Oral health benefits of chewing gum

Wessel, Stefan

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Magnolia bark extract increases adhesion of oral Gram-negative bacteria to a hydrophobic ligand

Stefan W. Wessel, Henny C. van der Mei, Amarnath Maitra, Michael W.J. Dodds and Henk J. Busscher

Submitted to Journal of Agricultural and Food Chemistry
Abstract

Cell surface hydrophobicity of oral bacteria can be altered by adsorption of components from oral health care products. A more hydrophobic cell surface enhances bacterial removal by hydrophobic ligands from the oral cavity. Here we investigate whether exposure of oral Gram-positive and Gram-negative bacteria to magnolia-bark-extract alters their hydrophobicity to enhance their removal from an aqueous suspension by adhesion to hexadecane. Eleven oral bacterial strains were exposed to aqueous solutions containing different concentrations of magnolia-bark-extract. Subsequently their removal from the aqueous phase by hexadecane was measured using the kinetic "Microbial Adhesion To Hydrocarbons" assay. Exposure of bacteria to magnolia-bark-extract in aqueous solution yielded changes in hydrophobicity of Gram-negative oral bacteria in a dose responsive manner, enhancing removal by hexadecane. This suggests that a combination of magnolia-bark-extract and hydrophobic ligands may find applications in nutraceuticals to reduce the prevalence of Gram-negative bacteria and associated oral diseases in the oral cavity.
Introduction

Maintenance of oral health is largely achieved by regular toothbrushing, the use of mouthrinses, dental floss or other interdental cleaning devices. In addition, chewing of gum has been promoted as an adjunct to regular oral hygiene (1,2). The oral cavity is comprised of more than 700 bacterial species (3) many of which contribute to oral health (4) rather than to disease. Yet, conventional maintenance of oral health is geared toward removal of as many bacteria as possible, irrespective of whether they are known contributors to oral health or disease.

The composition of the oral microbiome is not only of importance in maintaining oral health but also relates with the occurrence of several other, more general diseases such as diabetes mellitus, cardiovascular disease, preterm birth and obesity (4–6). Key to prevention of disease is symbiosis of bacteria with the host (7) and preventing overgrowth by specific oral pathogens. Accordingly, alternative oral hygiene measures that aim toward the removal of specific oral pathogens from the oral cavity rather than untargeted bacterial removal are currently looked for more than ever (8–11). As early as 1967, it was already demonstrated that the composition of the oral microbiome could be shifted towards a composition of solely Gram-negative bacteria by rinsing with vancomycin (12). Goldberg and Rosenberg described that of various mouthrinses tested, only rinses that contain hydrophobic ligands in aqueous formulations, can effectively bind and remove bacteria from aqueous suspensions or desorb bacteria from solid surfaces (13,14). A sufficiently high bacterial cell surface hydrophobicity is crucial for bacterial removal by hydrophobic ligands and these observations suggest that making oral bacteria more hydrophobic will facilitate their removal from the oral cavity by hydrophobic ligands. Exposure of oral bacteria to cetylpyridinium chloride and chlorhexidine (13) as well as low concentrations of amoxicillin, penicillin, metronidazole (15) changed the cell surface hydrophobicity of oral bacterial strains such as Porphyromonas gingivalis and Fusobacterium nucleatum. Also triclosan, a common oral antibacterial, has been demonstrated to make bacteria more hydrophobic (16). An oral health care regimen consisting of brushing with a triclosan containing toothpaste followed by rinsing with a mouthrinse containing hydrophobic ligands yielded a significant reduction in the prevalence of Streptococcus mutans on orthodontic retention wires after only 1 week of use (17). The reduction in the prevalence of specifically S. mutans was attributed to a slight increase in the cell surface hydrophobicity of S. mutans strains relative to other members of the oral microbiome upon exposure to triclosan,
although others (18) could not demonstrate effects of exposure to triclosan on *S. mutans* cell surface hydrophobicity.

There is a rising interest worldwide in natural products for the maintenance of health. Magnolia bark extract (MBE) has been widely used in medicine for 2,000 years, and lately MBE received scientific attention as an anti-inflammatory, anti-platelet and even chemopreventive agent (19–23). Moreover, inhibitory effects of MBE on the growth of specific oral bacterial strains have been reported (24). When applied in a chewing gum, this resulted in a reduction of mutants streptococcal prevalence in saliva and decreased biofilm acidogenicity after 30 days of use (25). Also in candy applications, such as compressed mints, incorporation of MBE was effective in reducing the total amount of bacteria in saliva (26). MBE is harvested from the *Magnolia officinalis* tree and its two active components, magnolol and honokiol, are hydrophobic (19,27).

In this study we aim to investigate whether exposure of different oral Gram-positive and Gram-negative bacteria to MBE alters their hydrophobicity in a direction that enhances their removal from an aqueous suspension by a hydrophobic ligand. To this end, a wide array of both Gram-positive and Gram-negative oral strains was exposed to aqueous solutions with different concentrations of MBE and subsequently their removal by a hydrophobic ligand was determined using the MATH (Microbial Adhesion To Hydrocarbon) assay (28) in its kinetic mode (29).

**Materials and methods**

**Preparation of bacterial strains**

A total of 11 oral bacterial strains were used in this study. All strains were grown on blood agar plates from frozen stocks in dimethylsulfoxide and subsequently inoculated in a 10 ml pre-culture. Next, 100 µl of the pre-culture was used to inoculate 100 ml of a main culture. *S. mutans ATCC 25175*, *Streptococcus oralis J22*, *Streptococcus mitis* ATCC 9811, *Streptococcus salivarius* HB, *Streptococcus sanguinis* ATCC 10556 and *Streptococcus sobrinus* HG 1025 were grown aerobically at 37 °C in Todd-Hewitt broth (Oxoid, Basingstoke, UK). *Actinomyces naeslundii* T14V-J1, *P. gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 43046, *Veillonella parvula* BME1 and *F. nucleatum* BME1 were grown anaerobically at 37 °C in Brain Heart Infusion broth (Oxoid) supplemented with sterile 0.5% hemin and 0.1% menadione. Bacteria were harvested by centrifugation at 1700 g for 10 min and washed twice in sterile buffer (1 mM calcium chloride, 2 mM potassium phosphate, 50 mM potassium chloride, pH 6.8) with an ionic strength and composition similar to saliva (30) and suspended to a concentration of 5 x 10^8 bacteria/ml.
Magnolia bark extract
A 1% (w/v) solution of MBE (95% magnolol (5,5’-di-2-propenyl-(1,1’biphenyl)-2,2’-diol), 5% honokiol (3’,5-di-2-propenyl-(1,1’-biphenyl)-2,4’-diol), Honsea Sunshine Biotech Co. Ltd., Guangzhou, China) in 100% ethanol was mixed with sterile buffer to yield concentrations of MBE of 25, 50, 100 and 200 µg/ml. Buffer without MBE and with an equal amount of ethanol added as in the solution containing 200 µg/ml MBE, were used as a control. During the course of the experimental period, MBE was stored at -20 °C and all solutions were freshly prepared for each experiment.

Microbial adhesion to hydrocarbons
Microbial Adhesion to Hydrocarbons (MATH) measures the removal of microorganisms from aqueous suspensions by quantifying their adhesion to a hydrophobic ligand after mixing (28). As the original MATH assay was criticized for not being sufficiently quantitative, we here use the MATH assay in its kinetic mode (31). First, 3 ml of bacterial suspension was combined with MBE solutions for 10 min and its optical density (A₀) measured (Spectronic 20 Genesys, Thermo Scientific, Waltham MA, USA). Subsequently, 150 µl of hexadecane was added to each glass tube and mixed for 10 s using a vortex mixer set at a fixed rotation speed. The resulting emulsion was allowed to settle for 10 min for phase separation before the optical density of the aqueous phase was measured again (Aₜ). This process was repeated 6 times yielding a total mixing time of 60 s. Next, log((Aₜ/A₀) x 100) was plotted against mixing time and the initial removal rate (R₀) was calculated from the tangent of the curve at time zero. R₀ accordingly represents bacterial removal rate per min from the suspension by hexadecane. All experiments were performed in triplicate for each bacterial strain.

Statistics
Removal rates of each bacterial strain were averaged for the different concentrations of MBE applied, analyzed for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests (p < 0.05) and compared using an ANOVA followed by LSD post-hoc analysis to identify differences between MBE concentrations (p < 0.05). Next, removal rates of all Gram-negative and Gram-positive strains were averaged at the different MBE concentration, again analyzed for normality and equality of means was compared using an ANOVA followed by LSD post-hoc analysis to identify differences between MBE concentrations (p < 0.05). Statistical analysis was performed using SPSS v20.0 (IBM Corp., Armonk, USA).
Results

As an example, Fig. 1 presents the decrease in the optical density as a function of time during mixing with hexadecane of bacterial suspensions prior to and after bacterial exposure to different concentrations of MBE. Clearly, exposure of Gram-positive *S. sobrinus* to MBE has no significant effect on its removal by a hydrophobic ligand, while *P. gingivalis* removal increases with increasing MBE concentration.

![Graphs showing removal of bacteria](image)

**Figure 1**
Removal by hexadecane, expressed as $\log(A_t/A_0 \times 100)$ as a function of the mixing time for a Gram-positive (*S. sobrinus* HG 1025) and Gram-negative (*P. gingivalis* ATCC 33277) oral bacterial strain after exposure to different concentrations of MBE. Note: bacterial removal rates $R_0$ (min$^{-1}$) are derived from the initial linear part of the curves. Error bars represent standard error of the mean over three experiments with separately grown bacteria.

Initial removal rates $R_0$, as derived from the graphs such as presented in Fig. 1, are summarized in Fig. 2 for all strains and MBE concentrations. Initial removal rates prior to exposure to MBE vary ten-fold across the strains and are relatively low for *S. mutans* ATCC 25175, *S. oralis* J22 and *P. intermedia* ATCC 43046, whilst being high for *S. mitis* ATCC 9811 and *S. salivarius* HB. Initial removal rates of *F. nucleatum* were extremely high compared to the other strains. In general initial removal rates increase upon exposure to solutions with increasing concentrations of MBE and increases are statistically significant compared to 0 µg MBE/ml at 200 µg MBE/ml for all Gram-negative strains with the exception of *F. nucleatum*. Initial removal rates of *F. nucleatum* also increase with increasing concentrations of MBE in the exposure solution, but a maximal removal rate is reached at 100 µg MBE/ml after which a minor decrease sets in.
MBE increases adhesion of oral Gram-negative bacteria to a hydrophobic ligand

Figure 2
Initial removal rate by hexadecane ($R_0$) for 11 oral bacterial strains prior to and after exposure to aqueous solutions of MBE at different concentrations. Bacterial names indicated in bold are Gram-negative strains. Asterisks (*) indicate a significant difference compared to 0 µg MBE/ml, i.e. prior to exposure to MBE. Error bars denote standard error of the mean over three experiments with separately grown bacteria.

Next, initial removal rates were averaged for all Gram-negative (with the exception of F. nucleatum as this would yield a skewed data distribution) and Gram-positive strains (Fig. 3), revealing that the initial removal rates of Gram-negative bacteria significantly increase by up to a factor of four with increasing concentrations of MBE, while removal of Gram-positive bacteria is not affected at all by the exposure to MBE.
Discussion

The development of new oral health care products is currently aimed towards maintaining the oral microbiome at health, targeting at the removal of oral pathogens (8,10). Here we demonstrate that bacterial exposure to MBE in aqueous solution yields changes in hydrophobicity of Gram-negative oral bacteria that enhance their removal by a hydrophobic ligand but not of Gram-positive strains. This suggests that an oral health care regimen consisting of exposure of the oral microbiome to MBE followed by exposure to a hydrophobic ligand can selectively remove Gram-negative bacterial strains from the oral cavity. Such a health care regimen may be used to reduce the prevalence of Gram-negative associated oral diseases.

Gram-negative bacterial strains differ from Gram-positive strains by the possession of a double lipid membrane. Every bacterial strain is decorated with a variety of different surface appendages (32), but Gram-positive strains mainly expose a thick peptidoglycan layer as their outer surface, whilst the outer surface layer of Gram-negative bacteria mainly consists of the outer lipid membrane. Hydrophobic compounds adsorb...
more readily to hydrophobic lipids than to more hydrophilic peptidoglycan, which explains why magnolol and honokiol are known to bind more tightly to the surface of Gram-negative bacteria (lipid content of cell wall 25% dry weight) than to the Gram-positive bacterial cell surface (lipid content of cell wall 0-3% dry weight) (33). Accordingly, it can be understood why bacterial exposure to solutions containing MBE enhances removal by a hydrophobic ligand of Gram-negative strains and not of Gram-positive ones.

In general, anaerobic Gram-negative bacteria, such as *P. intermedia*, *P. gingivalis* and *F. nucleatum* are considered causative in the etiology of halitosis (34,35) as well as in oral diseases like gingivitis and periodontitis. Previously it was shown that a small, but significant four-fold increase in removal rate of *S. mutans* after exposure to triclosan by hydrophobic mouthrinse components could invoke a change in oral biofilm composition (17). Our current study shows a similar potential of MBE to affect the Gram-negative bacterial cell wall and enhance its removal from the oral cavity when applied in two-component health care products to target Gram-negative bacteria associated oral diseases. For experimental reasons, we could not expose bacteria for shorter times to MBE solutions than the 10 min used, which does not necessarily imply that shorter exposure times would have given different results. Nevertheless, mouthrinses or toothpastes with typical applications times of less than 2 min, may not be the most suitable vehicles for a two-component product suggested here. Chewing gums or candies typically have residence times in the oral cavity around 10 min and may therefore be more suitable to exploit the potential of MBE in two-component variants.

The current data provide proof of principle for an effect of a two-component oral health care product containing MBE and a hydrophobic ligand on selective removal of Gram-negative bacteria from the oral cavity. Subsequent clinical testing should be conducted to establish whether such a product will also enhance bacterial removal from enamel surfaces, leading to an *in vivo* reduction of the prevalence of Gram-negative pathogens in the oral cavity, potentially associated with reductions in disease prevalence.

Concluding, in this study we demonstrate that exposure to MBE of Gram-negative oral bacteria enhances their removal from an aqueous phase by a hydrophobic ligand, while not affecting removal of Gram-positive oral bacterial strains. To the best of our knowledge this is the first time that enhanced microbial adhesion to a hydrophobic ligand for Gram-negative oral bacteria by MBE is demonstrated, showing the potential of MBE as an active ingredient in oral care products and nutraceuticals that contain a hydrophobic ligand.
Acknowledgements
We would like to thank Minne Koopmans for assisting and performing a part of the MATH experiments.

Conflict of interest
This work was funded by Wm. Wrigley Jr. Co, Chicago, USA and SASA BV, Thesinge, NL. Authors were employed by their own organizations. HJB is also director-owner of SASA BV, AM, MWJD are employees of the Wm. Wrigley Jr. Company. Opinions and assertions contained herein are those of the authors and are not meant to be construed as the representing views of the organizations to which the authors are affiliated. This study relates to a pending patent application.

References


27. William Wrigley Jr. company. Application for the approval of magnolia bark supercritical carbon dioxide
extract (MBSE) from magnolia officinalis under regulation (EC) No 258/97 of the European parliament and of the council of 27th January 1997 concerning novel foods and novel food ingredients. 2009; 1–79


