Journal of Functional Foods 47 (2018) 48–55

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Combinatorial effect of magnolia bark extract and ethyl lauroyl arginate against multi-species oral biofilms: Food additives with the potential to prevent biofilm-related oral diseases

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ARTICLE INFO

Keywords:
Confocal laser scanning microscopy
Saliva
Dental plaque
Antimicrobial agents
Dental caries
Periodontal disease

ABSTRACT

The use of novel antimicrobial food additives that display anti-biofilm properties offers an alternative approach to prevent biofilm-related oral diseases. We evaluated the individual and combined effect of two food additives, magnolia bark extract (MBE) and ethyl-lauroyl-arginate (LAE), against multi-species oral biofilms. Using static and microfluidic *in-vitro* biofilm models, multi-species oral biofilms were treated with different concentrations of MBE, LAE or combinations of both. Biofilms were imaged using confocal laser scanning microscopy, and biomass, thickness and viability were determined. Both actives significantly reduced biomass, thickness and viability of developed biofilms, although some antimicrobial/anti-biofilm effects differed between the static and microfluidic models. When both actives were combined, the association of 0.005% MBE and 0.0025% LAE was the most effective. In conclusion, MBE and LAE exhibited anti-biofilm and antimicrobial effects. The combination of MBE and LAE improved their individual effect.

1. Introduction

Microbial dysbiosis of oral biofilm communities on hard and soft tissues may promote oral disease, such as dental caries and periodontal diseases (Kilian et al., 2016; Marsh, Head, & Devine, 2015), which are a global public health burden (Kassebaum et al., 2015; Marcenes et al., 2013; Petersen & Ogawa, 2012). Unfortunately, mechanical removal of oral biofilms is difficult and highly dependent upon ability and motivation of the subjects for oral health compliance (Warren & Chater, 1996). Thus, antimicrobials/anti-biofilm agents are added to oral care products such as toothpastes and mouthrinses to prevent or reduce biofilm accumulation (Giertsen, 2004; Sanz, Serrano, Iniesta, Santa Cruz, & Herrera, 2013). Less explored strategies, such as the use of antimicrobial/anti-biofilm food additives (Campus et al., 2011; Greenberg, Urnezis, & Tian, 2007) are gaining attention to potentially augment the efficacy of common oral care products.

The control of oral biofilms is challenging since biofilms exhibit 100–1000-fold higher resistance to antimicrobial interventions than planktonic bacteria (Gilbert, Maira-Litran, McBain, Rickard, & Whyte, 2002; Mah & O'Toole, 2001). Furthermore, some antimicrobials such as chlorhexidine (CHX) can have undesirable/adverse effects (Babich, Wurzburger, Rubin, Sinensky, & Blau, 1995; Pemberton, 2016; Van Strydonck, Slot, Van der Velden, & Van der Weijden, 2012). Therefore, less problematic antimicrobial/anti-biofilm alternatives, which may be arguably more biologically compatible, may be appropriate to add to products to improve oral health (Gallob et al., 2015; Tartaglia, Kumar, Fornari, Corti, & Connelly, 2016). Novel and/or natural compounds, such as those approved for food use, have received increasing research interest (Cheng, Li, He, & Zhou, 2015; Freires, Denny, Benso, de Alencar, & Rosalen, 2015). In particular, magnolia bark extract (MBE) and ethyl lauroyl arginate (LAE) are compounds with anti-biofilm potential that have been accorded *generally recognized as safe* (GRAS) status by the Food and Drug Administration (FDA, 2005; Smith et al., 2009). Both compounds are conceivable candidates to be integrated

Abbreviations: MBE, magnolia bark extract; LAE, ethyl-lauroyl-arginate; CHX, chlorhexidine; GRAS, generally recognized as safe; FDA, food and drugs administration; CLSM, confocal laser scanning microscopy; CFS, cell-free saliva

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https://doi.org/10.1016/j.jff.2018.05.012
Received 2 January 2018; Received in revised form 4 May 2018; Accepted 8 May 2018
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into foods for controlling oral diseases, based on their reported antimicrobial action and safety (Chang & But, 1987; FDA, 2005; JECFA, 2009; Smith et al., 2009).

MBE is a natural compound isolated from *Magnolia officinalis* used in traditional Chinese medicine (Chang & But, 1987). When used as a food additive, beneficial oral outcomes have been observed (Campus et al., 2011; Greenberg et al., 2007). MBE and the bioactives it contains, specifically magnolol and honokiol (Bang et al., 2000; Maruyama, Kuribara, Morita, Yuzurihara, & Weintraub, 1998), have antimicrobial activity against numerous oral bacteria involved in oral malodor, periodontal disease (Chang, Lee, Ku, Bae, & Chung, 1998; Greenberg et al., 2007; Ho, Tsai, Chen, Huang, & Lin, 2001), and dental caries (Feng, Li, & Zhou, 2007; Namba, Tsunezuka, & Hattori, 1982; Sakaue et al., 2016). In comparison, LAE is a cationic surfactant derived from lauric-acid, arginine and ethanol, which are all naturally occurring substances (Infante, Dominguez, Erra, Julia, & Prats, 1984). LAE has been widely used as a food additive (JECFA, 2009) due to its broad-spectrum of activity (Infante et al., 1984) and safety (Hawkins, Rocabayera, Ruckman, Segret, & Shaw, 2009). In addition to food preservation (Pezo, Navascues, Salafranca, & Nerin, 2012; Terjung et al., 2014; Woodcock, Hammond, Ralyea, & Boor, 2009), LAE has been also shown to possibly prevent dental erosion (Bonvila, 2010) and help treat periodontal disease (Gallob et al., 2015). Because LAE is a cationic molecule (FDA, 2005) and MBE is non-ionic (Sakaue et al., 2016), they may have a cooperative antimicrobial effect. For example, the high affinity of LAE for bacterial cells and its effect on membrane potential and structure (Rodriguez, Seguer, Rocabayera, & Manresa, 2004) may enhance the antimicrobial activity of MBE. To date, however, the individual and combined effect of MBE and LAE on *in vitro* oral multi-species biofilms have not been thoroughly described.

Considering the potential detrimental effect of oral biofilms and that such microbial communities are not easily controlled by traditional personal oral hygiene measures (tooth brushing and/or dental floss), effective antimicrobial/anti-biofilm actives are a prime target for development of novel therapies. Thus, the aim of this work was to evaluate the antimicrobial and anti-biofilm effect of MBE and LAE on preformed *in vitro* multi-species biofilms.

2. Materials and methods

2.1. Summary of experimental design

The experimental design consisted of two stages. **(i)** *Testing actives individually* against saliva-derived multispecies biofilms using two different *in vitro* models (static and microfluidic) (Section 3.1), and **(ii)** *Testing actives in combination* against saliva-derived and dental plaque-derived biofilms grown in a microfluidic system (Section 3.2). Based on stage (i) data, specific concentrations of MBE and LAE were mixed together. In both stages developed biofilms (n = 9–11) were treated for 20 min with the actives, and with negative and positive controls. Treated biofilms were evaluated using confocal laser scanning microscopy (CLSM) and biomass, thickness and viability were determined by image analysis. After three independent assays (3–4 samples per assay per group), a total of 9 to 11 samples contributed to each experimental group. Each sample was the average of data from three different CLSM analysis locations.

2.2. Saliva and dental plaque collection

Eleven healthy individuals participated as donors of saliva and dental plaque for this study (Ethical approval by the University of Michigan - ID#HUM00101254). The patient selection criteria included a requirement for healthy adults with no chronic general diseases, who were in good oral health, and who have not received antibiotic therapy for at least 3 months prior to collection. Selected participants were asked to refrain from ingesting food and brushing their teeth the morning before collection. Sampling was performed as described previously (Fernandez, Aspiras, Dodds, Gonzalez-Cabezas, & Rickard, 2017). Briefly, visible supragingival dental plaque was collected with sterile-curettes from all accessible surfaces (buccal, lingual and interproximal) and pooled to generate the dental plaque inoculum. Stimulated saliva was collected by mastication of parafilm®, then, pooled and used as a saliva-inoculum. Cell-free saliva (CFS) was used as a natural nutrient source to develop oral biofilms and prepared as previously described (Nance et al., 2013; Samarian, Jakubovics, Luo, & Rickard, 2014).

2.3. Biofilm models

2.3.1. Static model system

A static microplate biofilm model previously described (Kolderman et al., 2015) was used to test the effect of MBE and LAE independently. Briefly, saliva-derived biofilms were developed in 24-well glass-bottom plates using CFS as the sole nutrient source. Plates were incubated at 37 °C, 5.5% CO_2 for 22 h. After growth, biofilms were washed with phosphate-buffered saline (PBS; pH 7.4) to remove unattached bacteria, then treated with 200 μL of the respective treatment for 20 min, and carefully rinsed in PBS. Treated biofilms were stained immediately with BacLight Live/Dead® viability stain (Invitrogen, Carlsbad, CA, USA) diluted in PBS (containing 3.34 μM Syto 9 and 20 μM propidium iodide) during 45 min. Stained biofilms were carefully washed with PBS, and imaged using CLSM.

2.3.2. Microfluidic biofilm model system

Multi-species oral biofilms were grown in a 24-channel Bioflux™ microfluidic system (Fluxion, South San Francisco, CA, USA) as previously described (Nance et al., 2013; Samarian et al., 2014). Saliva was used as inoculum when actives were independently tested (Section 3.1), and either saliva or dental plaque was used as an inoculum when MBE and LAE were mixed together (Section 3.2). Briefly, after formation of the acquired pellicle, channels were inoculated and incubated for 45 min at 37 °C to promote initial bacterial attachment. Subsequently, CFS was flowed for 20 h at 0.2 dyn/cm^2 while plates were maintained at 37 °C under aerobic conditions. After 20 h, biofilms were washed with PBS (pH 7.4) for 20 min. The respective treatments were applied for 20 min at 0.2 dyn/cm^2 and samples were washed with PBS for an additional 20 min. Treated biofilms were immediately stained as described above (Section 2.3.1), but in this model, the stain flowed for 45 min at 0.2 dyn/cm^2. Then, biofilms were washed with PBS for 20 min to remove stain excess.

2.4. Treatments

Solutions of MBE (Honsea Sunshine Biotech Co., Ltd, China) and LAE (Vedeqsa-Lamirsa, Spain) were prepared at different concentrations based upon preliminary studies of the actives (unpublished data). MBE was always dissolved in 6% ethanol, while LAE was dissolved in de-ionized water. For the combinatorial test, MBE and LAE were combined at different ratios (Table 1) in 6% ethanol. For all assays, the negative control was the respective solvent used to dilute the actives. Chlorhexidine (CHX) gluconate at 0.12% (Spectrum Chemical MFG. Corp., Garde, CA, USA) was used as a positive control. In both model systems, all treatments were applied for 20 min as described above.

2.5. Confocal laser scanning microscopy and image analysis

In each well (static model) or channel (microfluidic model), three random representative image stacks were taken using a Leica inverted Confocal Laser Scanning Microscope (CLSM) using a HCX PL APO 40X/0.85 CORR CS dry microscope objective lens (Leica, Exton, PA, USA). Using the Leica LAS AF software (Leica Mannheim; Wetzlar, Hesse, German), Live/Dead stained biofilms were imaged by exciting the

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