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Antibacterial effects of blackberry extract target periodontopathogens

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Background and Objective: Antimicrobial agents provide valuable adjunctive therapy for the prevention and the control of oral diseases. Limitations in their prolonged use have stimulated the search for new, naturally occurring agents with more specific activity and fewer adverse effects. Here we sought to determine the antibacterial properties of blackberry extract (BBE) *in vitro* against oral bacterial commensals and periodontopathogens.

Material and Methods: The effects of whole and fractionated BBE on the metabolism of 10 different oral bacteria were evaluated using the colorimetric water-soluble tetrazolium-1 assay. The bactericidal effects of whole BBE against *Fusobacterium nucleatum* were determined by quantitating the numbers of colony-forming units (CFUs). Cytotoxicity was determined in oral epithelial (OKF6) cells.

Results: BBE at 350–1400 μg/mL reduced the metabolic activity of *Porphyromonas gingivalis, F. nucleatum* and *Streptococcus mutans*. The reduced metabolic activity observed for *F. nucleatum* corresponded to a reduction in the numbers of CFUs following exposure to BBE for as little as 1 h, indicative of its bactericidal properties. An anthocyanin-enriched fraction of BBE reduced the metabolic activity of *F. nucleatum*, but not of *P. gingivalis* or *S. mutans*, suggesting the contribution of species-specific agents in the whole BBE. Oral epithelial cell viability was not reduced following exposure to whole BBE (2.24–1400 μg/mL) for ≤ 6 h.

Conclusion: BBE alters the metabolic activity of oral periodontopathogens while demonstrating a minimal effect on commensals. The specific antibacterial properties of BBE shown in this study, along with its previously demonstrated anti-inflammatory and antiviral properties, make this natural extract a promising target as an adjunct for prevention and/or complementary therapy of periodontal infections.

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Periodontal disease results from chronic infection and inflammation of the tissues that support the teeth (1). This oral inflammatory disease affects about 50% of the United States population (2) and is driven by pathogenic succession characterized by a shift in microbial species in the gingival sulcus from gram-positive, facultative and fermentative microorganisms to predominantly gram-negative, anaerobic, chemo-organotrophic and proteolytic microorganisms (1). Bacteria such as *Actinomyces viscosus, Actinomyces naeslundii* and *Streptococcus* spp. are associated with gingivitis and dental caries, whereas *Porphyromonas gingivalis, Tannerella forsythia, Treponema*

denticola, Prevotella intermedia and *Fusobacterium nucleatum* are later colonizers associated with periodontopathic biofilms (3). Periodontal disease also is implicated in several systemic diseases, including preterm/low birthweight deliveries, cardiovascular events, diabetes and other systemic conditions (4,5). Thus, development of new cost-effective measures to prevent and control periodontitis is important for improving oral and systemic health.

Successful treatment of periodontal disease involves elimination of the microbial burden and associated clinical signs of inflammation through mechanical disruption of biofilms, as well as the use of antimicrobial agents in aggressive and unresponsive forms of the disease (6). Use of mouth rinses with antimicrobial activity [e.g. chlorhexidine (CHX)] has been effective for controlling the colonization of oral bacteria, including periodontopathogens, and reducing gingival inflammation (7–9). Nevertheless, topical antimicrobial rinses containing CHX have restrictions owing to potential side effects, including staining and erosive and abrasive effects, as well as limitations on dosage scheduling (10,11). As bacterial growth as an oral biofilm is a continuous event important for biological equilibrium within the oral cavity, and few commercial products exist that inhibit this process, it is important to develop new and more efficient therapeutic strategies to control the constant emergence of bacterial pathogens related to oral disease.

Use of natural agents in chewing gums is an important approach for addressing this issue. Previous observations have suggested that blackberry extract (BBE) exhibits anti-inflammatory, anticancer and antiviral properties (12–15), and a limited number of reports have demonstrated antibacterial activity of blackberries and raspberries against skin and enteric pathogens (14,16). However, the antimicrobial effect of BBE as a topical agent against periodontal pathogens has not yet been demonstrated. In this study we sought to determine the antibacterial properties of BBE *in vitro* against oral bacteria associated with gingivitis and periodontal disease, with the potential for this material to be used as a topical agent for treating these infections.

Material and methods

Plant material, preparation and fractionation of BBEs

Hull blackberries (*Rubus eubatus* cv. ''Hull'') were grown at WindStone Farms (Paris, KY, USA). Seeds and skin were removed using a Langsenkamp type 161 Colossal Pulper (Langsenkamp Manufacturing LLC, Indianapolis, Indiana) and the resultant puree was stored at −20°C. Extracts were obtained from the puree (12,13,17). Briefly, blackberry puree (10 g) was sonicated for 30 min with 25 mL of an extraction solvent of ethanol containing 0.01% HCl (v/v). The supernatants were collected after filtration and dried by rotary evaporation at 40°C. The dried extract was resuspended in deionized water, filtered through 20–25 μm pore-size filter paper and lyophilized to obtain dried BBE. Dried BBE was dissolved in deionized water as a stock solution (140 mg/mL) and stored at −80°C until use, and is referred to as ''whole BBE''. The whole BBE was further fractionated by solid phase extraction modified from Skrede *et al.* (18). Whole BBE was applied to a preconditioned Discovery DSC-18 tube (Supelco, Bellefonte, PA, USA) and eluted sequentially with water, ethyl acetate and finally 50% aqueous methanol. As described previously in Murapa *et al.* (22), the water fraction contained > 98% of the total substances and comprised < 0.01% phenolic compounds. In contrast, > 99% of the total phenolics in the extract were concentrated in the ethyl acetate and methanol fractions, and > 97.3% of the anthocyanins were obtained in the methanol fraction. The methanol fraction (anthocyanin-enriched) was dried by rotary evaporation, reconstituted in dimethylsulfoxide as stock solution and stored at −80°C until use, and is referred to as ''anthocyanin-enriched fraction BBE''. Note that the anthocyanin-enriched fraction BBE does not contain methanol. As reported in Dai *et al.* (12), the polyphenols and anthocyanins are very stable when stored at −80°C, with no loss of activity for at least 90 d. Once thawed, the thawed sample was used once and then discarded.

Bacterial strains and growth conditions

Streptococcus mutans (ATCC 25175), *Streptococcus oralis* (ATCC 10557), *A. naeslundii* (ATCC 49340), *A. viscosus* (ATCC 43146), *Veillonella parvula* (ATCC 17745), *P. intermedia* (ATCC 25611), *F. nucleatum* (ATCC 25586), *P. gingivalis* (ATCC 381), *Aggregatibacter actinomycetemcomitans* (JP2) and *Streptococcus gordonii* (ATCC 10558) were used. All bacteria were grown in brain–heart infusion broth supplemented with 5 μg/mL of hemin and 1 μg/mL of menadione (BHI with supplements), at 37°C under anaerobic conditions (80% N_2, 10% H_2 and 10% CO_2).

Effect of BBE on bacterial metabolism

Bacteria were seeded at a density of 1×10^7 cells/well into 96-well plates with 135 μL of BHI with appropriate growth supplements. Fifteen microlitres of test reagent (i.e. different concentrations of whole BBE or anthocyanin-enriched fraction diluted in sterile water) was added to each well. As controls, 15 μL of medium, water (BBE vehicle) or 1% CHX gluconate freshly prepared from 20% stock solution (Sigma Chemical Co., St Louis, MO, USA) was added to select wells. The final concentration of CHX was selected based on its known antibacterial properties *in vitro* and *in vivo* (19,20) and its utility in the metabolic assays without adversely affecting the optical density (OD) readings. In these experiments, the colorimetric water-soluble tetrazolium-1 (WST-1) assay was used as the metabolic assay, and it served as a surrogate marker of cell proliferation and viability. Here, metabolic activity is defined as the ability of the viable cells to convert the stable tetrazolium salt to a visible dye (formazan) in the WST-1 assay. The assay was performed by incubating plates under

anaerobic conditions for 24 h, then 15 μL of cell-proliferation reagent WST-1 (Roche, Mannheim, Germany) was added to each well, as described in previous reports using this type of metabolic indicator (21). Finally, the plates were incubated for 1–2 h under the same assay conditions, then the absorbance was measured at 420–480 nm, with a reference wavelength of 600 nm, using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage inhibition was calculated using the ODs of bacterial metabolic activity under different experimental conditions according to the following formula: $100 \times [\text{OD (control)} - \text{OD (experimental)} / \text{OD (control)}]$, where control cultures were treated with media alone and experimental cultures were cultures treated with BBE, vehicle or CHX, as experimental, negative and positive controls, respectively.

Bactericidal effect of BBE

Bacteria were seeded into 96- well plates at a density of 1×10^7 cells/well in a total volume of 135 μL of BHI with supplements, and were incubated with 15 μL of different concentrations of whole BBE or water (vehicle control) for 1 h or 24 h, at 37°C under anaerobic conditions. After treatment, 100 μL of bacterial culture dilutions (2–4 log range) were spread onto blood agar plates using glass microbeads, and, after 48–72 h of incubation under the same experimental conditions, the numbers of colony-forming units (CFUs) were enumerated.

Cytotoxic effect of BBE in oral epithelial cells

The immortalized keratinocyte cell line OKF6/hTERT-2 (OKF6), established by ectopic expression of the telomerase catalytic subunit (hTERT) in cells from normal oral mucosal epithelium, was obtained from Dr James Rheinwald, Harvard Medical School, and used for viability assays, as previously shown (22,23). Cells were cultured in keratinocyte-SFM supplemented with bovine pituitary extract (25 μg/mL), recombinant epidermal growth factor (0.2 ng/mL) and penicillin–streptomycin (Ker-SFM) and were maintained at 37°C in a humidified incubator with 5% CO_2. For viability assays, cells were plated in 96-well plates at 9400 cells/well in Ker-SFM. The following day, cells were treated with vehicle control (water) or with varying concentrations of BBE for 3, 6 or 24 h. Cells were exposed to BBE at dilutions ranging from 2.24–1400 μg/mL final concentration. OKF6 cell viability was determined by quantifying the conversion of resazurin to resorufin using the CellTiter-Blue viability assay (Promega, Madison, WI, USA) (23).

Statistical analysis

Significant differences between the means for the experimental groups were determined using the Student's *t*-test (SigmaStat 3.5; Systat Software, Point Richmond, CA, USA). In cell-viability studies, differences among treatment groups were determined using analysis of variance and Fisher's least significance test, using STATVIEW software (SAS Institute, Cary, NC, USA). *p* values were considered significant at < 0.05.

Results

Effects of the whole and an anthocyanin-enriched fraction of BBE on bacterial metabolic activity were evaluated against several oral commensal and pathogenic periodontal bacteria. In these assays, 24 h of exposure to BBE significantly reduced the metabolic activity of a small group of oral bacteria particularly associated with periodontal disease and dental caries, but not that of other pathogens or commensal microorganisms (Table 1). We observed that 700 μg/mL of whole BBE inhibited the metabolic activity of *F. nucleatum* and *P. gingivalis* by about 40%. The same concentrations of whole BBE reduced the metabolic activity of *S. mutans* by approximately 30%. Higher concentrations of whole BBE (1400 μg/mL) showed a greater effect only against *F. nucleatum*, reducing its metabolic activity by about 84%. A similar range of metabolic inhibition (30–57%) was observed for *F. nucleatum* and *P. gingivalis* after 24 h of exposure to 0.1% CHX.

To understand the pharmacologic properties of BBE in greater detail, the anthocyanin-enriched fraction of BBE with known antiproliferative and anti-inflammatory properties (13), was next investigated. This enriched fraction exhibited antimicrobial effects against *F. nucleatum* similar to those seen with whole BBE, reducing metabolic activity by about 70% after 24 h of exposure (Fig. 1). Of note, the enriched BBE fraction did not significantly

Table 1. Effect of whole blackberry extract (BBE) on the metabolic activity of common oral bacteria

	Range of BBE concentrations				
Strains	Water	175 μg/mL	350 μg/mL	700 μg/mL	1400 μg/mL
Streptococcus oralis	−3.1 (8.6)	3.6 (2.7)	−0.5 (9.3)	−7.9 (10.5)	8.6 (7.8)
Streptococcus gordonii	2.3 (3.6)	−1.0 (10.2)	−1.6 (4.5)	−11.1 (13.3)	−11.6 (5.1)
Streptococcus mutans	5.2 (9.6)	16.1 (5.9)	**23.9 (4.0)***	**31.4 (3.4)***	**27.5 (1.7)***
Actinomyces naeslundii	3.3 (12.2)	6.5 (13.3)	12.9 (7.0)	11.7 (10.8)	14.0 (8.7)
Actinomyces viscosus	−7.1 (7.0)	0.4 (5.2)	3.8 (4.9)	−3.4 (8.1)	10.3 (7.3)
Veillonella parvula	−0.2 (2.1)	−0.4 (2.4)	−0.4 (2.6)	−0.03 (2.6)	−0.003 (2.1)
Prevotella intermedia	0.3 (2.3)	0.1 (2.3)	0.4 (2.1)	0.2 (2.6)	0.3 (2.2)
Aggregatibacter actinomycetemcomitans	7.6 (20.0)	−7.2 (17.8)	−14.2 (14.3)	−19.5 (10.3)	−9.6 (10.4)
Porphyromonas gingivalis	1.6 (6.9)	13.8 (5.5)	**27.0 (5.3)***	**40.7 (2.0)***	**43.6 (2.5)***
Fusobacterium nucleatum	1.7 (5.7)	0.0 (7.0)	4.5 (9.4)	**40.9 (18.5)***	**83.5 (5.2)***

Data are mean (standard deviations) percentage reductions of metabolic activity following treatment with a range of BBE concentrations and represent the results from at least two independent experiments performed in triplicate for each strain of bacteria. Values significantly different ($p \leq 0.05$) between water treatment vs. BBE extract are identified with an asterisk (*). The percentages of metabolic-activity inhibition for *F. nucleatum* and *P. gingivalis* exposed to 0.1% chlorhexidine for 24 h were 29.1 (13.7) and 57 (5.9), respectively.

inhibit *P. gingivalis* or *S. mutans*. These findings suggest that the antibacterial activity against *F. nucleatum* was retained in the anthocyanin-enriched fraction, whereas the specific inhibitory component(s) against *P. gingivalis* and *S. mutans* were eliminated from BBE during the fractionation process.

Lastly, we determined whether the reduction in bacterial metabolic activity observed for *F. nucleatum* as a result of treatment with whole BBE was caused by a reduction in cell viability. This periodontopathogen was selected for these experiments because *F. nucleatum* was most susceptible to inhibition by BBE. Consistent with the findings for the metabolic-based assay, the numbers of CFUs of *F. nucleatum* were significantly reduced (by approximately 4- to 13.6-fold) by BBE concentrations of ≥ 350 μg/mL (Fig. 2A). Shorter exposure of *F. nucleatum* (1 h) to whole BBE also induced a similar significant reduction in the numbers of CFUs (by approximately three-fold) (Fig. 2B). Of note, oral epithelial (OKF6) cell viability was not reduced following exposure to whole BBE (2.24–1400 μg/mL) for ≤ 6 h, suggesting that at these concentrations and exposure times, whole BBE was not harmful to oral epithelial cells. In contrast, viability was reduced by 15% and 43% following 24 h of exposure to 280 and 1400 μg/mL of whole BBE, respectively (Fig. 3).

Discussion

Overgrowth of specific bacterial species within oral biofilms is associated with periodontal disease (24,25). Thus, the infectious nature of periodontitis makes the use of topical antimicrobial agents that interfere with bacterial replication and co-aggregation an important strategy for preventing periodontal disease (26–28). To date, CHX has been one of the most effective antimicrobial agents for the adjunctive control of gingivitis and periodontitis. However, its broad spectrum of activity and adverse-effect profile limits its prolonged use as a topical oral antimicrobial agent by the general population (10). Therefore, there is a need to develop additional active agents with higher specificity and limited adverse effects that can control the growth of oral pathogens on dental and mucosal surfaces, while limiting the effect on commensals.

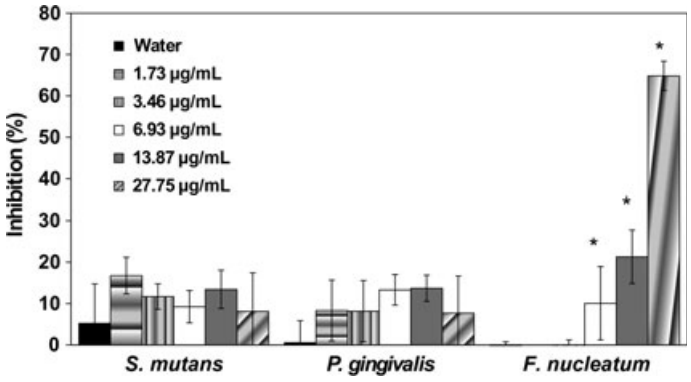

Fig. 1. Effect of the anthocyanin-enriched fraction of blackberry extract (BBE) on the metabolic activity of bacterial oral pathogens (*Streptococcus mutans*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*). The bars denote the mean percentage inhibition of different fraction concentrations and the standard deviation of at least two independent experiments performed in triplicate for each species of bacteria. Values significantly different ($p \leq 0.05$) between water treatment vs. BBE anthocyanin-enriched fraction are identified with an asterisk (*).

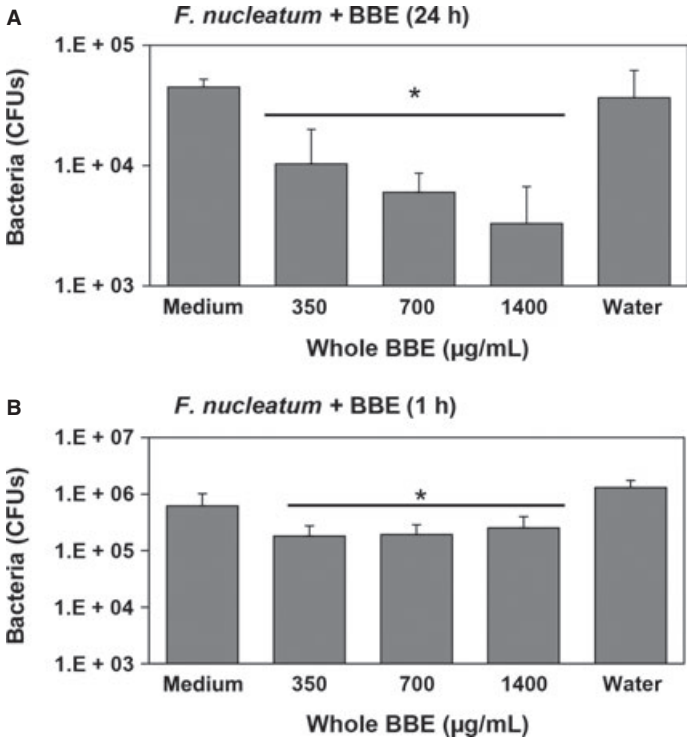

Fig. 2. Bactericidal effect of the whole blackberry extract (BBE) against *Fusobacterium nucleatum*. Bacteria were seeded into 96-well plates in brain–heart infusion (BHI) broth, in either the presence or the absence of several concentrations of whole BBE extract diluted in water for (A) 24 h or (B) 1 h. The numbers of colony-forming units (CFUs) were enumerated after bacteria were spread into agar plates and cultured, as described in the Material and methods. The bars are representative of at least two independent experiments performed in triplicate. Data are expressed as mean ± standard deviation. Values significantly different ($p \leq 0.05$) between untreated bacteria (control) vs. bacteria exposed to BBE or water are identified with an asterisk (*).

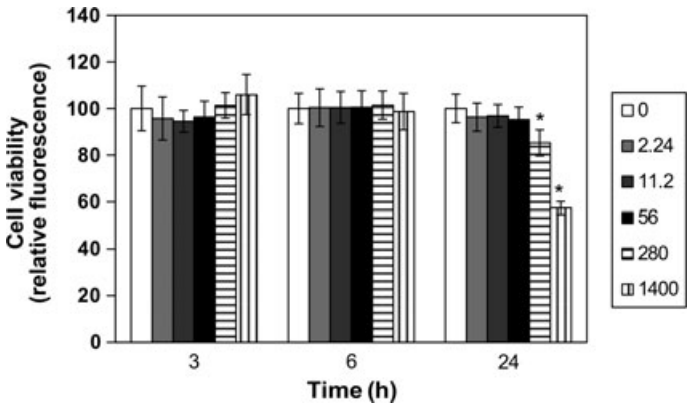

Fig. 3. Cytotoxic effect of blackberry extract (BBE) in oral epithelial (OKF6) cells. OKF6 cells were plated in 96-well plates (at a density of 9400 cells/well) in Ker-SFM and cultured for 24 h as described in the Material and methods. Cells were treated with vehicle control (water/white bar) or with varying concentrations of BBE for 3, 6 or 24 h. Cell viability was evaluated using the CellTiter-Blue viability assay. Data for BBE-treated cultures were normalized to the mean for water-treated cultures. Values significantly different ($p \leq 0.05$) between water-treated cells vs. OKF6 cells exposed to BBE are identified with an asterisk (*).

The anti-inflammatory and antimicrobial properties of BBE, as well as its biochemical characteristics, make it a promising candidate for the development of topical therapeutic strategies focused on controlling bacterial growth in relation to periodontal diseases (13,15). Accordingly, we tested the antimicrobial properties of BBE against a group of oral bacteria including commensals and periodontopathogens. Using the whole extract from Hull blackberries, we found that the antimicrobial effect was specific, with significant inhibition of metabolic activity occurring in a select group of microorganisms associated with periodontitis (*F. nucleatum* and *P. gingivalis*) and dental caries (*S. mutans*), but not in the oral commensal microorganisms, as occurs with CHX (29). Although the mechanism for the antibacterial specificity shown by BBE needs to be elucidated, it could involve a critical metabolite of anthocyanins (i.e. gallic acid) (30), which is an iron chelator that forms stable complexes with these metal ions and decreases their availability to bacterial species (31). Thus, the differential iron requirements for sustaining normal bacterial growth and/or the ability to persist under iron-deficient conditions exhibited by oral bacterial species, could make BBE-induced iron starvation a selective process for controlling pathogenic oral strains. The selective targeting demonstrated by BBE is important because growing evidence suggests a critical role for the commensal bacteria colonizing the mucosal surfaces in maintaining the homeostasis and modulating the innate immune response, which results in a protective effect from pathogens (32). Therefore, the specificity exhibited by the BBE in this study would be an important characteristic to consider for controlling oral pathogens in the context of polymicrobial communities.

In addition, our studies show that BBE not only impacts bacterial metabolism, but also has the ability to kill oral bacteria, as observed for *F. nucleatum* at BBE concentrations of ≥ 350 μg/mL. Although the cellular and molecular mechanisms associated with the antimicrobial effects are not fully understood, evidence suggests that berry-derived polyphenols (e.g. anthocyanins) could be involved (33–36). This is consistent with recent evidence that polyphenolic beverages, such as red wine, cistus tea and black tea, can reduce the total count of adherent oral bacteria (37).

It is noteworthy in our studies that short exposure times (1 h) of *F. nucleatum* to BBE had a significant bactericidal effect, and the same range of BBE concentrations did not affect oral epithelial cell viability within the first 6 h. Thus, these antimicrobial properties would be consistent with the short-term topical use of BBE in oral products for bacterial oral diseases. Also, a significant change in *F. nucleatum* metabolic activity was not observed following 24 h of exposure to 350 μg/mL of BBE, yet this concentration conferred bactericidal activity, as determined by the significant reduction of CFUs. Although this finding may seem counterintuitive, we speculate that at this concentration BBE-derived constituents may interfere more with bacterial metabolism necessary for survival than with events required for bacterial replication, as shown by others (38). Thus, the metabolic activity of the ''surviving'' cells could have increased proportionally, such that the overall metabolic activity of the cultures remained unchanged.

It is important to mention that the efficiency in recovering anthocyanins from a natural source depends on several factors, such as the culture, growth conditions and storage of raw material (39). Similarly, the solvent system used for extraction plays a crucial role because it has been shown that the recovery of anthocyanins can be substantially improved by selective enrichment processes (40). In this research, the antimicrobial effect of previously titrated concentrations of the enriched portion of BBE (13) was evaluated against three oral pathogens. Interestingly, when the anthocyanin-enriched fraction was tested, the metabolism of *F. nucleatum*, but not that of either *P. gingivalis* or *S. mutans*, was affected in a manner similar to that observed with whole BBE. These results suggest that the components within the whole BBE that have antimicrobial activity against *P. gingivalis* and *S. mutans* could be different (i.e. nonphenolic components) and would be present in the unused portion of the fraction, in contrast to the situation observed for *F. nucleatum*. Thus, future studies are being directed toward the development of additional chemical isolation strategies that will identify those specific antimicrobial BBE components.

In summary, this study showed that noncytotoxic BBE concentrations

exhibit antimicrobial properties against important periodontal pathogens as well as *S. mutans*. The minimal effect of BBE on early oral colonizers considered as commensal bacteria (i.e. *Streptococcus* and *Actinomyces*) is also a promising observation for its potential utility as a product in controlling oral disease. Although more studies are clearly needed, including further characterization of BBE, the assessment of BBE against oral biofilm formation and maintenance, its effect on other pathogenic strains from the same species, as well as its potential for tooth staining, suggest that this natural extract has the potential to be used as an antibacterial topical agent for the prevention and control of periodontitis as well as dental caries. Chewing gums containing a variety of agents (e.g. sorbitol, xylitol and CHX) are recognized to be effective adjunctive approaches for maintaining oral health (41–43). Incorporation of BBE in oral- release devices, such as chewing gum, is a long-term goal.

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Conflict of interest

The authors report no conflicts of interest related to this study.

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