Dental plaque regrowth studies to evaluate chewing gum formulations incorporating magnolia bark extract

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ARTICLE INFO

ABSTRACT

The plaque inhibiting properties of magnolia bark extract (MBE) were assessed in a volunteer trial following the consumption of various sugar-free chewing gum formulations over a period of 4-days. Paired t-tests demonstrated significant ($p < 0.15$) differences between the placebo and a gum containing MBE (0.4%) plus lauramide arginine ethyl ester (LAE) (0.5%) with respect to% plaque coverage (36.3% vs 34.0%) and area of plaque fluorescence (109.4 mm^2 vs 75.2 mm^2). These findings were supported by microbiological counts of total salivary bacteria (7.77 $\log_{10}$ cfu/ml vs 7.45 $\log_{10}$ cfu/ml) as well as *Streptococcus* spp. (6.76 $\log_{10}$ cfu/ml vs 6.29 $\log_{10}$ cfu/ml). MBE (0.4%) + LAE (0.5%) delivered by chewing gum had a moderate inhibitory effect on plaque formation and salivary bacteria. Limiting the formation of dental plaque and salivary bacteria, specifically oral streptococci, could contribute towards an improvement in oral health with respect to gum disease and caries.

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1. Introduction

A panoply of phytochemicals have received interest on account of their potential pharmacological or antimicrobial activity (Abreu, McBain, & Simoes, 2012), including those purported to be active against oral bacteria (Ciric et al., 2011). Magnolia Bark Extract (MBE) is one such natural product from the stem bark of *Magnolia officinalis* (Magnoliaceae) that is isolated by CO_2 super critical fluid extraction (Modey, Mulholland, & Raynor, 1996). MBE is actually a mixture of neolignans, lignans, alkaloids and sesquiterpenes (Shen et al., 2009), including over a dozen polar molecules (Yu, Yan, Liang, Wang, & Yang, 2012); but its primary components

(45–97%) are magnolol [4-Allyl-2-(5-allyl-2-hydroxy-phenyl)phenol] and its isomer honokiol [2-(4-hydroxy-3-prop-2-enyl-phenyl)- 4-prop-2-enyl-phenol] (Fig. 1). Both magnolol and honokiol are potent antioxidants (Lo, Teng, Chen, Chen, & Hong, 1994) that have demonstrable broad spectrum antimicrobial activity against a number of different (non-oral) bacteria (Hu, Qiao, Zhang, & Ge, 2011; Park et al., 2004) as well as some fungi, including *Candida albicans* (Bang et al., 2000). Magnolol has also been shown to specifically inhibit biofilm formation in *Staphylococcus aureus* (Wang et al., 2011). With regard to the oral microbiota, MBE has an antimicrobial effect against a number of periodontopathic bacteria including *Porphyromonas gingivalis*, *Prevotella* spp., *Aggregatibacter actinomycetemcomitans* and *Capnocytophaga gingivalis* (Chang, Lee, Ku, Bae, & Chung, 1998; Ho, Tsai, Chen, Huang, & Lin, 2001) as well as bacteria associated with dental caries including *Streptococcus mutans* (Namba, Hattori, Tsunezuka, Yamagishi, & Konishi, 1982). Furthermore, MBE has been reported to exhibit anti-quorum sensing activity against non-oral bacteria

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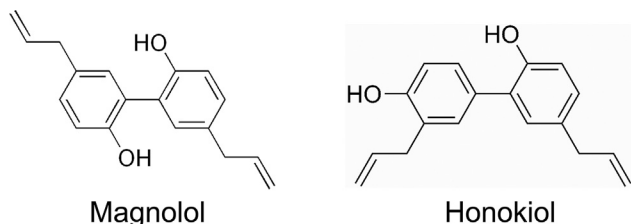
Fig. 1. Magnolol and honokiol.

which may indicate potential anti-biofilm activity (Yeo & Tham, 2012). MBE is a traditional Chinese medicine permitted food additive and is classified as 'generally regarded as safe' (GRAS) by the United States Food and Drug Administration following independent recommendation (Li et al., 2007; Liu et al., 2007), and has also received Novel Foods Approval status in the European Union (EC 258/97, Article 4.2). The antimicrobial effect of MBE has been recently attributed to magnolol and honokiol's ability to bind to surface appendages of the microbial cell, most notably of gram-negative bacteria, whereby they increase cell surface hydrophobicity (Wessel et al., 2016).

The antimicrobial properties of MBE suggest that it may be able to moderate the growth of oral bacteria and if such activity can be manifested in the mouth it could in turn contribute towards an improvement in oral health (American Academy of Periodontology. Research, 2005). One possible method of delivering antiplaque actives into the oral cavity is in a form of chewing gum (Simons, Kidd, Beighton, & Jones, 1997; Smith, Moran, Dangler, Leight, & Addy, 1996). However, the hydrophobic nature of magnolol and honokiol means that these molecules are only slightly water soluble, and as such they do not release well in an aqueous environment such as saliva from chewing gum. Work in the sponsor's laboratory suggests that approximately 50% of the loaded dose of MBE is released from the gum matrix over a 20-min chew period due to of the solubility limit of the active agents in saliva. The aim of this plaque regrowth experiment was to test MBE at a concentration of 0.4% with an expected delivery dose of 6 mg per gum serving and also in combination with lauramide arginine ethyl ester (LAE) as a surfactant to help release and disperse MBE in the oral environment. LAE is approved as a food additive by the European Food Safety Authority (EFSA-Q-2006-035) and the United States Food and Drug Administration (GRAS 000164).

A well-established, plaque regrowth protocol has been widely used to compare the effects of a range of oral interventions; including mouthwashes, toothpastes and chewing-gums containing; chlorhexidine, triclosan, probiotics and essential oils (De Siena, Del Fabbro, Corbella, Taschieri, & Weinstein, 2013; Keller, Hasslof, Dahlen, Stecksen-Blicks, & Twetman, 2012; Pizzo, Compilato, Di Liberto, Pizzo, & Campisi, 2013; Pizzo et al., 2007; Pretty, Edgar, & Higham, 2004). Such plaque regrowth studies, by definition, require the implementation of plaque planimetric techniques – one of which is quantitative light-induced fluorescence (QLF). This technique uses 405 nm light to induce red fluorescence in dental plaque (de Josselin de Jong, Higham, Smith, van Daelen, & van der Veen, 2009). The aim of this plaque regrowth study was to use QLF and microbiological techniques to determine if MBE containing chewing gums have any plaque inhibiting properties.

2. Materials and methods

2.1. Clinical trial

This clinical trial was a single-centre, double-blind, single-treatment, randomised, placebo-controlled, four-legged crossover trial taking place over a period of 8 weeks involving 12 subjects. The sequence of sugar-free gum (SFG) formulations allocation was predetermined using a Latin Square (Varma, Fertig, Chilton, & Mandel, 1974). Twelve healthy adult volunteers (aged 19–63) were recruited from staff and postgraduate students working at the Liverpool University Dental Hospital. Ethical approval was sought from the local ethics committee (Liverpool Adult Research Ethics Committee, reference number 09/H1005/45). All of the volunteers received verbal and written information concerning the study and informed written consent was taken. A washout period of 9 days preceded each experimental phase. At the beginning of each leg of the study (Monday morning) the subjects were given professional tooth-cleaning by a dentist after their teeth had been disclosed using PlaqueFinder™ (Pro-Dentec, Rota-Dent, Cambridge, UK). Baseline white-light digital images and QLF images were taken to confirm that the labial surfaces of the anterior teeth (maxillary and mandibular, canine to canine; 11–13, 21–23, 31–33, 41–43) were plaque free.

2.2. Chewing gum formulations and dosage

The exact methods used in preparing the gum by the manufacturer are proprietary, although they can be summarised here in brief. Due to its extreme hydrophobicity, MBE (Honsea Sunshine Bioscience and Technology Co., Ltd., Guangzhou, China) containing 95.42% magnolol and 1.94% honokiol (manufacturer's data; as determined by high-performance liquid chromatography) was added to the pellet coating, along with flavouring and LAE (as appropriate) rather than incorporating this into the gum core.

The subjects were provided with SFG containing one of the three test formulations or a placebo in the form of chewing gum in 5 heat sealed bags (each containing 10×1.5 g gum pellets), one for each day Monday through Friday. The volunteers were asked to self-administer a serving of two pellets, five times per day in the absence of other oral hygiene procedures. The chewing gum was consumed over a period of 10 min at unspecified time points spread as evenly as possible throughout the day. The four SFG formulations, used in the study were; placebo gum (wintergreen flavour), MBE (0.4%), MBE (0.4%) plus LAE (0.5%) and MBE (0.4%; suck-then-chew method). The 'suck-then-chew' method denoted specific instructions to the subjects to suck the gum pellets for the first 2 min followed chewing for the remaining 8 min. The subjects were provided with a generic stopwatch timer to ensure adherence to these protocol.

2.3. Microbiological sampling and analysis

After the end of each experimental leg (Friday afternoon) the subjects returned to the dental clinic. Firstly, the subjects were asked to drool saliva (i.e. unstimulated) over a period of 5 min into a sterile container. After collection, the saliva sample was placed into a container of crushed ice and immediately transported from the clinic to the laboratory for processing within 10 min of collection. The saliva samples were vortex mixed and then serially diluted in phosphate-buffered saline (Oxoid, Basingstoke, UK). 20 μl aliquots of these dilutions were then spread in quadruplicate onto fastidious anaerobic agar (FAA) containing 5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) as a non-selective solid growth medium (Lab M, Heywood, UK). Replicate aliquots were similarly spread onto *Mitis salivarius* agar (MSA) supplemented with 1% Chapman tellurite (Beckton Dickinson, Oxford, UK) to isolate the growth of *Streptococcus* spp. All of these plates were then incubated at 37 °C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for 72 h before the number of colony forming units (cfu) was determined. The cfu results were $\log_{10}$ transformed prior to statistical analysis by *t*-test (paired samples for means).

2.4. Plaque imaging

Dental plaque was disclosed after saliva donation and a second set of QLF images was captured. The subjects were then provided with further dental prophylaxis before undergoing a nine day washout period during which they resumed their normal oral hygiene procedures using the toothbrush and toothpaste (Tesco Daily Care 1090 ppm F, Cheshunt, UK) provided as part of this study. They were then asked to present the following Monday for the next phase of the trial. This was repeated until each subject has used all four SFG formulations. Subjects followed their usual diet including any regular snacks they normally consumed in the course of a day.

The QLF images were analysed using proprietary software (InspektorPro™; Inspektor Research Systems BV, The Netherlands) which incorporates tools for measuring the intensity of red fluorescence (ΔR) in comparison to a plaque-free reference region of the tooth. The software also returned the area of red fluorescence (mm^2). These analyses were performed for each of twelve images of the anterior teeth and used to calculate an average for each individual volunteer. The images were also analysed using freely available image analysis software (ImageJ v1.43; National Institutes of Health, Bethesda, USA) to calculate the percentage of the tooth covered with plaque. Briefly, the QLF image was split into its component colours to isolate the green and the red colour channels. These two, 8-bit greyscale images (0–255; 0 = black, 255 = white) were then processed by the 'Image Calculator' function to divide the red pixel values by the green pixel values. The total number of pixels within the area of the tooth was used to calculate the percentage plaque coverage by allocating pixels with a value of '0' as 'plaque-free' and those with a value ≥ 1 as 'plaque covered'. The superimposition of a number of representative original images with the binary results of the image calculator showed good conformity and reproducibility for this technique (Hope et al., 2014).

2.5. Statistical analysis

Three planimetric methods were used to assess plaque regrowth. The intensity of red fluorescence ($\Delta R\%$) and the area of red fluorescence (mm^2) were assessed by QLF, whereas the percentage area of disclosed dental plaque was determined by image analysis. The planimetric analyses were supported by two microbiological analyses of the total number of viable bacteria and streptococci present in volunteers' saliva. The sample size was twelve in all instances.

The alpha level of significance used in the planimetric and microbiological analyses was set at p < 0.15 to detect a 'moderate effect' as delivered by a functional foodstuff as opposed to a 'clinically significant' effect at p < 0.05 that one would perhaps wish to achieve from a medicament.

3. Results

3.1. Plaque planimetrics

Analysis of variance (ANOVA) revealed a statistically significant 'moderate effect' (i.e. p < 0.15) between experimental groups in terms of $\Delta R\%$ and area of red fluorescence (mm^2) (p = 0.07) (Fig. 2). Paired t-tests for post hoc comparisons demonstrated significant differences between the placebo gum (36.3%) and MBE + LAE (34.0%) (p = 0.08). A stronger significance was observed with respect to the area of red fluorescence (ANOVA; p = 0.04), whilst the corresponding paired *t*-test showed significance between the placebo gum (109.4 mm^2) and MBE + LAE (75.2 mm^2) (p = 0.03) (Fig. 2). The image analysis methods to calculate percentage
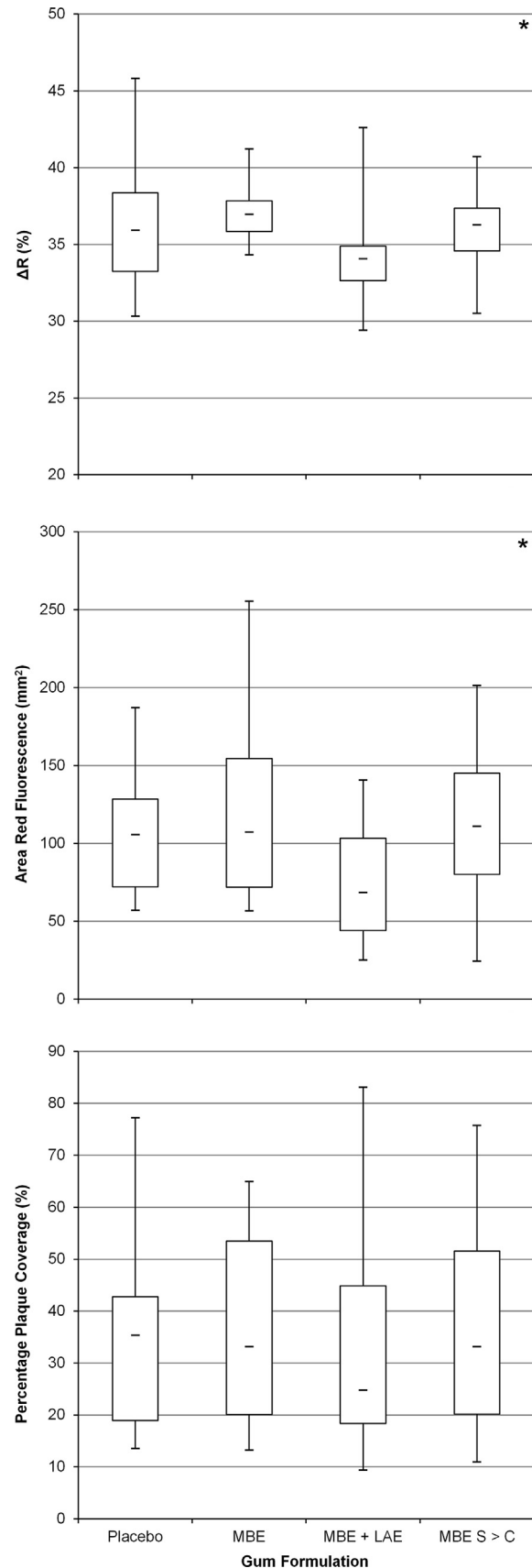

Fig. 2. Box plots of plaque planimetrics; percentage of red fluorescence ($\Delta R\%$), area of red fluorescence (mm^2) and percentage plaque coverage (%) following consumption of various chewing gum formulations; placebo, MBE (0.4%), MBE (0.4%) + LAE (0.5%) and MBE S > C (0.4%) consumed by sucking for two minutes before chewing. * Indicates p < 0.15 (ANOVA, n = 12).

(disclosed) plaque coverage did not detect any significant variance between the SFGs (ANOVA; p = 0.76).

3.2. Salivary microbiology

The log_{10} mean counts of total salivary bacteria enumerated as colony forming units per millilitre on FAA, revealed significant differences between the four SFG formulations (ANOVA; p = 0.046) (Fig. 3). Paired t-tests revealed that the difference between placebo (7.77 log_{10} cfu/ml) and MBE + LAE (7.45 log_{10} cfu/ml) was significant (p = 0.046). Likewise, the log_{10} mean counts of Streptococcus spp. as enumerated on MSA revealed a similar trend (ANOVA; p = 0.135) together with a significant difference between placebo (6.76 log_{10} cfu/ml) and MBE + LAE (6.29 log_{10} cfu/ml) (p = 0.066).
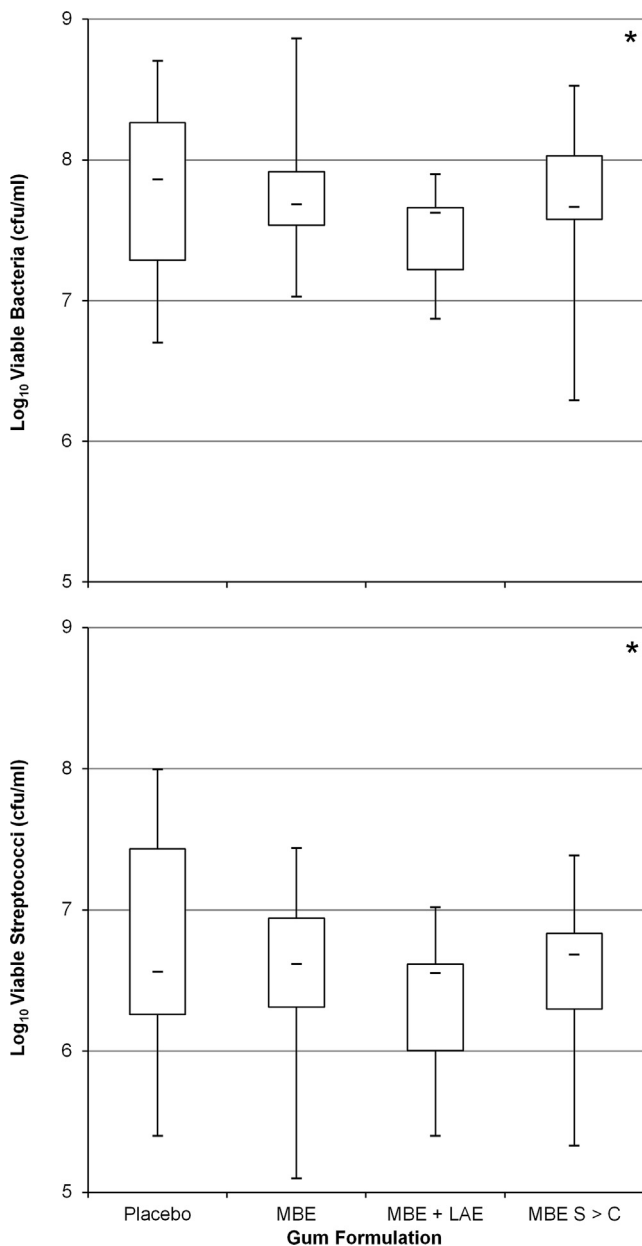

Fig. 3. Box plots of salivary microbiology; counts of total viable bacteria and streptococci (log_{10} cfu/ml) following consumption of various chewing gum formulations; placebo, MBE (0.4%), MBE (0.4%) + LAE (0.5%) and MBE S > C (0.4%) consumed by sucking for two minutes before chewing. * Indicates p < 0.15 (ANOVA, n = 12).

3.3. MBE + LAE

Overall, the mean values for all five measurements of plaque regrowth demonstrated lower values in the MBE + LAE leg of the experiment compared to the placebo gum. These differences were significant (p < 0.15) in all instances with the exception of percentage plaque coverage (Fig. 4 and Table 1). A similar result was found when comparing the MBE (0.4%) vs MBE (0.4%) + LAE in that the addition of the surfactant had a statistically significant (p < 0.15) effect in reducing plaque indices by all measures with the exception of percentage plaque coverage (Table 1).

4. Discussion

The described protocol is based upon a well-established methodology for assessing plaque regrowth which has sufficient power to discriminate at the alpha = 0.15 level with a sample size of twelve volunteers (Moran, Addy, & Newcombe, 1997; Pretty, Gallagher, Martin, Edgar, & Higham, 2003). Although in vitro experiments are useful for screening possible antimicrobial agents and determining their minimum inhibitory concentrations (MIC), plaque regrowth studies such as these are more representative of adjunct oral hygiene measures that might be taken throughout the day, such as chewing SFG or sucking sugar-free confectionary.

Magnolol and honokiol are highly hydrophobic and relatively insoluble with a log P (partition coefficient; a measure of solubility) of 5.25 (details provided by the sponsor's laboratory). This presents a challenge when it comes to dispersing these adjuncts in saliva. It was for this reason that the surfactant LAE was included in one of the gum formulations as was the 'suck-then-chew' variable. The results suggest that sucking the active ingredient-containing coating did not measurably enhance the effects of MBE, probably because this approach did nothing to address the problem of hydrophobicity/solubility. However, the inclusion of the surfactant LAE did significantly increase the efficacy of MBE when compared to MBE alone (Table 1).

A previous study involving chewing sugar-free gum for a period of twenty minutes elicited an increase in subjects salivary flow rate from a baseline of 0.6 ml min^{-1} to a peak of 5.3 ml min^{-1} and continued to be stimulated at a rate of 1.6 ml min^{-1} at the cessation of chewing. This study also revealed that there was also a corresponding increase in salivary pH of approximately 0.5 units during chewing together with an immediate increase in sodium concentration from 5.8 mmol l^{-1} to 32.3 m mol^{-1} which then fell to 11.6 after twenty minutes (Dawes & Dong, 1995). The ecological plaque hypothesis (Marsh, 2003) proposes that such changes in saliva composition, being part of the plaque's 'local environment', could potentially bring about a corresponding shift in the plaque's microbial composition. However a more likely cause for changes in plaque composition upon exposure to a (phyto)chemical agent would be due to the selective inhibition (or enrichment) of certain bacterial species. Subtle changes in specific members of the oral microbiota can elicit much larger dynamic shifts in the microflora on the whole (Hajishengallis et al., 2011; Marsh, Head, & Devine, 2015).

A recent study into the effects of sugar-free chewing gum containing MBE corroborates our findings by reporting a reduction in salivary mutans streptococci (Campus et al., 2011). There were however a number of important differences in the experimental designs between their study and the present study. Firstly, although they used a much larger sample size (n = 120) they did not employ a cross-over design (n = 38–40 per group). Secondly, the active dose was lower as the gum contained 0.17% MBE, without the addition of a surfactant, and was taken as five pieces, three times a day for 30 days. Thirdly, they enumerated mutans streptococci on Mitis salivarius agar supplemented with bacitracin; as a
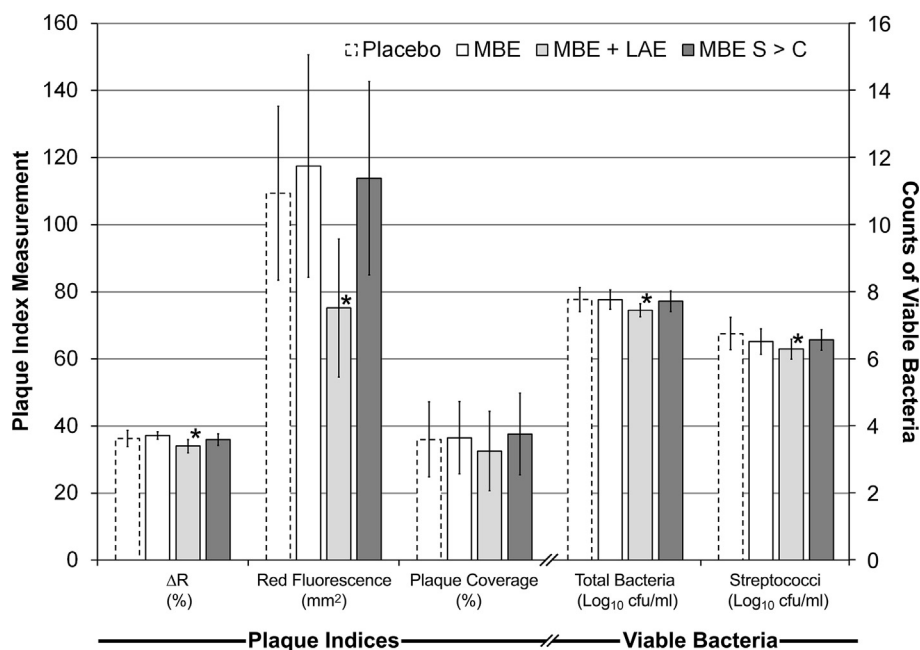
Fig. 4. Plaque indices; ΔR (%), area of red fluorescence (mm^2), percentage plaque coverage (%) and numbers of viable bacteria (log_{10} cfu/ml; total bacteria and Streptococci) for the different gum formulations; placebo, MBE (0.4%), MBE (0.4%) + LAE (0.5%) and MBE S > C (0.4%) consumed by sucking for two minutes before chewing. * and + symbols indicate statistical significance; *p < 0.15; (*t*-test paired samples, n = 12).

Table 1
Summary of the mean values (n = 12) of plaque indices and viable counts of bacteria with respect to plaque regrowth following consumption of four different chewing gum formulations.

Gum formulation		ΔR%	Red fluorescence (mm^2)	Plaque coverage (mm^2)	Log_{10} viable bacteria (cfu/ml)	Log_{10} viable streptococci (cfu/ml)
Placebo		36.29	109.40	36.02	7.77	6.76
MBE (0.4%)		37.16	117.48	36.50	7.77	6.51
MBE (0.4%) + LAE (0.5%)		34.01	75.16	32.53	7.45	6.29
MBE (0.4%); suck then chew		35.94	113.85	37.62	7.72	6.56
ANOVA	P-value	0.073	0.041	0.738	0.136	0.135
Placebo vs MBE (0.4%) + LAE (0.5%)		0.080	0.026	0.260	0.046	0.066
MBE (0.4%) vs MBE (0.4%) + LAE (0.5%)		0.005	0.007	0.225	0.022	0.070

result, the viable counts for mutans streptococci were of the order of 1.5 log lower than the present study which reported total streptococci. Perhaps most importantly though, the Campus study was not a 'plaque re-growth' study in that the volunteers continued with their existing oral hygiene measures in the form of a standardised fluoridated (1450 ppm) toothpaste. Other interesting findings of the study were that MBE elicited a reduction in the acidogenicity of plaque together with a reduction in bleeding score. These findings corroborate those reported in a study which reported that 0.2% MBE delivered in the form of chewing gum or pressed mints reduced levels of salivary bacteria (Greenberg, Urnezis, & Tian, 2007). The Greenberg study also challenged a panel of three oral bacteria; *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *S. mutans* with MBE and reported MICs of 8, 31 and 16 μg/ml respectively. A more recent laboratory study reported that a methanol extract of magnolia bark had an MIC against planktonic *S. mutans* of 30 μg/ml whilst the purported antimicrobial components, magnolol and honokiol, both had an MIC of 10 μg/ml (Sakaue et al., 2016). This study also demonstrated an antibiofilm effect although a minimum biofilm eradication concentration was not given. The ability of MBE to increase cell surface hydrophobicity causes the affected bacteria to form larger cell aggregates (Krasowska & Sigler, 2014) that would be less likely to be incorporated into the existing plaque mass by co-aggregative mechanisms than smaller aggregate/individual cells.

Future work should seek to determine the effects that MBE and/or its components have upon a developing multispecies biofilm community either by using complex *in vitro* models (Hope et al., 2012) or expanded *in vivo* studies.

5. Conclusions

The results of the present study suggest that MBE (0.4%) + LAE (0.5%) delivered by chewing gum under the regimen described above had a significant inhibitory affect upon plaque formation. MBE without surfactant (LAE) did not have a plaque-inhibiting effect which suggests that the surfactant improved the bioavailability of the MBE components. The regular consumption of an MBE containing chewing gum between toothbrushings could possibly contribute towards good oral health.

contained herein are those of the authors and are not meant to be construed as representing views of the organisations to which the authors are affiliated. The author TI was in the employment of the funder at the time the experiments were conducted.

Author Contributions

Organisation of the clinical trial, imaging and plaque planimetric analysis; GNK. Experimental design and project management; SMH, CKH. Manuscript preparation; CKH, GNK, SMH, TI. Microbiological analysis; QW. Statistical analysis; GB. Preparation of gum formulations and supporting data; TI. Dental prophylaxis and clinical support; PWS, AAA.

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