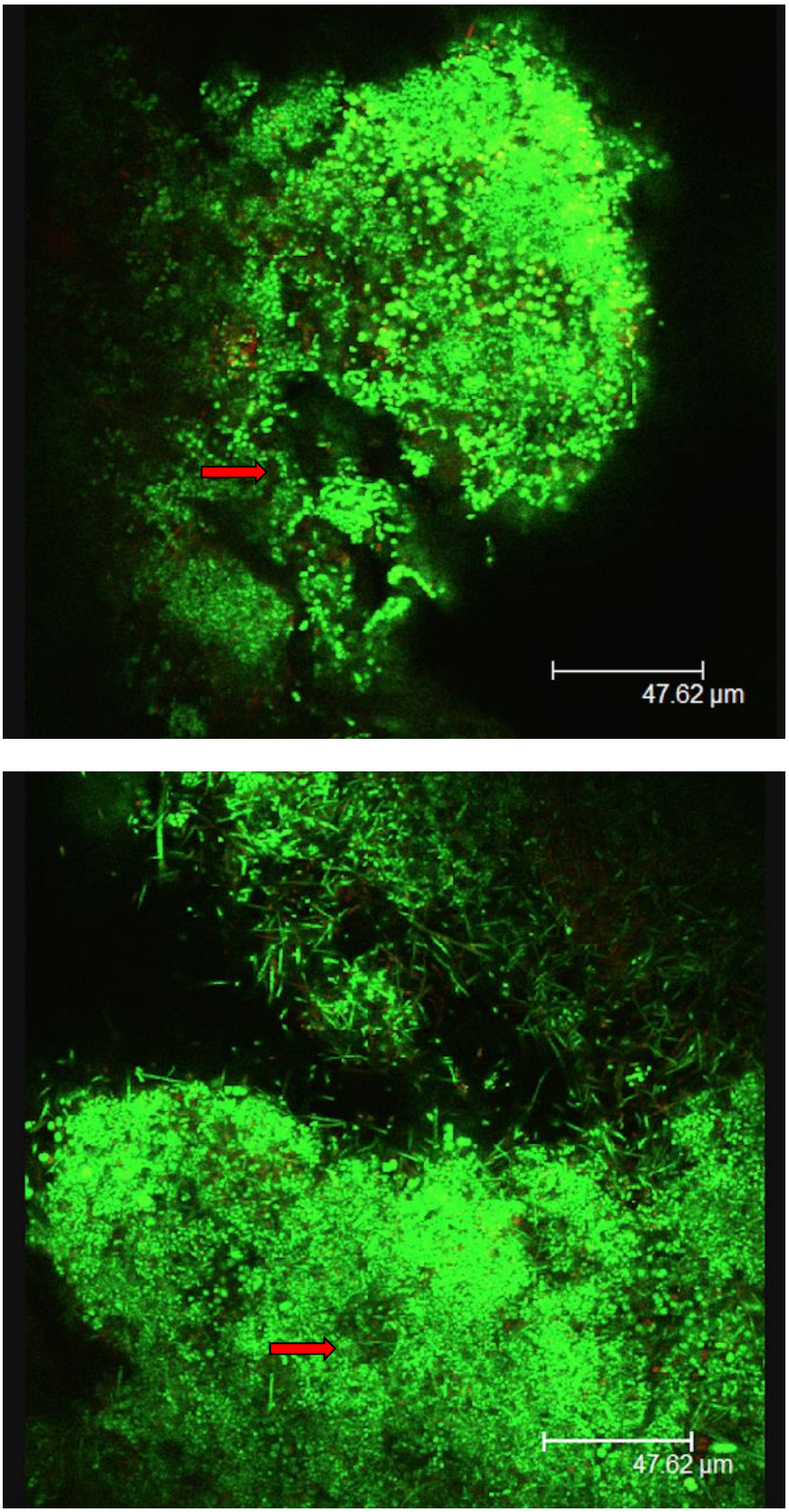


Figure 30 CLSM images showing the architecture of untreated (control) 14-days *in vivo* formed biofilm samples. Aggregates of bacteria were separated by fluid filled voids (black holes-red arrow). Images taken with x63 lens.

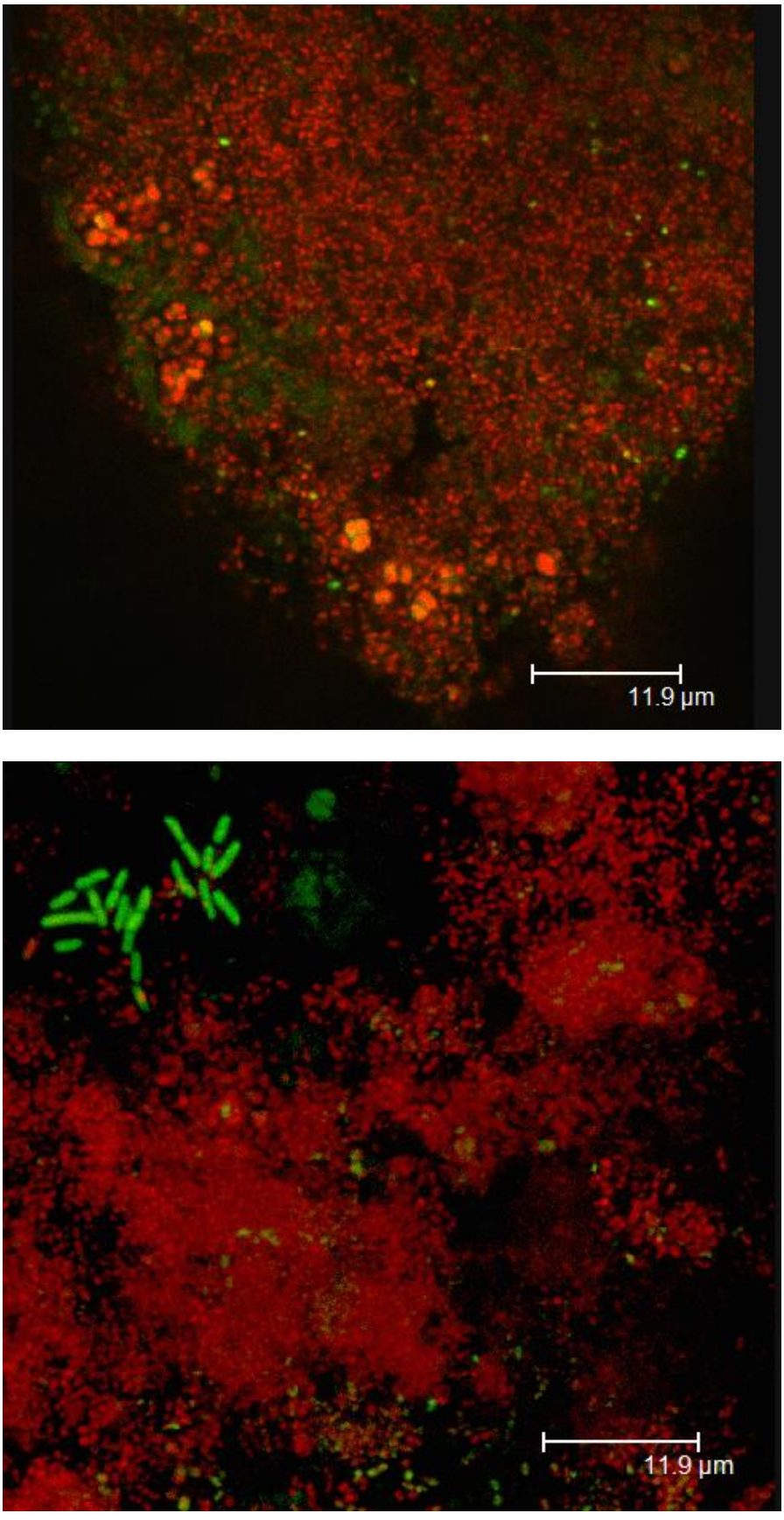


Figure 31 CLSM images of *in vivo* dental plaque biofilm samples incubated with erythrosine (220µM) for 15min (Arm-1) and irradiated for continuous 15min. Images taken with x63 lens.

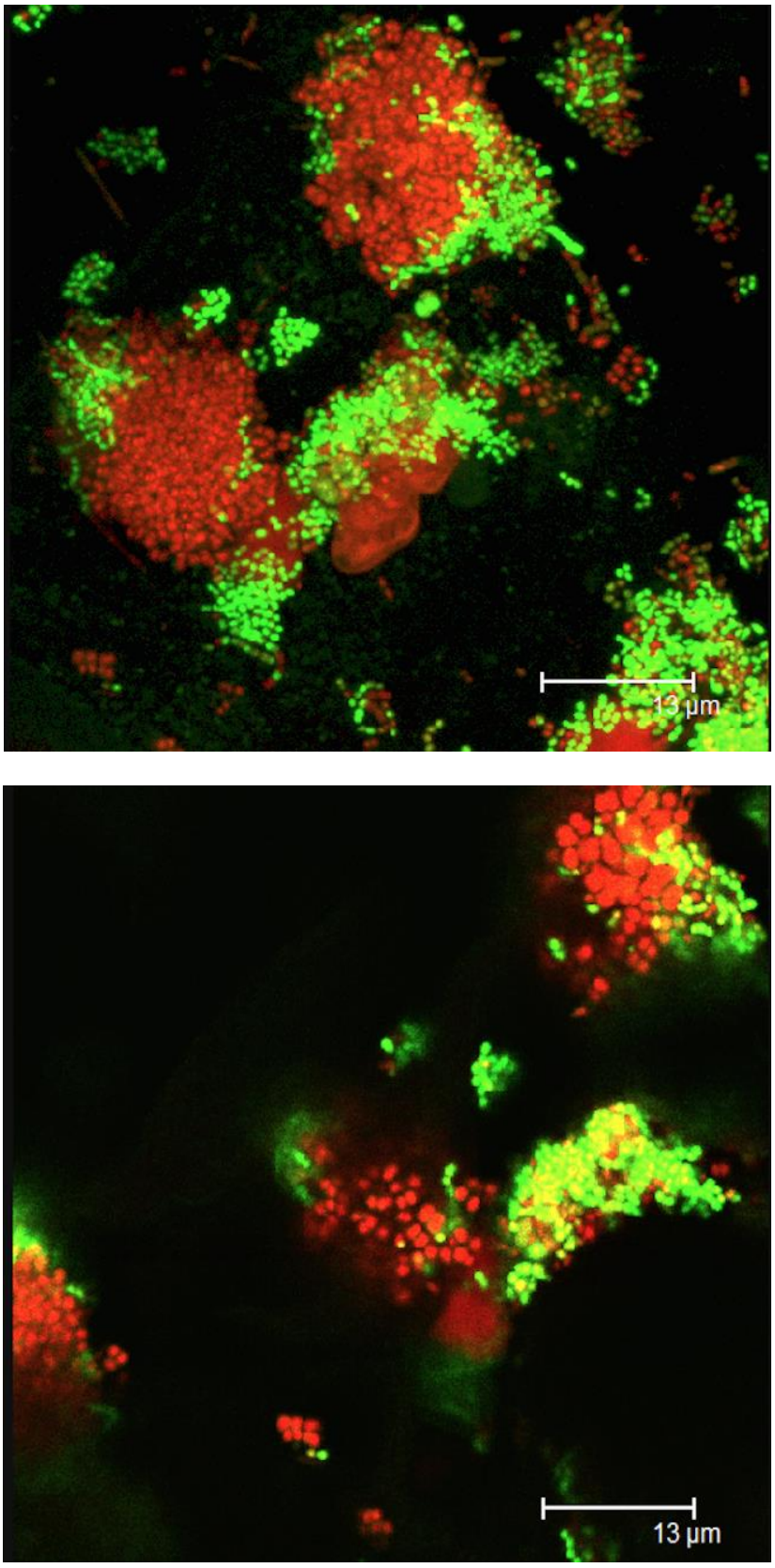


Figure 32 CLSM images of *in vivo* dental plaque biofilm samples incubated with erythrosine (220µM) for 15min (Arm-1) and irradiated for fractionated 5*30sec with 1min dark recovery periods. Images taken with x63 lens.

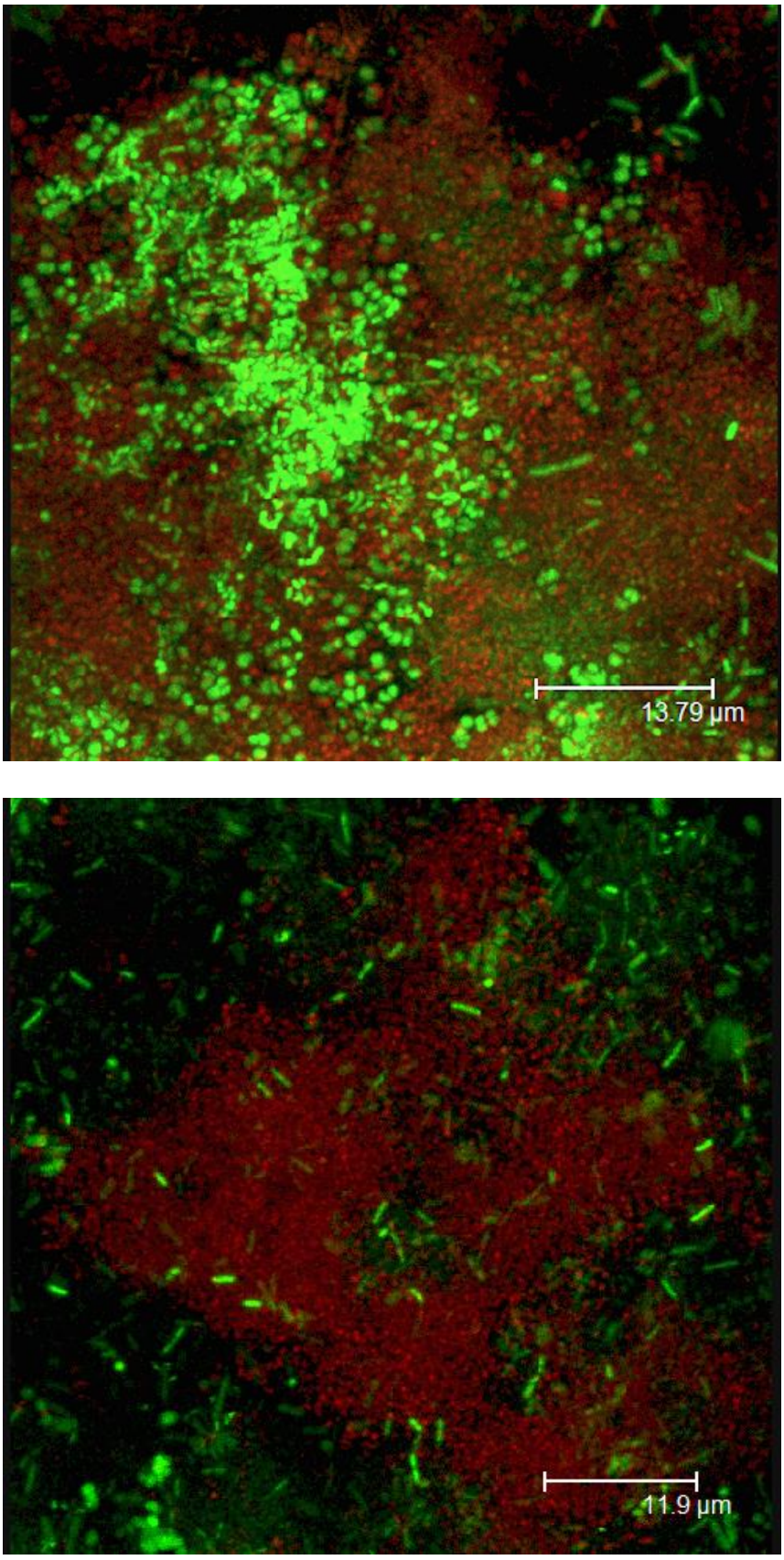


Figure 33 CLSM Images of *in vivo* dental plaque biofilm samples incubated with erythrosine (220µM) for 2min (Arm-2) and irradiated for continuous 15min. Images taken with x63 lens.

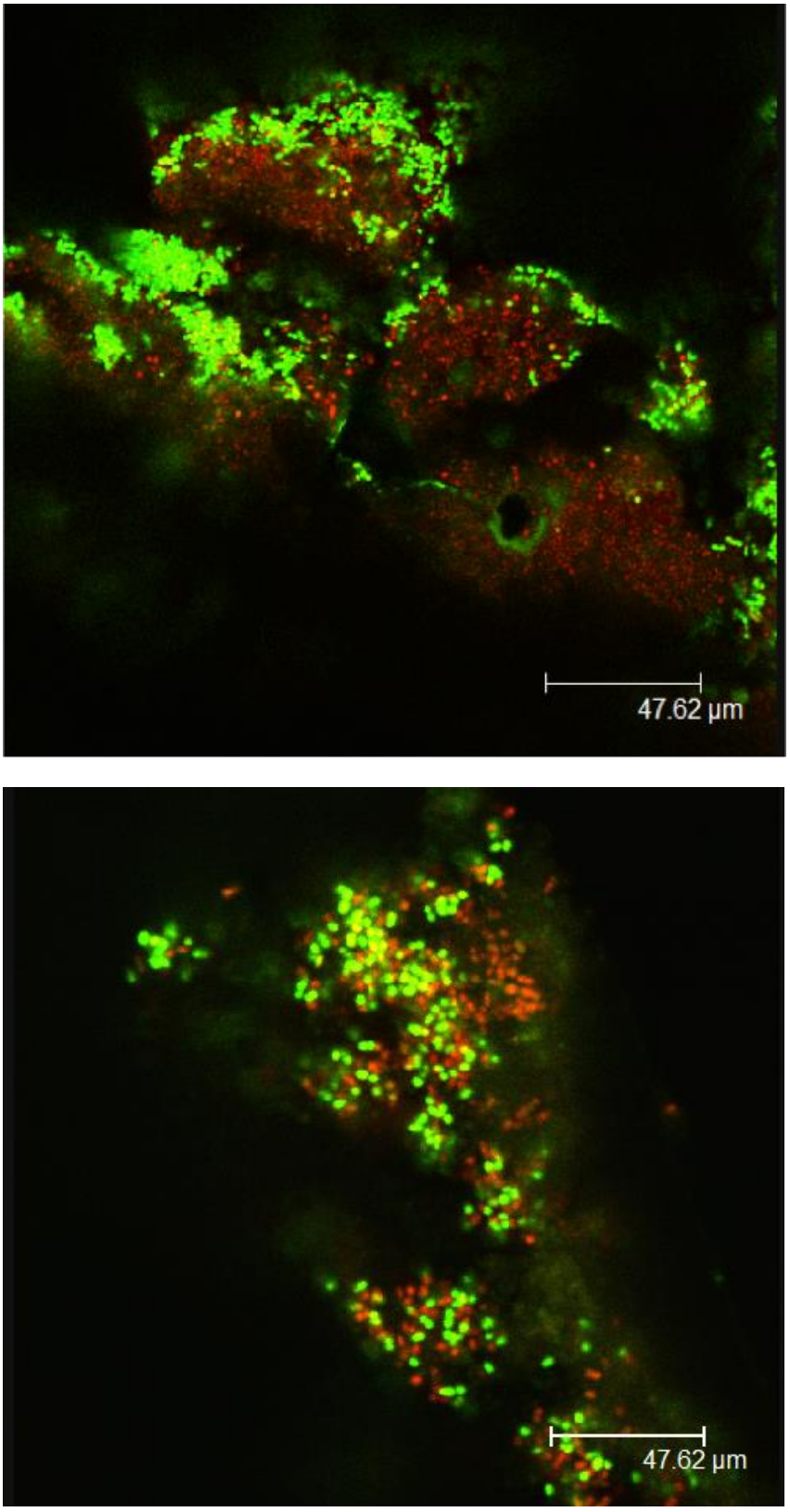


Figure 34 CLSM images of *in vivo* dental plaque biofilm samples incubated with erythrosine (220µM) for 2min (Arm-2) and irradiated for fractionated 5*30sec with 1min dark recovery periods. Images taken with x63 lens.

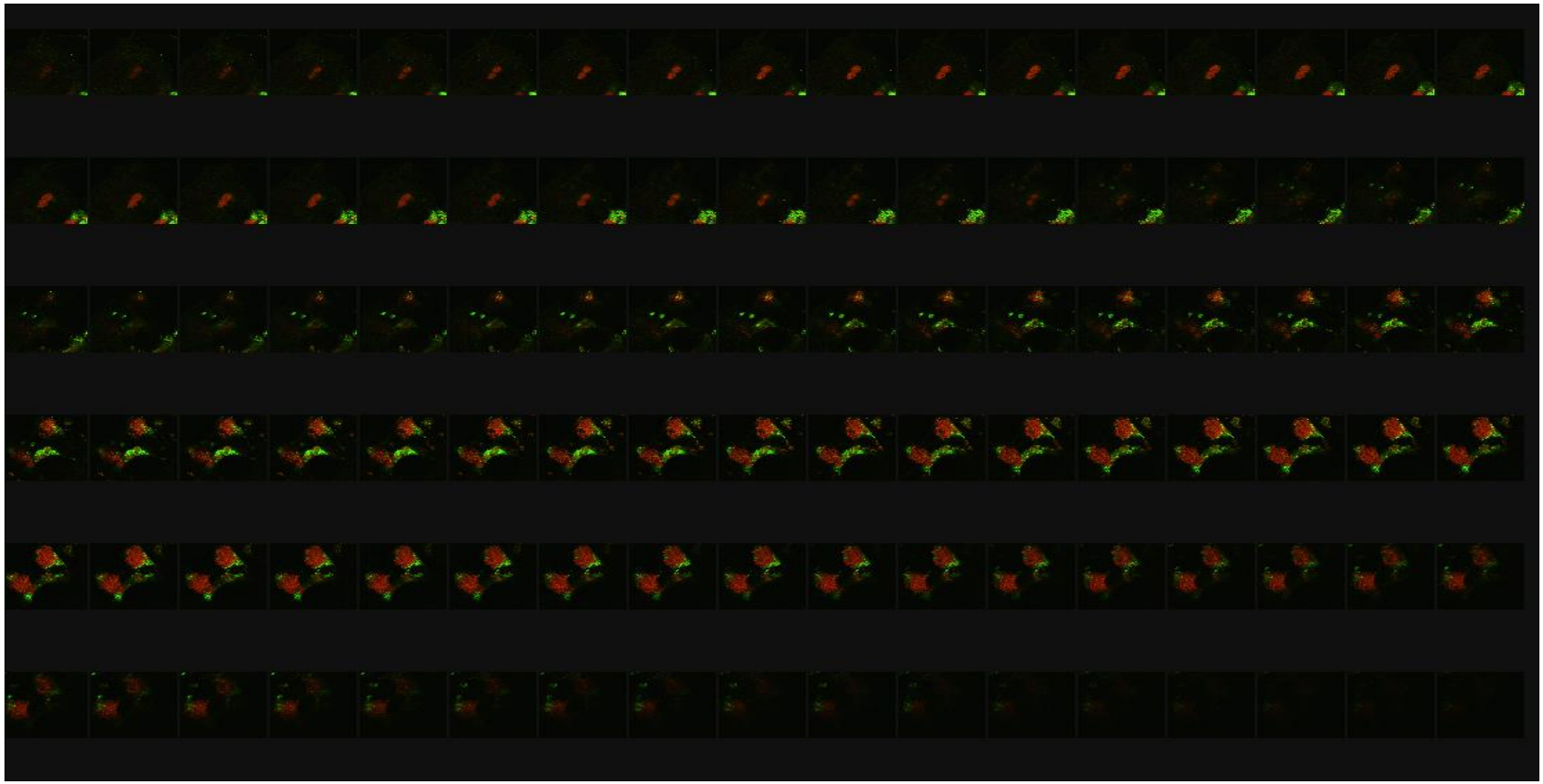


Figure 35 Confocal optical sections of 14-days-old dental plaque biofilm on an enamel slab worn *in situ* and treated with PDT. Sequence of 102 horizontal (x-y) sections at 0.5µm increments, from the enamel slab (top left) to the outer layers (bottom right).

In the studied samples, as shown in Figure 35, the proportion of vital cells were lower adjacent to the enamel surface (top left), increased in the z-axis towards the central parts, and decreased again towards the outer layers (bottom right). The thickness of the *in vivo* formed biofilms for 14-days as assessed by CLSM ranged from 395µm to 610µm (control samples) depending on the individual. Even with one sample, the plaque height varied. While the biofilm thickness following PDT ranged from 51µm to 491µm.

Chapter 4 Discussion

In the present study, the antimicrobial effect of PDT on *in vivo* formed plaque biofilms was investigated using erythrosine and a tungsten filament lamp (white light). This was based on the previous studies by Tahmassebi *et al.* (2015), Wood *et al.* (2006) and Metcalf *et al.* (2006).

Clinical treatment time is an important factor in delivering care to patients. This is particularly necessary for special needs patients and children with short attention span. PDT effect on bacterial killing is well documented in the literature. However, it might involve a long treatment time. Therefore, investigating a more clinically acceptable treatment time is required, while still ensuring effective killing of bacteria.

As mentioned earlier in the introduction, different irradiation regimes including continuous and fractionated irradiations with different lengths have been used and compared in the previous PDT studies. For example, Metcalf *et al.* (2006) study revealed that application of fractionated light (1min light pulses for 5 times separated by 5min dark periods or 30sec light pulses for 10 times separated by 2min dark periods) to *S.mutans* biofilms grown *in vitro* has an increased cell-killing efficacy compared with continuous irradiation at the same overall light dose (5min) and, also, compared to longer periods of continuous light (10, 15, and 30 min). Whereas, Tahmassebi *et al.* (2015) reported that both 15min continuous light and fractionated light of similar overall treatment dose were equally effective in bacterial killing and both were more effective than continuous irradiation of 2 and 5min lengths.

However, an irradiation time of 15min or more would not be realistic in a clinical setting. As a comparison with previous work (Tahmassebi *et al.*, 2015) in the present study we looked at bacterial killing following 15min continuous irradiation and, also, following a fractionated regime of 30sec light pulses for 5 times separated by 1min dark periods (Total light treatment

length= 7.5min), which is half the previous time, in an attempt to reduce the overall PDT treatment time to enhance its clinical usefulness.

Additionally, among other factors, the effectiveness of antimicrobial PDT has been reported to be dependent on the incubation time, which is the period required by the photosensitizer to remain in contact with the microorganisms before irradiation (Andrade *et al.*, 2013). Various erythrosine-mediated PDT studies have used different incubation times prior to irradiation. However, there were no reports in the literature that have compared the efficacy of different incubation times on *in vivo* formed biofilms. Therefore, in the current study, bacterial killing following different incubation times, including 2, 5, and 15min, were looked at, attempting to further reduce the overall treatment time for PDT.

Thus, in the first phase of the study, PDT effect on *L.casei* spp. in planktonic form was investigated using different incubation times to determine the most effective bactericidal time. Once this was determined, this finding was tested on *in vivo* formed plaque biofilms to investigate whether it has any effect on its bacterial viability, and then the results were compared with the results of the 1st arm of the *in situ* study, where 15min incubation time was used, attempting to reduce the overall PDT time.

4.1 *In vitro* study

4.1.1 PDT on planktonic cultures of *L.casei*

L.casei bacteria was chosen to be tested under PDT as it is one of the most commonly isolated species from oral samples (Bagg *et al.*, 2006) and at the same time they are associated with advanced caries lesions and carious dentine (Marsh *et al.*, 2009). As shown in the results section (3.1.1.2), different incubation times (2, 5 and 15min) of these bacteria with erythrosine presented no statistical differences in their bacterial viability counts.

Additionally, the samples that were exposed to erythrosine alone and no irradiation found to have some effect on their bacterial viability counts. This confirmed the antimicrobial property of erythrosine that is well documented in the literature against Gram-positive and Gram-negative oral bacteria (Marsh *et al.*, 1989a; Baab *et al.*, 1983; Caldwell and Hunt, 1969; Begue *et al.*, 1966). However, the combined treatment of light irradiation in the presence of erythrosine caused complete death of these bacterial cells. These results were in agreement with Andrade *et al.* (2013) whose study reported that a long incubation time is not required for Curcumin-based PDT on planktonic cultures of *Candida* spp. In their study, 5, 10, and 20min incubation times with Curcumin were used. However, there were no reports in the literature that have compared different incubation times with erythrosine to compare our results with.

As a result of these findings, it was important to test if this finding was also applicable on the *in vivo* formed biofilms. Therefore, 2min incubation time was used in the 2nd arm of the *in situ* study to investigate whether it has any effect on bacterial viability and then compare the results with the 1st arm of the *in situ* study, where 15min incubation time was used, attempting to reduce the overall PDT time.

4.2 *In situ* study

4.2.1 Sample size

The sample size of this study was based on the previous pilot study (Tahmassebi *et al.*, 2015) and it was determined following statistical advice by a qualified biostatistician, University of Leeds. Fifteen participants were recruited in the previous pilot study where their plaque samples were treated with erythrosine-based PDT and, then, the total bacterial counts were measured. Whereas the sample size used in other PDT studies on *in vivo* formed biofilms, using different types of photosensitizer and/or light source,

were 8, 20 and 21 participants (Teixeira *et al.*, 2012; Lima *et al.*, 2009; Wood *et al.*, 1999). Therefore, based on the previous literature and the time available for patient recruitment, 18 participants were recruited to ensure a sufficient sample size that would successfully complete the study.

4.2.2 *In situ* appliance

A mandibular removable appliance, which was introduced by Koulourides and Volker (1964) was used in this present study with some modifications to collect the undisturbed layer of plaque. Similar design was used in the previous pilot study (Tahmassebi *et al.*, 2015) without causing severe problems or discomfort to participants. This appliance did not interfere with the participants' daily oral hygiene procedures, allowing them to remove it during tooth brushing as well as during eating and drinking. This is also allowed the accumulation of dental plaque with no disruption, since the analysis of undisturbed human dental plaque biofilms has been considered as the best method for studying the architecture and physiology of biofilm formation on dental materials and, also the effect of antimicrobial therapies on the biofilm structure (Tomás *et al.*, 2010).

The appliance used in the current study contained three enamel slabs inserted in the right buccal flange, and another three in the left buccal flange in a way that biofilm growth is protected and not disturbed by the tongue and the cheeks (Figure 18). In order to ensure that the baseline measurements of the accumulated plaque in all the six slabs were comparable, we investigated whether there was a variation of bacterial viability counts among these slabs in one appliance. No difference in the total bacterial counts (CFU/ml) was found among the six *in vivo* formed biofilm samples for 14-days. This was in agreement with studies carried out by Tahmassebi *et al.* (2015) and Arweiler *et al.* (2004).

4.2.3 Results of Arm-1- Irradiation time

The aim of this arm of the *in situ* study was to determine the most effective irradiation time that would significantly reduce the total viable bacterial counts among the control and treatment groups when using 15min incubation time in 220μM erythrosine. Two irradiation regimes were used: a) 15min continuous light; b) 30sec light pulses for five times separated by 1min dark periods (fractionated irradiation), aiming to reduce the overall PDT clinical time.

The results demonstrated that incubation of the biofilm samples with erythrosine alone and no irradiation had a small effect on the CFU values compared with the control group (0.26 $\log_{10}$). Whereas, in the treatment groups that were treated with erythrosine and irradiation resulted in a bacterial reduction of 1.33-1.36 $\log_{10}$ when the 15min continuous light was applied and 1.02-1.13 $\log_{10}$ reduction when the fractionated light was applied.

In terms of the percentage reduction of total bacterial counts, both irradiation regimes, 15min continuous light and 30sec*5 fractionated light separated by 1min dark periods, were found to be statistically significantly different than the control groups. In addition, the bacterial killing following these two irradiation regimes were found to be not statistically significantly different, up to 95% and 91% bacterial killing, respectively. Therefore, both these irradiation regimes were found to be equally effective in bacterial killing when 15min incubation time with 220μM erythrosine was used.

These findings agreed with the previous studies of Tahmassebi *et al.* (2015); Wood *et al.* (2006) and Metcalf *et al.* (2006). Tahmassebi *et al.* (2015) evaluated the photodynamic activity of erythrosine (220μM), irradiated with a tungsten filament lamp on a 14-days *in vivo* formed biofilms. Irradiation regimes of 15min continuous light and fractionation regime of 1min light pulses for 5 times separated by dark periods of 2min were both found to be superior in bacterial killing compared to 2min and 5min continuous irradiation

when 15min incubation time with erythrosine was used. Both of these irradiation regimes were also equally effective in bacterial killing (≈ 96% and 93%, respectively). Their results suggested that bactericidal effect of PDT was dose-dependent, in which the higher the concentration of photosensitizer and the longer the duration of the light that is used, the more the damage to the target tissues is expected (Tahmassebi *et al.*, 2015; Konopka and Goslinski, 2007; Metcalf *et al.*, 2006). Whereas, Metcalf *et al.* (2006) demonstrated that fractionation of light (1min light pulses for 5 times separated by 5min dark periods and 30sec light pulses for 10 times separated by 2min dark periods) during irradiation of *S.mutans* biofilms grown *in vitro,* treated with 15min incubation with 22μM erythrosine, resulted in a significantly increased bacterial killing by 1 to 1.7 $\log_{10}$ when compared to 5, 10, 15 or 30min continuous light regimes.

Interestingly, in the current study, when the irradiation time was reduced to 2.5min, which was applied in 30sec light pulses for 5 times separated by 1min dark periods, the bacterial killing was effective and equal to the bacterial killing obtained from the 15min continuous irradiation. This can be due to the replenishment of oxygen during the dark periods for erythrosine to initiate more photochemical reactions or maybe due to the replenishment/redistribution of the erythrosine itself within the biofilm during these dark periods as the photodynamic activity results to lessen erythrosine levels due to photo-bleaching (Metcalf *et al.*, 2006; Wood *et al.*, 2006).

Furthermore, Wood *et al.* (2006) evaluated the effectiveness of erythrosine, irradiated with tungsten filament lamp on *S.mutans* biofilms grown *in vitro*. Different photosensitizers were compared, including 22μM erythrosine, methylene blue and photophrin. Biofilms were incubated for 15min in their solutions then irradiated for continuous 15min. Erythrosine was found to be the most effective photosensitizer, resulting in a 2.2 $\log_{10}$ reduction of bacterial counts for 24h biofilms and a 3.0 $\log_{10}$ reduction for 288h biofilms. The possible explanation for this finding was that *S.mutans* are photo-inactivated mainly by membrane damage that resulted from lipid peroxidation, which is the likely mechanism of action of erythrosine,

whereas, methylene blue causes damage to bacterial cell DNA and to a lesser extent the outer cell membrane (Wood *et al.*, 2006).

Overall, the bactericidal effect of 15min incubation time with erythrosine and irradiation with either 15min continuous light or different regimes of fractionated light resulted in a significant reduction of total bacterial counts in both *in vitro* and *in vivo* formed biofilms. However, the bacterial reductions observed in the previous studies on *S.mutans* biofilms grown *in vitro* were higher (3-3.7 $\log_{10}$) (Metcalf *et al.*, 2006; Wood *et al.*, 2006) than those found in the current study on *in vivo* formed biofilms (1-1.36 $\log_{10}$). This can be explained by the fact that *in vitro* formed biofilms have a limited number of species and have a composition and structure that is different to those in *in vivo* formed biofilms (Watson *et al.*, 2005). As a result the analysis of undisturbed human dental plaque biofilms has been considered as the best method for studying the effect of antimicrobial therapies on the biofilm structure (Tomás *et al.*, 2010).

4.2.4 Results of Arm-2- Incubation time

The aim and the protocol for the 2nd arm was exactly the same as the 1st arm, except; the incubation time of samples was different (2min), which was determined from the initial *in vitro* study on planktonic cultures of *L.casei* bacteria, aiming to investigate whether this shorter incubation time has any effect on bacterial viability of *in vivo* formed biofilms and then compare the results with the 1st arm of the *in situ* study, where 15min incubation time was used, attempting to reduce the overall PDT time.

The results of this arm showed that incubation of the biofilm samples with erythrosine alone and no irradiation had a minor effect on the CFU values compared with the control group (0.1 $\log_{10}$). Whereas, in the treatment groups that were treated with erythrosine and irradiation resulted in a bacterial reduction of 1.14-1.24 $\log_{10}$ when the 15min continuous light was

applied and 0.63-0.81 log_{10} reduction when the fractionated light was applied.

In terms of the percentage reduction of total bacterial counts, both irradiation regimes, 15min continuous light and 30sec*5 fractionated light separated by 1min dark periods, were found to be statistically significantly different than the control groups. But, when comparing the bacterial killing following these two irradiation regimes, they were statistically significantly different, up to 93% and 64% bacterial killing respectively. This finding was not observed in arm-1 of the study where 15min incubation time was used. Therefore, 15min continuous light was found to be the most effective for bacterial killing when 2min incubation time with 220μM erythrosine was used.

4.2.5 Comparison of cell death percentage in *in vivo* formed biofilms between Arm-1 (15min incubation time) and Arm-2 (2min incubation time)

Surprisingly, when applying continuous light with either incubation time, 2 or 15min, there was no significant difference in the viability of bacteria. Whereas, when applying fractionated light, there was more cell death when the 15min incubation time was used, which was up to 91% compared to 64% with the 2min incubation time. As stated earlier, there were no reports in the literature that have compared the efficacy of different incubation times on *in vivo* formed biofilms to compare our findings with. Instead, some erythrosine-based PDT studies have used 5min incubation time (Pereira *et al.*, 2013; Rolim *et al.*, 2012; Chibebe Junior *et al.*, 2010) while others have used 15min incubation time (Tahmassebi *et al.*, 2015; Metcalf *et al.*, 2006; Wood *et al.*, 2006) and both times showed significant reduction in the bacterial viability. However, the reasons for this difference in the present study are unclear, but may be the less contact time with the photosensitizer could have limited the uptake of its molecules into the bacterial cells and as a consequence less cell death have occurred. Therefore, it would be good to

look at the uptake of the photosensitizer into the bacterial cells in future work.

Moreover, these findings definitely have supported the fact that the bactericidal effect of PDT works in a dose-dependent manner. However, even though there was a statistical difference in the percentage reduction of total bacterial counts between the two irradiation regimes, there was a 64% reduction compared to the control, which still means that PDT was not unsuccessful but was less effective.

This outcome partly contradicted the results found in the *in vitro* phase of the current study, where the different incubation times (2, 5 and 15min) presented no statistical differences on the percentage reduction of *L.casei* counts. Therefore, this showed and supported the fact that the effect of PDT on dental biofilms is different than on planktonic cells. This is either due to the structural variation in the bacterial cell membranes or the presence of other components, such as extracellular matrix and quorum-sensing factors in dental biofilms, rendering the photosensitizer-microorganism interaction (Huang *et al.*, 2012).

4.2.6 Assessment of bactericidal effects of PDT by CLSM

In the present study, CLSM was used to visualize PDT effects on *in vivo* formed biofilms. Numerous researchers have also used CLSM to study biofilm architecture and PDT effects on dental plaque biofilms (Wood *et al.*, 2006; Wood *et al.*, 1999; Netuschil *et al.*, 1998). It provides detailed compositional information such as differentiating between different morph-types of bacteria, like cocci, rods and filaments, especially when used in combination with fluorescent probes (Dige *et al.*, 2007; Lawrence *et al.*, 2003). The present CLSM analysis on *in vivo* formed biofilm sample (Figure 30) agreed with previous studies' findings on biofilm structure as it showed a heterogeneous architecture in terms of types of cells present, such as cocci,

rods and filaments, and also in terms of the overall structure where clumps of bacteria were surrounded by extracellular matrix and separated by voids/channels (black holes). These voids were thought to be filled with biological substances, such as extracellular polymeric substances (EPS) and glycoproteins and act as a circulatory system where oxygen and nutrients were available in the biofilm (Wood *et al.*, 2000). It extended through the whole thickness of the biofilm sample providing a link between the oral environment and the tooth surface (Auschill *et al.*, 2001; Wood *et al.*, 2000).

In terms of visualizing PDT effects on bacterial viability, CLSM provides images for instantaneous observation of surviving bacteria and affected bacteria. Few previous studies evaluated the antibacterial effect of PDT using erythrosine by CLSM analysis. However, these studies evaluated the effect of PDT against *in vitro* formed *S.mutans* biofilms (Lee *et al.*, 2013; Wood *et al.*, 2006). The antibacterial effect of erythrosine and a white light source (tungsten filament lamp) against *in vivo* formed dental plaque biofilm had never been tested under CLSM. In the present investigation, CLSM images showed that there was a marked difference in the bacterial viability between the control and the treated samples with PDT against *in vivo* formed biofilm. In the control samples (Figure 30) almost all cells were stained green, indicating viable bacteria with undamaged cell membranes. Whereas in the treated samples, either with continuous light or fractionated light, there was uneven spatial distribution of vital and dead cells with higher proportion of dead cells that were stained red/yellow. These findings corresponded with the findings of Lee *et al.* (2013) when PDT effects was investigated against *in vitro* formed biofilms using erythrosine and dental halogen curing light. However, in our investigation, we couldn't determine which treatment group was superior in terms of bacterial killing as no quantitative technique was performed. Nevertheless, there are several explanations for the uneven distribution of these vital and dead cells throughout the biofilms such as the availability of oxygen for PDT to have an effect or may be due to having diverse populations of cells that have different susceptibility to PDT because of their different structures. Interestingly, this was seen in the plaque samples

in Figure 31 and Figure 33, where the rod shaped bacteria is seem to be less susceptible to PDT than the cocci shaped bacteria as evidenced by the green fluorescence (undamaged membranes).

Moreover, in the studied sample (Figure 35) the proportion of vital cells were increased in the z-axis towards the central parts. This was in agreement with previous studies' findings by Auschill *et al.* (2001). This high proportion of viable bacteria found in the central part of the biofilm sample and surrounding the pores and channels has been explained to be due to having a direct access to nutrients diffusing through these voids (Auschill *et al.*, 2001). These voids also has been considered as a significant factor in relation to the penetration and distribution of molecules, including antimicrobial agents. However, on the other hand, biofilm matrix, which contains polysaccharides, proteins and other metabolites, is thought to protect the microorganisms from the environment and could prevent diffusion of these antimicrobial agents in the biofilm (Marsh, 2005; Marcotte *et al*., 2004; Robinson *et al*., 1997). This also may explain the high proportion of viable bacterial surrounding the pores and channels. As a result, microorganisms in dental plaque biofilms are much more resistant to antimicrobial agents than planktonic bacteria, which require less antimicrobial agent to be killed than that is needed to destroy dental biofilms (Marsh *et al*., 2011; Auschill *et al*., 2001).

In terms of the thickness of the plaque biofilm formed for 14-days in the present investigation, as assessed by CLSM, this ranged from 395μm to 610μm depending on the individual. Even with one sample, the plaque height varied. However, the plaque thickness following PDT was less, ranging from 51μm to 491μm. This was in agreement with findings published by Wood *et al.* (1999) where the thickness of *in vivo* formed biofilm samples for 7-days following PDT, using phthalocyanine photosensitizer, were around half the thickness of the controls under their CLSM analysis. The researchers used both TEM and CLSM in their study. They reported that the

reason for the reduced thickness of plaque in the treated samples was due to damage of bacterial membranes, as evident in TEM, which lead to reduce cell-to-cell or cell-to-matrix binding and subsequent loss of bulk of biofilm.

In summary, the bactericidal effects of PDT as seen by CLSM has corresponded with our findings when using the microbiological analysis of viable bacterial count, indicating that a combination of microbiological techniques and microscopic techniques can help to achieve a realistic representation of PDT effect on *in vivo* formed dental plaque biofilms.

4.3 Future research

Evidently erythrosine-based PDT can cause significant reduction of *in vivo* formed plaque biofilms and might be useful for controlling dental plaque related diseases such as caries and periodontal disease. In addition, we have shown that improving the clinical usefulness of PDT by reducing the incubation time with erythrosine form 15min to 2min resulted in an equally effective bactericidal killing with 15min of continuous irradiation (≈95% and 93%, respectively). However, when we attempted to reduce the irradiation time further by fractionating the light dose, the results showed less effectiveness in total bacterial killing (≈64%) when a 2min incubation time was used. However, in order to determine the true effectiveness of PDT, we need to identify which bacteria within the plaque are most affected by the treatment.

Therefore, future work should investigate the bactericidal effect of PDT on specific bacteria, which have been identified as playing a major role in dental caries, such as *S.mutans.* Targeting of disease-causing bacteria would also be beneficial as it would not be appropriate to kill the entire oral microflora, as this would leave the patient open to opportunistic infections. This specific targeting can be achieved by coupling the photosensitizers with antibodies

specific for bacterial cell wall components (Soukos and Goodson, 2011; Meisel and Kocher, 2005).

Further work is now being undertaken to test the combined effect of 220μM erythrosine and white light on epithelial cells in order to minimize any possible normal tissue damage in the patient. Following this, well conducted *in situ* studies/trials are required to assess the effect of PDT clinically if they exist and compare it with the current best practice. This can be done by running a randomised controlled clinical trial investigating, for example, the effect of PDT as an adjunct to non-surgical treatment of deep periodontal pockets, comparing the periodontal clinical parameters such as plaque index, bleeding index, probing depths and clinical attachment levels at baseline and post-treatment in monthly intervals to assess for improvements. Additionally, the PDT effect, *in situ*, on the number of bacteria causing dental caries in patients with high caries risk, can be assessed by measuring the salivary levels of these bacteria before and after the PDT intervention on dental plaque biofilms. However, prior to the transition to clinical trials, the mode of delivery of PDT in oral cavity needs to established.

Although the tungsten filament lamp showed to be effective in the past and the current investigations using erythrosine-based PDT, the drawback of this light source is that it is bulky and, also, generates heat that might cause burning sensation *in vivo*, especially if applied for long times. Therefore, for clinical relevance, tungsten filament lamp might not be convenient for clinical use in patients, especially in a small area as the oral cavity and alternatives need to be considered to improve the delivery of light. One such alternative is the LED light source. It is already been used in dentistry as a curing light for restorative materials and, also, it is available in the market as in a chair-side bleaching light trays. It is small, portable and safe. In addition, it doesn't generate heat as the tungsten filament lamp. Several erythrosine-based PDT studies have used LED on the *in vitro* dental plaque biofilms and planktonic cultures and it showed a successful outcome (Pereira *et al.*, 2013; Rolim *et*

al., 2012; Chibebe Junior *et al.*, 2010). Therefore, the combination of the LED and erythrosine varnish should be considered and studied for PDT clinical trials in near future, especially that LED has a wavelength close to the wavelength that corresponds to the maximum absorption of erythrosine. Again, as both erythrosine and LED are commonly used in dental clinics, PDT will be available without additional cost. However, its cost effectiveness should be measured against the current best practice.

Chapter 5 Conclusions

From the results of this study on the antimicrobial effect of PDT on *in vivo* formed dental plaque biofilms using 220μM erythrosine and a tungsten filament lamp (white light), it can be concluded that:

1. There was no significant difference in the percentage reduction of total bacterial counts when using either 2min or 15min incubation times, plus an irradiation regime of continuous light (15min).

2. There was no significant difference in the percentage reduction of total bacterial counts when using either 15min continuous light (~95%) or 30sec*5 fractionated light separated by dark periods of 1min (~91%) when using a 15min incubation time with erythrosine.

3. There was a significant difference in the percentage reduction of total bacterial counts when using either 15min continuous light (~93%) or 30sec*5 fractionated light separated by dark periods of 1min (~64%) when using a 2min incubation time with erythrosine.

Therefore, improving the clinical usefulness of PDT by reducing its overall treatment time seems to be promising and effective in killing *in vivo* formed dental plaque biofilms.

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List of Appendices

Appendix A REC approval

NHS
Health Research Authority
NRES Committee South Central - Berkshire B
Whitefriars
Level 3, Block B
Lewins Mead
Bristol
BS1 2NT
Telephone: 01173 421383

19 August 2014

Mrs Aysha S Alsaif
Paediatric Dentistry Department, School of Dentistry, University of Leeds
level 6, Worsley Building
Clarendon Way, Leeds, W.Yorkshire
LS2 9LU

Dear Mrs Alsaif

Study title:	**Treatment of dental plaque biofilms using photodynamic therapy**
REC reference:	**14/SC/1226**
IRAS project ID:	**149271**

Thank you for your letter of responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the Chair.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Miss Lauren Allen, nrescommittee.southcentral-berkshireb@nhs.net.

Confirmation of ethical opinion- Favourable Opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations

NHS
Health Research Authority

involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Poster Advertisment]	2	14 August 2014

NHS
Health Research Authority

Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [University of Leeds insurance]	1	14 August 2014
Other [Jinous Tahmassebi CV]	1	12 August 2014
Other [Participants' instructions and restrictions of wearing the appliance]	1	12 August 2014
Participant consent form [Consent form]	2	20 June 2014
Participant information sheet (PIS) [PIS]	5	14 August 2014
REC Application Form [REC_Form_08082014]		08 August 2014
Research protocol or project proposal [Research Protocol]	6	20 June 2014
Summary CV for Chief Investigator (CI) [CI's CV]	1	20 May 2014
Summary CV for student [CI's CV]	1	20 May 2014
Summary CV for supervisor (student research) [Simon Wood's CV]	1	20 May 2014

Statement of compliance

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

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

14/SC/1226	**Please quote this number on all correspondence**

NHS
Health Research Authority

With the Committee's best wishes for the success of this project.

Yours sincerely

PP

Dr John Sheridan
Chair

Email: nrescommittee.southcentral-berkshireb@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*

Copy to: *Mrs Clare Skinner*

Ms Anne Gowing, Leeds Teaching Hospitals NHS Trust

Appendix B R&I approval

The Leeds Teaching Hospitals NHS
NHS Trust

Amanda Burd

Research & Innovation
Leeds Teaching Hospitals NHS Trust
34 Hyde Terrace
Leeds
LS2 9LN

Tel: 0113 392 0162
Fax: 0113 392 0146

r&d@leedsth.nhs.uk
www.leedsth.nhs.uk

14/01/2015

Mrs Aysha S Alsaif
Paediatric Dentistry
Level 6, Worsley Building
University of Leeds

Dear Mrs Aysha S Alsaif

Re: NHS Permission at LTHT for: Treatment of dental plaque biofilms using photodynamic therapy
LTHT R&I Number: DT14/11310 (149271/WY):
REC: 14/SC/1226

I confirm that *NHS Permission for research* has been granted for this project at The Leeds Teaching Hospitals NHS Trust (LTHT). NHS Permission is granted based on the information provided in the documents listed below. All amendments (including changes to the research team) must be submitted in accordance with guidance in IRAS. Any change to the status of the project must be notified to the R&I Department.

Permission is granted on the understanding that the study is conducted in accordance with the *Research Governance Framework for Health and Social Care*, ICH GCP (if applicable) and NHS Trust policies and procedures available at http://www.leedsth.nhs.uk/research/

This permission is granted only on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet Conditions of Approval.

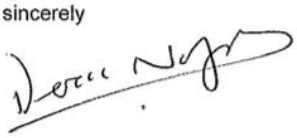
If you have any queries about this approval please do not hesitate to contact the R&I Department on telephone 0113 392 0162.

Indemnity Arrangements

The Leeds Teaching Hospitals NHS Trust participates in the NHS risk pooling scheme administered by the NHS Litigation Authority "Clinical Negligence Scheme for NHS Trusts" for: (i) medical professional and/or medical malpractice liability; and (ii) general liability. NHS Indemnity for negligent harm is extended to researchers with an employment contract (substantive or honorary) with the Trust. The Trust only accepts liability for research activity that has been managerially approved by the R&I Department.

The Trust therefore accepts liability for the above research project and extends indemnity for negligent harm to cover you as investigator and the researchers listed on the Site Specific Information form. Should there be any changes to the research team please ensure that you inform the R&I Department and that s/he obtains an appropriate contract, or letter of access, with the Trust if required.

Yours sincerely

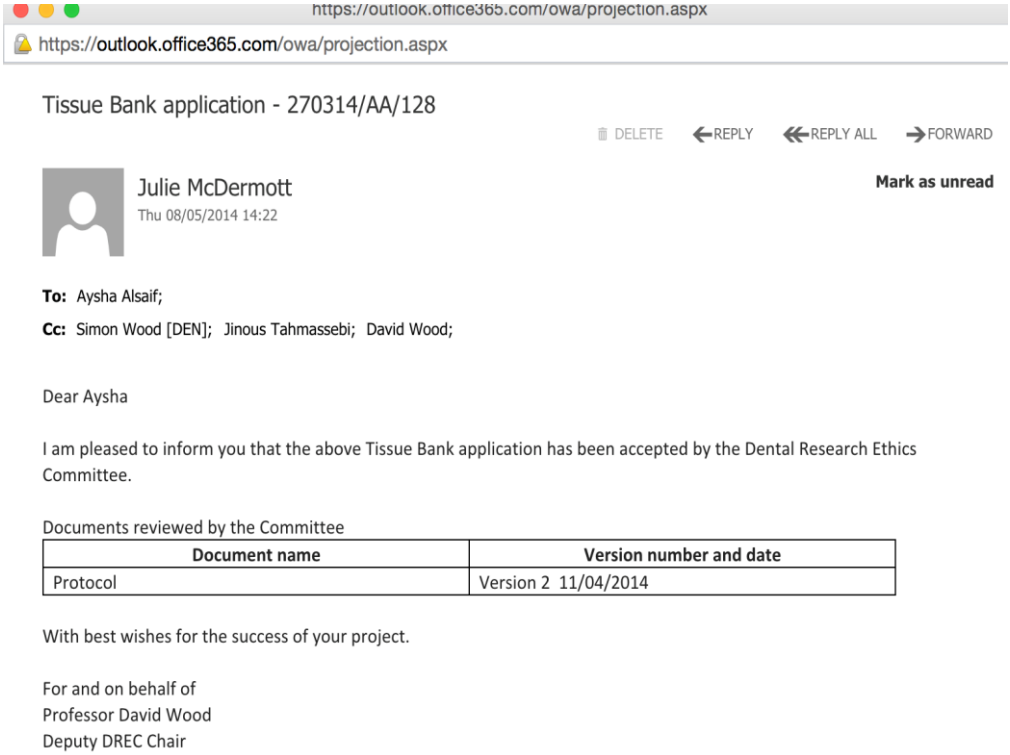


Dr D R Norfolk
Associate Director of R&I

Approved documents
The documents reviewed and approved are listed as follows:-

Document	*Version*	*Date of document*
NHS R&D Form	3.5	09/10/2014
SSI Form	3.5	13/01/2015
Directorate Approval	AS	13/01/2015
Evidence of Insurance	U of L	14/08/2014
Poster	2	14/08/2014
Participant Instructions	1	12/08/2014
Consent Form	2	20/06/2014
Participant information sheet	5	14/08/2014
REC Letter confirming favourable opinion		19/08/2014
Protocol	6	20/06/2014

Appendix C Tissue bank approval



https://outlook.office365.com/owa/projection.aspx

https://outlook.office365.com/owa/projection.aspx

Tissue Bank application - 270314/AA/128

DELETE REPLY REPLY ALL FORWARD

Julie McDermott
Thu 08/05/2014 14:22

Mark as unread

To: Aysha Alsaif;

Cc: Simon Wood [DEN]; Jinous Tahmassebi; David Wood;

Dear Aysha

I am pleased to inform you that the above Tissue Bank application has been accepted by the Dental Research Ethics Committee.

Documents reviewed by the Committee

Document name	Version number and date
Protocol	Version 2 11/04/2014

With best wishes for the success of your project.

For and on behalf of
Professor David Wood
Deputy DREC Chair

Appendix D Poster advertisement

Are you 18+ years old or older?

Would you be interested in participating in a research study?

We are investigating a new method to control accumulation of dental plaque.

What we will need from you is to wear an appliance, which will allow us to collect samples of your plaque.

You will receive £130 for taking part in the study.

For further information, please contact Aysha Alsaif, at the University of Leeds
Tel: 07922883889 or
Email: dnasa@leeds.ac.uk

Appendix E Consent Form

UNIVERSITY OF LEEDS

Patient Identification Number for this trial:

CONSENT FORM

Title of Research Project: Treatment of dental plaque biofilms using photodynamic therapy

Name of Researcher: Aysha Alsaif

Initial the box if you agree with the statement to the left

1. I confirm that I have read and understand the information sheet dated 14/08/2014 (Version 5) explaining the above research project, and I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without there being any negative consequences. In addition, should I not wish to answer any particular question(s), I am free to decline. ☐

3. I understand that my responses will be kept strictly confidential. I give permission for members of the research team to have access to my anonymised responses. I understand that my name will not be linked with the research materials, and I will not be identified or identifiable in the report(s) that result from the research. ☐

4. I understand that relevant sections of data collected during the study may be looked at by individuals from regulatory authorities (university of Leeds), where it is relevant to my taking part in this research. I give permission for these individuals to have access to my personal data. ☐

5. I agree to take part in the above research project and will inform the researcher should my contact details change. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

When completed: 1 copy for participant; 1 (original) to be kept with the project's main documents that must be kept in a secure location.

Appendix F Participant information sheet

Version 5, 14/08/2014

UNIVERSITY OF LEEDS

Participant Information Sheet

Thank you for expressing an interest in our forthcoming study. Please find below more information about the study, which we hope will answer some of the questions you may have.

Title of Study: **Treatment of dental plaque biofilms using photodynamic therapy**
Ethics Committee Ref No: 14/SC/1226

You are being invited to take part in the above research study, but before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully, and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of this study?
The aim of this study is to find out about a new method for the control of dental plaque. This method, called photodynamic therapy, uses a dye that is currently used widely by dental practitioners in the clinic to stain and visualise dental plaque. When the dye is exposed to light, it can kill the bacteria that make up the dental plaque. We aim to further characterise how we can effectively use this method to kill plaque bacteria.

Why have I been chosen?
All we ask for participants is that they are willing to take part in the study; that they are at least 18 years old; that they are in general good health, not pregnant or smokers and that they have at least 20 natural teeth. Before you are enrolled on the study, you will need to be 'screened'. This will involve a short dental examination including measuring saliva flow rate (by spitting in a plastic tube), to enable us to establish whether you are suitable for inclusion in the study. Also, we will ask you a few simple questions about your general health. Let us stress that this information is kept confidential and that your saliva samples will be disposed according to the Human Tissue Authority Code of Practice immediately following measurements of the salivary flow rate.

Do I have to take part?
Participation in this study is entirely voluntary. If you decide to participate, you will be given this information sheet to keep, and be asked to complete and sign a consent form to show that you agree to take part in the study. If after reading and thinking about the information given, you decide you would not like to take part that is fine. Even if you decide to take part in the study, you are still free to withdraw your permission at any time without giving a reason, unless you want to. If you do decide to withdraw from the study at any point, please let us reassure you that there won't be any negative consequences to that.

What will be involved if I take part in this study?

If you decide you would like to take part and our screening procedures identify you as a suitable participant, you will have a professional scaling and polishing and a removable appliance similar to an orthodontic plate will be constructed for you. You will have to wear the removable appliance, which contains six sterilised enamel pieces. The appliance should be worn at all times for two separate periods of two weeks each (total of four weeks), except when eating, drinking and tooth brushing. We will show you how to remove and re-insert the appliance, and you will have the opportunity to ask any questions, and to make sure you are happy and confident in using it before starting the study. The study consists of two periods and each period will last approximately two or three weeks, therefore the whole study time will be approximately two months. You will need to visit us two times in each period, a total of about once per week. These visits are to provide you with the appliance and collect it from you at the end of each study period. The appliance will be collected by the researcher (Aysha Alsaif), and the enamel pieces will be removed in the laboratory. Let us stress that this information is kept confidential and that your collected plaque samples will be disposed according to the Human Tissue Authority Code of Practice immediately following bacterial measurements.

What do I have to do?
You will need to agree to wear the appliance that is constructed for you, and agree to remove it when eating, drinking and brushing your teeth. You will also need to come into the School of Dentistry, University of Leeds, at the dates and times agreed. We realise that the whole procedure may cause you some discomfort initially, but once you get used to the routine, we feel you will find it easy to stick to. In recognition of any inconvenience, you will be paid £130 for taking part in the study. This money will be paid to you at the end of your last visit. In order for us to pay, you will need to complete a bank details form, and provide us with your National Insurance number. This information will be held confidentially.

What are the disadvantages?
The only disadvantage to you as a participant will be that you might find it slightly inconvenient wearing the appliance initially, however, this will disappear in the following day as you will get used to the appliance. If there is any pain, discomfort, or any other problems with the appliance at home (e.g. accidentally broken) please contact us on the telephone number **07922883899 (Mrs Aysha Alsaif-Researcher)** and we will arrange an appointment to adjust the appliance if needed.

What are the possible benefits of taking part?
There are no direct immediate benefits to you for participation in our study. However, you will be helping us to develop what we hope will be a new way of controlling dental plaque.

Will taking part in this study be kept confidential?
Yes, any information we gather will be kept strictly confidential. You will be not identified by name in any reports of the study.

What will happen to the results of the research study?
We will report on the results of this study as a research thesis for a higher degree. We hope to publish the results in an international journal and present our findings at both national and international meetings. You will not be identified by name in any reports we write.

What if something goes wrong?

If you have any concerns about any aspect of the study, please do not hesitate to speak to the researchers who will do their best to answer your question (Aysha Alsaif 07922883899). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the Leeds Dental Institute. In the event that something does go wrong and you are harmed during the study, and this is due to someone's negligence, then you may have grounds for a legal action for compensation against the University of Leeds, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

Who is organizing and funding the research?
This study is carried out as an educational project as part of a professional doctorate programme in Paediatric dentistry. It is carried out by the research team at the Cariology laboratory and Oral Microbiology laboratory at School of Dentistry, University of Leeds. More specifically, the research will be carried out by Mrs Aysha Alsaif and supervised by the following members of staff:

- Dr Simon Wood- Senior Lecturer, Deputy Director of Student Education and Programme Lead for the Professional Doctorate and MSc and Chair of the Postgraduate Framework Committee, School of Dentistry, University of Leeds.
- Dr Jinous Tahmassebi- Associate Professor and Specialist in Paediatric Dentistry, School of Dentistry, University of Leeds.

Who has reviewed this research project?
All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given a favourable opinion by the South-Central Berkshire B Research Ethics Committee.

Contact information:

Name	Telephone	E-mail address
Mrs Aysha Alsaif	07922883899	dnasa@leeds.ac.uk
Dr Simon Wood	01133438061	s.r.wood@leeds.ac.uk
Dr Jinous Tahmassebi	01133436138	j.tahmassebi@leeds.ac.uk

Thank you for taking the time to read this information sheet.

1 copy for participant; 1 (original) to be kept with the project's main documents that must be kept in a secure location.

Appendix G Participants' instructions and restrictions of wearing the appliance

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Participants' instructions and restrictions of wearing the appliance

1. Wear the appliance all the times except when eating, drinking and teeth brushing. At these times, place the appliance in damp gauze inside a plastic case that will be provided to you.

2. The appliance should be worn for two separate periods of two weeks each (total of four weeks).

3. Make sure that you fit the appliance as instructed by the researcher. When removing, pull only on the clasps at the side of your mouth with your fingers. Do not use your tongue to remove or insert the appliance as this can fracture the wire clasps.

4. You might find it slightly inconvenient wearing the appliance initially, however, this will disappear in the following day, as you will get used to the appliance. If there is any pain or discomfort, please contact us on the telephone number **07922883899 (Mrs Aysha Alsaif-Researcher)** and we will arrange an appointment to adjust the appliance if needed.

Appendix H Case Record Form

School of Dentistry

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Leeds LS2 9LU

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E dentistry@leeds.ac.uk

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Participant Initials: __ __ __ Participant DOB: __ __ / __ __/ __ __ __ __

Screening No.: __ __ __

Participant ID No.: __ __ __

Case Record Form

Treatment of dental plaque biofilms using photodynamic therapy

Ethics Committee Ref No: 14/SC/1226

Academic Supervisors

- Dr Simon Wood
  (Oral Biology department)
- Dr Jinous Tahmassebi
  (Paediatric Dentistry department)

Research Investigator

Mrs Aysha Alsaif

(Paediatric Dentistry department)

Participant Initials: __ __ __ Screening No.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

Screening Visit **Date of Visit: __ __ / __ __/ __ __ __ __**

Participant's Information Sheet **Version No. ___ Dated: __ __/__ __/__ __ __ __**	**Date Participant given copy of PIS:** **__ __/__ __/__ __ __ __**
Participant's Consent Form **Version No. ___ Dated: __ __/__ __/__ __ __ __**	**Date of informed consent :** **__ __/__ __/__ __ __ __** **Time of informed consent:** **__ __: __ __ (hh:mm)**

- **Medical History**

List any relevant previous and current medical conditions (including allergies) and surgeries that have been experienced by the participant in the table below*

Any medical conditions to report? □ Yes □ No

Medical Condition	Start Date	On-going	Stop date (If applicable)
	…/……/……	YES/ NO	…/……/……
	…/……/……	YES/ NO	…/……/……
	…/……/……	YES/ NO	…/……/……
	…/……/……	YES/ NO	…/……/……
	…/……/……	YES/ NO	…/……/……
	…/……/……	YES/ NO	…/……/……

*Note that if any treatment(s) is/are currently being taken for any of the above conditions this (these) must be recorded in the following table (Current/Concomitant medication)

- **Current/ Concomitant medication**

Drug's name	Reason for medication	Dosage	Date started	Date stopped (If applicable)

Researcher's Signature:…… Date __ __/__ __/__ __ __ __

Participant Initials: __ __ __ Screening No.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

SCREENING VISIT **Date of Visit: __ __ / __ __/ __ __ __ __**

Inclusion Criteria Checklist*

	Yes	No
1. Age ≥ 18 years	☐	☐
2. General Health		
• No relevant medical history and under no medication that is known to affect the oral cavity, the oral flora status and salivary flow rate	☐	☐
• Non-pregnant female volunteers	☐	☐
3. Dental examination		
i. Presence of at least 20 natural teeth	☐	☐
ii. Free from visual signs of untreated caries or periodontal disease or any other adverse dental oral health conditions that could be exacerbated by the study interventions	☐	☐
iii. Unstimulated salivary flow rate ≥ 0.25 ml/min	☐	☐
iv. DMFT ≥ 1	☐	☐
4. Compliance		
• Fully understand and is willing, able and likely to comply with the study procedures and restrictions	☐	☐
5. Consent		
• Demonstrate understanding of the study and willingness to participate as evidenced by voluntary written informed consent	☐	☐

***Note: If any of the above questions are answered "No", the participant should be discontinued from the study as a "Screen failure" on the study conclusion page.**

Researcher's Signature:..... **Date** **__ __ / __ __/ __ __ __ __**

Participant Initials: __ __ __ Screening No.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

SCREENING VISIT **Date of Visit: __ __ / __ __/ __ __ __ __**

Exclusion Criteria Checklist*

	Yes	No
6. Age: < 18 years	☐	☐
7. General Health:		
• Current or recurrent disease that could affect the oral cavity or interfere with the dental examination and/or wearing of oral appliance	☐	☐
• Severe psychiatric, physical and medical disorders requiring treatment or making the participant unlikely to give informed consent or to cope with the procedures required by the study protocol	☐	☐
• Pregnant/ intending to become pregnant/ lactating female participant	☐	☐
8. Medication		
• Medication that is known to affect the oral cavity, oral flora status and salivary flow rate	☐	☐
• Antimicrobial therapy within 14 days prior to screening or during the study	☐	☐
• Antibiotic treatment within 28 days prior to screening or during the study	☐	☐
9. Dental details		
• Dental disease that require immediate treatment	☐	☐
• Oral surgery or extraction 6 weeks prior to screening or during the study	☐	☐
• Wearing of prostheses or orthodontic appliances that could affect the study procedures	☐	☐
• Unstimulated salivary flow rate < 0.25 ml/min	☐	☐
10. Clinical trials		
• Participation in another clinical study or receipt of an investigational drug within 30 days of the screening and during the study	☐	☐
11. Other		
• Smokers	☐	☐
• Known or suspected intolerance/ hypersensitivity to study materials closely related to compounds or ingredients that will be used in the study	☐	☐

***Note: If any of the above questions are answered "Yes", the participant should be discontinued from the study as a "Screen failure" on the study conclusion page.**

Researcher's Signature:..... **Date** **__ __ / __ __/ __ __ __ __**

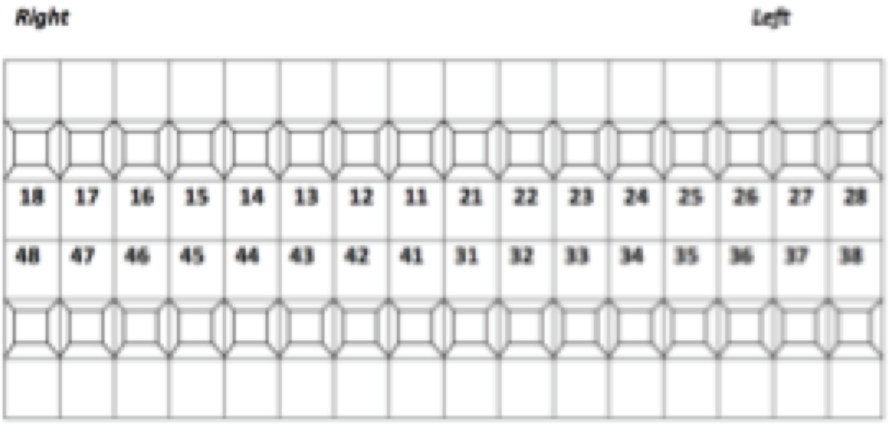
Participant Initials: __ __ __ Screening No.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

SCREENING VISIT **Date of Visit: __ __ / __ __/ __ __ __ __**
Time of Dental Examination: __ __: __ __

- **Oral Cavity Examination**

Soft tissue	
□ Normal □ Abnormal	**Describe abnormality:**…… …… ……

- **Dental Examination**



Total DMFT:	No. of teeth:

Note: Participant must have at least 20 natural teeth to be eligible for inclusion

- **Salivary Flow Rate**

Unstimulated: _. _ _ ml/5min _. _ _ ml/min	**(Must be ≥ 0.25 ml/min)**

Researcher's Signature:…… **Date** __ __/__ __/__ __ __ __

Participant Initials: __ __ __ Screening No.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

SCREENING VISIT **Date of Visit: __ __ / __ __/ __ __ __ __**

Fitness and Eligibility to Participate in the study

In the investigator's opinion, on the basis of the screening assessments and inclusion and exclusion criteria, is the Participant eligible to participate in the next part of the study?

Yes ☐ **No** ☐

Researcher's Signature:..... **Date** **__ __ / __ __/ __ __ __ __**

Participant Initials: __ __ __ **Screening No.: __ __ __**	**Participant DOB: _ _ / _ _ / _ _ _ _**

Date of Visit: __ __ / __ __/ __ __ __ __

Screening Visit Checklist

Personal Data sheet completed	Yes ☐	No ☐
Medical History checked	Yes ☐	No ☐
Dental Examination completed	Yes ☐	No ☐
Inclusion Criteria Sheet completed	Yes ☐	No ☐
Exclusion Criteria Sheet completed	Yes ☐	No ☐
Eligibility Sheet completed	Yes ☐	No ☐

Researcher's Signature:..... **Date** __ __/__ __/__ __ __ __

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

SCHEDULED STUDY VISITS

First Visit **Date**__ __ / __ __/ __ __ __ __	• Dental Scaling & polishing	YES ☐ N/A ☐
	• Upper/Lower alginate impression + Wax bite	YES ☐
Signature:_____	• Any problems? YES ☐ NO ☐ **If Yes, give details**……	
Second Visit **Date**__ __ / __ __/ __ __ __ __	Have there been any changes to the participant's medication record since the last visit? **(*If yes please complete the Concomitant Medication page)**	YES ☐ NO ☐
	Have there been any changes to the inclusion/exclusion criteria since the last visit?	YES ☐ NO ☐
	Is the participant still eligible to continue in the study? ****If no, please specify reason on the study conclusion page.**	YES ☐ NO ☐
	• Fitting of appliance	YES ☐
	• Any problems? YES ☐ NO ☐ **If Yes, give details**……	
Signature:_____	• Instructions & restrictions	YES ☐
	• Appointment arranged after 2 weeks?	YES ☐ NO ☐

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

Third Visit **Date__ __ / __ __/ __ __ __ __**	Have there been any changes to the participant's medication record since the last visit? **(*If yes please complete the Concomitant Medication page)**	YES ☐ NO ☐
	Have there been any changes to the inclusion/exclusion criteria since the last visit?	YES ☐ NO ☐
	Is the participant still eligible to continue in the study? ****If no, please specify reason on the study conclusion page.**	YES ☐ NO ☐
	Has the participant worn the appliance as per protocol (i.e. continuously except when eating, drinking & tooth brushing)?	YES ☐ NO ☐
	If no, is the participant still eligible to take part in the study?	YES ☐ NO ☐
	• Removal of appliance	YES ☐
Signature:_____	• Any problems? YES ☐ NO ☐ **If Yes, give details**……. …… …… …… …… ……	

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

Fourth Visit **Date**__ __ / __ __/ __ __ __ __	Have there been any changes to the participant's medication record since the last visit? **(*If yes please complete the Concomitant Medication page)**	YES ☐ NO ☐
	Have there been any changes to the inclusion/exclusion criteria since the last visit?	YES ☐ NO ☐
	Is the participant still eligible to continue in the study? ****If no, please specify reason on the study conclusion page.**	YES ☐ NO ☐
	• Fitting of appliance	**YES** ☐
	• Any problems? YES ☐ NO ☐ **If Yes, give details**……	
	• Instructions & restrictions	YES ☐
Signature:_____	• Appointment arranged after 2 weeks?	YES ☐ NO ☐

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

Fifth Visit **Date**__ __ / __ __/ __ __ __ __	Have there been any changes to the participant's medication record since the last visit? **(*If yes please complete the Concomitant Medication page)**	YES ☐ NO ☐
	Have there been any changes to the inclusion/exclusion criteria since the last visit?	YES ☐ NO ☐
	Is the participant still eligible to continue in the study? ****If no, please specify reason on the study conclusion page.**	YES ☐ NO ☐
	Has the participant worn the appliance as per protocol (i.e. continuously except when eating, drinking & tooth brushing)?	YES ☐ NO ☐
	If no, is the participant still eligible to take part in the study?	YES ☐ NO ☐
	• Removal of appliance	YES ☐
	• Any problems? YES ☐ NO ☐ **If Yes, give details**……	
Signature:_____	• Dental Scaling & polishing	YES ☐ N/A ☐

Participant Initials: __ __ __ **Participant ID no.: __ __ __**	**Participant DOB: _ _ / _ _ / _ _ _ _**

UNSCHEDULED VISIT - APPLIANCE MODIFICATION SHEET

Modifications

	Yes*	No
Has the participant required any modification to their appliance? **If yes*, please specify in the comments' space below.	☐	☐

Comments
(Record each date a modification was required and details of modification conducted)

Date	Reason	Clinician's Signature
	…… ……	

Researcher's Signature: …… **Date:** __ __ / __ __ / __ __ __ __

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

- **CURRENT/ CONCOMITANT MEDICATION**

Drug's name	Reason for medication	Dosage	Date started	Date stopped (If applicable)

Researcher's Signature:…….. **Date** __ __/__ __/__ __ __ __

Participant Initials: __ __ __ Participant ID no.: __ __ __	Participant DOB: _ _ / _ _ / _ _ _ _

Study Conclusion

Did the participant complete the entire study? **Yes** ☐ **No*** ☐

If " Yes", Date completed: __ __/__ __/__ __ __ __

If "No" is checked, please complete the following (Please check as an appropriate):

Screen Failure ☐

Adverse Event ☐

Lost to follow-up ☐

Protocol Deviation (please specify details) ☐

……
……
……
……

Withdrawal of Volunteer (please specify details) ☐

……
……
……
……

Other (please specify details) ☐

……
……
……
……

Researcher's Signature

I confirm that I have reviewed all the data collected in this Case Report Form and take responsibility that the information is accurate and complete.

Researcher's Name……

Researcher's Signature…… **Date** __ __/__ __/__ __ __ __

Subject code:	Randomisation no:

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Adverse Events

Adverse Event	Onset Date	End Date	Duration	Outcome	Pattern	Intensity	Relation to study	Action taken	Serious*

Duration (Units)	Outcome	Intensity	Relationship to study	Action taken (Regarding the study)	Serious
1. S-Seconds 2. M-Minutes 3. H-Hours 4. D-Days	1. Resolved 2. On-going	1. Mild 2. Moderate 3. Severe	1. Not related 2. Unlikely 3. Possible 4. Highly possible	1. None 2. Interrupted 3. Discontinued	1. No 2. Yes*

* All Serious adverse events must be reported to the study monitor within 24 hours and require special action