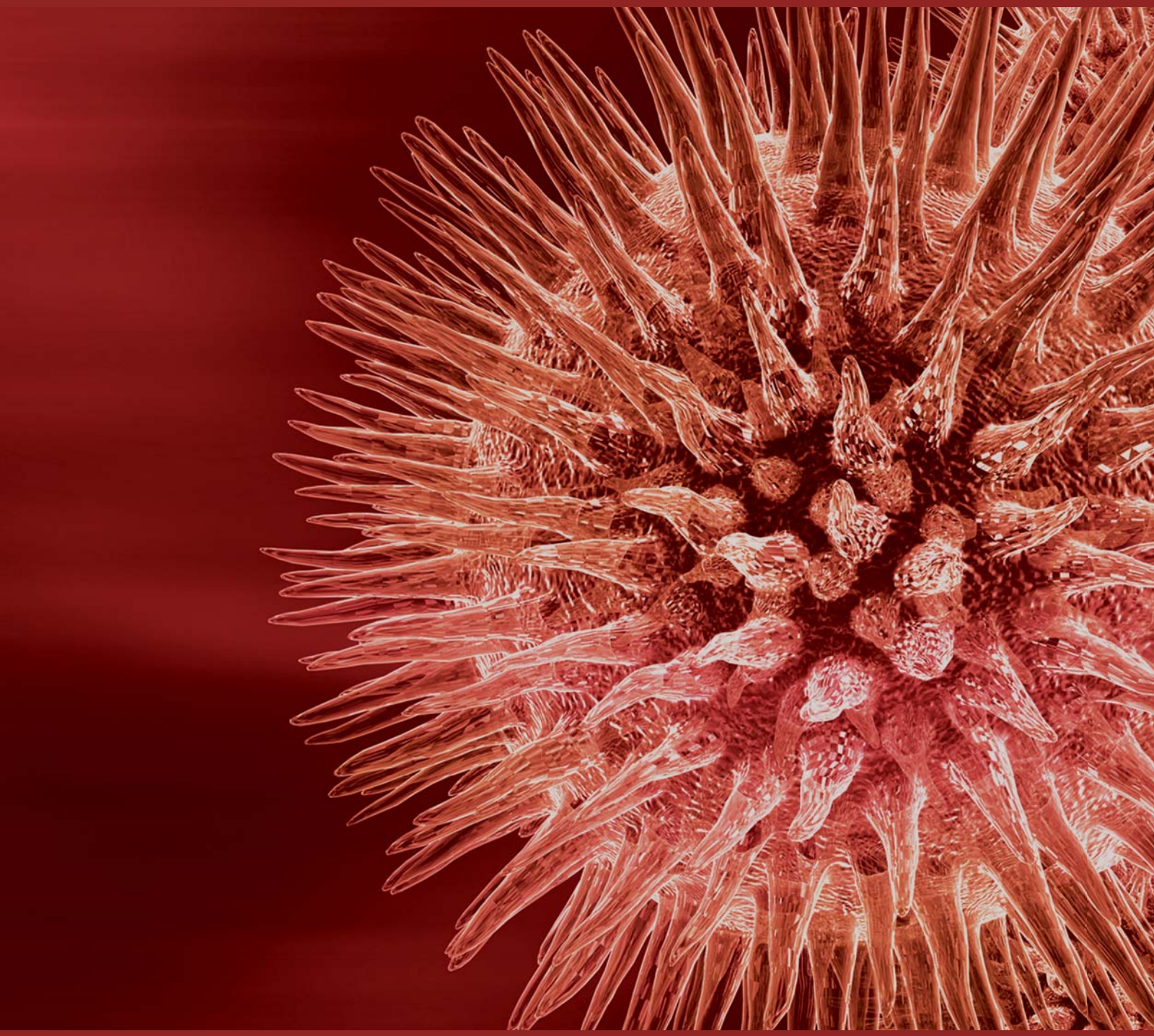
Journal of Biomedicine and Biotechnology

Functional Foods: Towards Improving Oral Health

Guest Editors: Itzhak Ofek, Carla Pruzzo, and David Spratt



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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 618314, 2 pages
doi:10.1155/2012/618314

Editorial

Functional Foods: Towards Improving Oral Health

Itzhak Ofek,[1] Carla Pruzzo,[2] and David Spratt[3]

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Received 24 November 2011; Accepted 24 November 2011

The aim of this special issue is to provide a foundation on which further research for the development of compounds from food as possible candidates for improving oral health could be based. The maintenance of oral health can be invariably achieved by manipulating the oral microbiota toward a population of mixed species that is less likely to induce diseases such as gingivitis or caries. Dental caries and gingivitis are the most prevalent infectious diseases of humans and are due to the accumulation of dental plaque (a bacterial biofilm) on the tooth surface and at the gingival margin, respectively.

There is evidence that certain beverages and foods can protect against caries and gingivitis. However the use of foodstuffs (functional foods) as a starting point for fractionation of compounds that influence the composition of the oral microbiota to one that is beneficial or at least nonpathogenic has been the focus of intensive research [1]. An advantage of this approach is that such natural agents are likely to be nontoxic. Furthermore, the identified active components can be used as food supplements negating the necessity to adhere to a particular diet as discussed by Shmuely et al. [2]. Perhaps most important advantage of searching for such dietary agents is that approval of clinical trials is easier to obtain, as toxicity is usually not an issue. Interestingly, dietary agents often lack bactericidal activity but retain their ability to manipulate the oral microbiota by exhibiting other important properties for example antiadhesion or antibiofilm activities. Such agents may be expected to decrease the selection pressure placed on resistant strains and therefore reduce their emergence in biofilm communities.

The collection of manuscripts in this special issue is mainly composed of research articles and one review article. The latter highlights the importance of food intake with emphasis on the effect of Mediterranean diet on oral health, discussed by G. A. Scardina and P. Messina. All the research articles represent methodological studies by a coherent group of investigators (Investigators from 7 academic institutions and an industrial partner representing 5 countries (University of Pavia, University of Tel Aviv, University of Genoa, Göteborg University, Academic Centre for Dentistry Amsterdam, and the University of Verona as well as an industrial partner, Givaudan). The consortium and project were named NUTRIDENT and was a € 2.2M specific targeted research project entitled "Towards functional foods for oral health care-isolation, identification and evaluation of beverage and food components with anti-caries and/or anti-gingivitis activities." It was funded from within the Framework Programme 6 (FP6)—Thematic Priority 5: Food Quality and Safety.)

Seven plant and fungal homogenates and extracts (green and black tea, cranberry juice, raspberries, shiitake mushrooms, red chicory, and beer) were subjected to a number of microbiological assays broadly related to survival and/or fitness of specific oral bacterial species usually associated with the development of either gingivitis or caries. This is discussed by D. A. Spratt et al. In this study it was found that the low-molecular-mass (LMM) fractions of shitake mushroom and chicory homogenates had the most significant anticaries and antigingivitis associated activities of the seven tested. These included inhibition of bacterial growth, adherence, coaggregation, biofilm formation, biofilm integrity,

and signal transduction. A significant find was that these two homogenates also inhibited proinflammatory cytokine production. Further in-depth studies by the investigators focusing on specific bacterial activities were carried out. Shiitake mushroom extract lowered the numbers of some pathogenic oral bacteria without affecting bacteria associated with oral health. This is demonstrated by L. Ciric et al. Moreover, the compounds in the LMM fraction from shiitake mushroom inhibited dentin demineralization usually caused by cariogenic bacteria and induced a shift in the microbiota to that associated with oral health; this is shown by E. Zaura et al. The LMM fraction in shiitake mushroom and chicory homogenates also inhibited the adverse induction of genes expression in the gingival KB cell line by gingivitis bacteria; this is exhibited by L. Canesi et al. Efforts to characterize the effect of the chicory and mushroom extracts revealed that the extracts induced elongation of gingivitis-associated bacteria, reminiscent to that induced by sublethal concentrations of quinolones and β-lactam antibiotics; this is discused by C. Signoretto et al. These *in vitro* findings prompted clinical trials with the LMM shiitake mushroom fraction. In one trial the total counts of plaque bacteria in volunteers rinsing with mouthwash supplemented with LMM mushroom fraction were significantly reduced to levels similar to those in samples of volunteers rinsing with Listerine; this is proven by C. Signoretto et al.). In another trial it was found that mouthwash containing the mushroom fraction reduced the metabolic activity of dental plaque, suggesting anticariogenic potential of the fraction; this is demonstrated by P. Lingström et al. These *in vivo* effects are reminiscent to the outcome of a clinical trial whereby rinsing with a cranberry fraction caused significant reduction in total oral bacteria including mutans streptococci [2]. Indeed as confirmation, and as shown in this special issue, the cranberry fraction reduced metabolic activity of preformed *Streptococcus* sp. biofilm; which is shown by J. Babu et al.

A detailed characterization of the plant and fungal extracts and the fractions arising from these was carried out by M. Daglia et al. and attempts to identify the active component(s) were initiated. All seven plant and fungal extracts were found to contain polyphenols; relatively high amounts were detected in beer, cranberry, and green and black tea by M. Daglia et al. Chicory and shiitake mushroom homogenates and raspberry contained the highest amount of zinc. Further studies are planned to identify the active components in these extracts especially those in the LMM fractions exhibiting the most favourable activity for maintaining a community associated with oral health.

In summary, promising foods/beverages have been identified using a range of *in vitro* and *in vivo* methods. These homogenates, or fractions of them, could be used in a number of ways to improve oral health, perhaps in mouthwashes or chewing gum.

Acknowledgments

We would like to thank the authors for providing such high-quality articles for this special issue of the Journal of Biomedicine and Biotechnology. We sincerely hope that this collection of papers will prompt further research and development of functional foods or compounds derived from these to be supplemented into various foods and beverages or as rinses to improve oral health.

Itzhak Ofek
Carla Pruzzo
David Spratt

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 510198, 12 pages
doi:10.1155/2012/510198

Research Article

Evaluation of Plant and Fungal Extracts for Their Potential Antigingivitis and Anticaries Activity

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Received 9 August 2011; Revised 7 November 2011; Accepted 10 November 2011

Academic Editor: Itzhak Ofek

The link between diet and health has lead to the promotion of functional foods which can enhance health. In this study, the oral health benefits of a number of food homogenates and high molecular mass and low molecular mass fractions were investigated. A comprehensive range of assays were performed to assess the action of these foods on the development of gingivitis and caries using bacterial species associated with these diseases. Both antigingivitis and anticaries effects were investigated by assays examining the prevention of biofilm formation and coaggregation, disruption of preexisting biofilms, and the foods' antibacterial effects. Assays investigating interactions with gingival epithelial cells and cytokine production were carried out to assess the foods' anti- gingivitis properties. Anti-caries properties such as interactions with hydroxyapatite, disruption of signal transduction, and the inhibition of acid production were investigated. The mushroom and chicory homogenates and low molecular mass fractions show promise as anti-caries and anti-gingivitis agents, and further testing and clinical trials will need to be performed to evaluate their true effectiveness in humans.

1. Introduction

During the last decade epidemiological studies have demonstrated a clear relationship between diet and health and this has resulted in new roles being ascribed to foods. Foods are now not only regarded as being an indispensable source of nutriment but are also considered to be beneficial in many ways. Foods that have some particular beneficial effects on health are generally defined as functional foods [1, 2]. Their activity is determined by a specific and selective interaction of their minor components with one or more physiological functions of the organism. Both simple foods and food products, meaning technologically treated foods in which their chemical composition and, therefore, their organoleptic, nutritional, or biological characteristics have been changed, are considered functional foods. Foods depleted in, or enriched with, specific components can also be considered to be functional foods.

Caries is one of the most prevalent chronic diseases of humans. It is an endogenous infection of the calcified tissues of the teeth and is a result of their demineralisation by organic acids produced by those plaque bacteria that ferment dietary carbohydrates. The most common aetiological microbiological agents of enamel caries are considered to be *Streptococcus mutans* and *Streptococcus sobrinus.* Additional associated microorganisms are lactobacilli and actinomyces,

the former being considered as secondary invaders, while the latter being responsible for root surface caries [3–6]. The pathogenesis of dental caries is dependent upon the presence of fermentable sugars in the diet and the presence of cariogenic bacterial species. The main virulence properties of *S. mutans* and *S. sobrinus* are their ability to adhere to the tooth surface together with their rapid metabolism of sucrose to organic acids and to extracellular polysaccharides.

Several approaches to caries prevention are possible: (i) elimination of dietary carbohydrates from the diet, (ii) elimination of the causative organisms, (iii) prevention of bacterial adhesion and/or plaque formation, (iv) interference with bacterial metabolism, for example, by fluorides, and (v) enhancing acid resistance of the tooth enamel, for example, by fluoride [7–12]. Chemicals able to achieve one or more of the above have been shown to be present in a number of foods. In some foods the presence of compounds with antibacterial activity against different pathogens has been detected; in other foods both antiadhesive activity and inhibitory activity against the extracellular polysaccharide have been demonstrated. Recently, the anticariogenic properties of food components have been verified *in vivo* using both animals and humans tests. For example, extracts obtained from different teas and their polyphenol components have been investigated thoroughly for their activity. Polyphenols in tea have been shown to reduce caries development in animals because they decrease the cell surface hydrophobicity of *S. mutans* and the ability of the organism to synthesize adherent water-insoluble glucan from sucrose [13–17]. Additionally propolis [18] has been shown to possess both antimicrobial and GTF-inhibitory activities. The extract from *Lentinus edodes*, an edible mushroom, was studied in rats [19] and found to have an inhibitory effect on water-insoluble glucan formation by GTF. The same inhibitory effects have been shown by apple procyanidins [20]. High molecular weight components of hop bract inhibit adherence of water-insoluble glucan synthesis by *S. mutans* [21]. The cariostatic activity of cacao mass extract has been observed *in vitro* and in animal experiments. In this case, high molecular weight polyphenolic compounds and unsaturated fatty acids were shown to be the active constituents. The former, which showed strong anti-GTF activity, were polymeric epicatechins in an acetylated form. The latter showed bactericidal activity against *S. mutans* [22, 23]. An interesting antibacterial activity has been detected in coffee that is effective against *S. mutans* as well as other Gram-positive bacteria and some Gram-negative species [24–26]. In particular, it has been shown that roasted coffee interferes with streptococcal sucrose-independent adsorption to hydroxyapatite (HA) beads. Such activity may be due to not only small molecules occurring naturally, such as trigonelline, nicotinic and chlorogenic acids, but also to coffee components containing condensed polyphenols or melanoidins that occur during the roasting process [27].

Periodontal diseases are a heterogeneous group of inflammatory conditions that involve the supporting tissues of the teeth. They include gingivitis, in which only the gingiva is involved, and the various forms of periodontitis in which destruction of alveolar bone occurs. Characteristically, in these diseases, the junctional epithelial tissue at the base of the gingival crevice migrates down the root of the tooth with the result of the formation of a periodontal pocket. The initiation and progression of periodontal diseases is attributed to the presence of elevated levels of pathogenic bacteria within the gingival crevice. Any of several hundred bacterial species may inhabit the gingival crevice; however, it has been shown that only a few play a significant role in the aetiology of the various periodontal diseases. Indeed, it is generally accepted that a consortium of bacteria, not a single species, is involved in these diseases.

Gingivitis is the most prevalent form of periodontal disease and a disease which can be prevented and alleviated by the topical application of suitable agents in oral hygiene products such as toothpastes and mouthwashes. Accumulation of dental plaque at gingival margins due to inadequate dental hygiene leads to the inflammation of the gingivae, defined as gingivitis [28]. It can be defined as a nonspecific inflammatory process of the gingivae (gums) without destruction of the supporting tissues. This is a reversible condition as a return to meticulous dental hygiene practices will restore gingival health [29]. The plaque biofilm on the surfaces of teeth at the gum margin can cause inflammation. Several bacterial species have been implicated as aetiological agents of this disease. These include *Actinomyces israelii*, *A. naeslundii*, *A. odontolyticus*, *Lactobacillus* spp., *Prevotella* spp., *Treponema* spp., and *Fusobacterium nucleatum*. A key trend observed during gingivitis is the ascendancy of *Actinomyces* spp. and Gram-negative rods at the expense of *Streptococcus* spp. Gingivitis affects 100% of the adult population at some point during their lives, and, in some cases, it can lead to the development of periodontitis (although this can occur in individuals without any gingivitis) which results in loss of attachment of the gingivae to the teeth, a condition causing major discomfort and tooth loss, and necessitates extensive and costly dental treatment.

In comparison with caries, there is considerably less information available regarding the effects of beverages/foods on periodontal diseases. Possible ways in which such materials could prevent or alleviate gingivitis would be by directly killing the causative organisms, interfering with the formation of plaque at the gingival margin, disrupting preformed plaque, attenuating the virulence of the causative organisms, and acting as free radical scavengers thereby reducing the plaque-induced inflammation. Diets rich in vitamin C have long been known to protect against gingivitis [30]. Folate also appears to protect against the disease [31]. Green tea polyphenols have *in vitro* inhibitory effects on the adhesion of oral bacteria to epithelial cells [32]. Furthermore, it has been shown that the high molecular weight material of cranberry juice is effective in inhibiting coaggregation between different causative bacteria and *Fusobacterium nucleatum* [33]. Adhesion of streptococci is inhibited by hop bract polyphenols [34] and by several tea materials [16, 35] that have also been shown to inhibit water-insoluble glucan synthesis and bacterial amylases. An interesting antibacterial activity has been detected in coffee [24–27].

The aim of this work was to select foods and beverages from the folk literature and using the knowledge of

the authors that had anti-caries and anti-gingivitis activities and to characterize and identify the active ingredients.

2. Materials and Methods

2.1. Test Materials. The selection of which foods and beverages investigated was based on an extensive literature search (PubMed, Web of Science, Medline) together with the expertise and knowledge of the authors. The following foods and beverages were selected for investigation: Green and Black tea (*Camellia sinensis*), Cranberry juice (*Vaccinium macrocarpon;* Ocean Spray, Lakeville-Middleboro, MA), Raspberries (*Rubus idaeus*), Shiitake mushrooms (*Lentinula edodes*), Red chicory (*Cichorium intibus. var. Silvestre—Radicchio di Treviso tardivo IGP*), and Beer (Guinness; Diageo PLC, London).

Homogenates/extracts of the selected foods/beverages were prepared and chemically analysed to provide material suitable for subsequent investigations. For each food/beverage an operative protocol was prepared. This enabled the identification of the critical points that could influence the chemical composition of the extracts and therefore their biological properties [36].

2.2. Initial Fractionation of Homogenates. Fractionation of raspberry, shiitake mushroom, and chicory extracts was carried out by ultrafiltration or dialysis to provide a low molecular mass (LMM) fraction and high molecular mass (HMM) fraction for subsequent bioassay [36].

The HMM and LMM fractions of all three homogenates were subjected to a range of *in vitro* microbiological assays selected to determine their potential antigingivitis or anticaries properties (as described below).

2.3. Assessment of the Homogenates, Extracts for Antigingivitis Activities and Anticaries Activities. In order to evaluate the extracts for their potential anti-gingivitis activities, a number of high-throughput assays were designed for use in the study. These involved organisms associated with gingivitis and with oral health (*Streptococcus sanguinis, Actinomyces naeslundii, Fusobacterium nucleatum*, *Prevotella intermedia, Veillonella dispar,* and *Neisseria subflava*).

Additionally, to test the extracts for their potential anticaries activities, a number of high-throughput assays were designed for use in the study. These involved organisms associated with caries and with oral health (*S. sanguinis, S. mutans, L. casei, V. dispar,* and *N. subflava*).

The tests used to evaluate the homogenates/extracts were as follows.

The assays used for anti-gingivitis and anti-caries effects were as follows:

(1) prevention of biofilm formation by the target organisms,

(2) determining antibacterial effect against the target organisms,

(3) prevention of coaggregation by the target organisms,

(4) disruption of preexisting biofilms of the target organisms.

The assays used to specifically evaluate anti-gingivitis activity were as follows:

(5) prevention of adhesion to, and invasion of, gingival epithelial cells by those target organisms associated with gingivitis,

(6) inhibition of bacteria-induced host cell pro-inflammatory cytokine production by those target organisms associated with gingivitis.

The assays used to specifically evaluate anti-caries activity were as follows:

(7) prevention of adhesion of the target organisms to, and induce detachment from, hydroxyapatite,

(8) disruption of signal transduction in *S. mutans,*

(9) inhibition of acid production by caries-associated organisms.

2.3.1. Prevention of Biofilm Formation by the Target Organisms. The capability of the selected homogenates/extracts, at different concentrations, to prevent biofilm formation was evaluated by the microtitre plate assay described here in after.

All bacteria were cultured in Brain Heart Infusion broth (BHIB) except for *S. mutans* which was grown in BHIB ($\times 0.5$) supplemented with sucrose (final concentration, 0.2%). Cultures were incubated at 37°C in 5% CO_2/air (*S. mutans*, *S. sanguinis,* and *L. casei*) or under anaerobic conditions (*P. intermedia, A. naeslundii,* and *V. dispar*).

Bacterial suspensions were prepared in the appropriate growth medium containing different concentrations of the test material (pH adjusted to 7). The final concentration of bacteria was either 3–5 $\times$ 10^5 cfu mL^{-1} (*S. mutans, S. sanguinis, L. casei, V. dispar,* and *A. naeslundii*) or 5–8 $\times$ 10^6 cfu mL^{-1} (*P. intermedia*). Aliquots (200 μL) of the cell suspensions were inoculated into the wells of 96-well polystyrene microtiter plates. For each strain, test material-untreated controls were included. Plates were then incubated at 37°C up to one week in either 5% CO_2/air (*S. mutans*, *S. sanguinis,* and *L. casei*) or anaerobic conditions (*P. intermedia, A. naeslundii,* and *V. dispar*), with incubation media changed every 24 h and every 48 h for aerobic and anaerobic bacteria, respectively. Biofilm formation was quantified after 48 h and 7-day incubation. To this end, the growth medium was removed by aspiration; wells were gently washed with water and air dried; adherent bacteria were then stained with 0.01% crystal violet (100 μL). After 15 min incubation at room temperature, wells were gently washed with water, and bound dye was extracted from stained cells by adding 200 μL of ethanol: acetone (8 : 2). Biofilm formation was quantified by measuring the absorbance of the solution at 540 nm. Biofilm inhibitory activity was evaluated as a proportion of untreated controls (100%). Experiments were run in triplicate and were performed twice.

2.3.2. Antibacterial Activity of Homogenates/Extracts. All the extracts were assayed for their antibacterial activities in a standard Minimum Inhibitory Concentration (MIC) assay.

Bacteria were grown in 5 mL tubes at 37°C either aerobically at ambient air or under anaerobic conditions (GasPack Anaerobic System, Becton, and Dickinson) in BHIB. After overnight growth, the bacterial culture was diluted in broth to contain 10^5 cfu/mL. Twofold dilutions of test samples and fractions in 0.1 mL of BHIB were placed into wells of flat-bottomed microtitre plates (Nunc 96-well flat-bottomed microtitre plates). A 10 μL volume of bacterial culture was then added. Following incubation of the plates for 18 h at 37°C in ambient air or anaerobically as described previously, the MICs were determined. The MICs were recorded as the lowest concentration or dilution of test sample or fraction that completely inhibited visible growth of the bacteria.

2.3.3. Prevention of Coaggregation of Target Organisms. All combinations of the strains used were tested for coaggregation activity and the following is used in subsequent assays: *S. sanguinis* snd *P. intermedia, S. sanguinis* and *F. nucleatum, N. subflava* and *F. nucleatum, S. sanguinis* and *V. dispar, S. sanguinis* and *N. subflava, S. sanguinis* and *S. mutans and S. mutans* and *L. casei*.

The homogenates/extracts were assayed for their ability to inhibit coaggregation as described as follows.

Bacteria were grown in 5 mL tubes at 37°C either aerobically at ambient air or under anaerobic conditions (GasPack Anaerobic System, Becton, and Dickinson) in BHIB. After overnight growth, cells were harvested, washed with coaggregating buffer (1 mM tris (hydroxy-methyl) aminomethane; 0.1 mmol/L magnesium chloride; 0.1 mmol/L sodium chloride; 0.02 percent sodium azide adjusted to pH, 8.0), adjusted to an optical density of 1.5 at 400 nm (UV-Vis. Spectrophotometer), and stored at 4°C until use. The ability of test sample or fraction to inhibit coaggregation of selected pairs of bacteria was tested by adding equal volumes (0.05 mL) of bacterial suspension of one pair to equal volume of serial twofold dilution of test sample or fraction in coaggregating buffer followed by adding equal volume of the bacterial suspension of the other coaggregating member in 12×75 mm test tube. After vigorous vortex of the mixture and further incubation at room temperature for 2 min coaggregation was scored according Cisar et al. [37]. The last dilution of the sample causing complete inhibition of coaggregation was recorded and expressed either as final concentration (w/v) or as per cent of undiluted sample.

2.3.4. Disruption of Preexisting Biofilms of the Target Organisms. Mature biofilms of each of the test organisms were grown on cellulose nitrate membrane filters and incubated with the test compounds for 1 min. The number of live and dead cells disrupted from the biofilm was assessed as well as the number of live and dead cells remaining following the protocol described by Bryce et al. [38].

2.3.5. Prevention of Adhesion to, and Invasion of, Gingival Epithelial Cells

Adherence to KB22 Cells. The capability of the selected food/beverages to inhibit bacterial adherence to KB22 monolayers was evaluated using three experimental approaches: (a) labeled bacteria in PBS with the tested compounds were added to monolayers; (b) monolayers were pretreated with the tested compounds and then incubated at 37°C; (c) labeled bacteria, grown in medium supplemented with the tested compound, were added to the monolayers. Before performing the described experiments, the toxicity of the tested compound at 2x and 1x concentrations towards KB cells after 1 and 2 h incubation was tested by trypan blue exclusion.

Bacterial Growth and Labeling. All bacteria were cultured in BHIB except for *S. mutans* which was grown in BHIB (0.5x) supplemented with sucrose (final concentration, 0.2%). Cultures were incubated at 37°C in 5% CO_2/air (*S. mutans*, *S. sanguinis,* and *L. casei*) or under anaerobic conditions (*P. intermedia, A. naeslundii,* and *V. dispar*). To radiolabel bacteria, 10 μCi [methyl-^{3}H]thymidine (25 Ci mmol^{-1}) mL^{-1} was added to the growth medium. Cells were harvested at stationary phase by centrifugation (5,000 × g for 10 min at 4°C) and washed twice with 10 mM phosphate buffer (PB), pH 7.0; pellets were resuspended in either 10 mM PB, pH 7.0, or BHIB or phosphate buffered saline (PBS: 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.2 to 7.4), depending on the test to be performed. Cell bound radioactivity was quantified with a liquid scintillation counter. Cell labeling efficiency (number of bacteria per count per min) was then determined.

Cell Culture. Gingival fibroblast KB cell line (accession number ICLC HTL96014) obtained from Cell bank Interlab Cell Line Collection (ICLC) of IST-Istituto dei Tumori di Genova (Genoa, Italy) was cultured in a complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) high glucose, with 4500 mg L^{-1} glucose and sodium bicarbonate supplemented with 10% foetal calf serum, penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), and 2 mM L-glutamine. Cells were incubated at 37°C in a 5% CO_2 atmosphere to about 90% confluence and used after 5–10 passages. For bacterial adherence experiments, monolayers prepared in 96 well, flat bottom microtitre plates, were washed twice before use with PBS.

Adherence to KB Cell Line. The effect on bacterial adherence to KB cells of unfractionated whole material was tested. KB monolayers were prepared in 96 well, flat bottom microtiter plates, using Dulbecco's Modified Eagle's Medium (DMEM) high glucose prepared as described previously without antibiotics; before the assay, monolayers were washed twice with PBS (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.2 to 7.4). Suspensions of labeled bacteria (*A. naeslundii*) were prepared in PBS containing different concentrations of test materials (pH adjusted to 7) (final bacterial concentration, 4–6 × 10^8 cfu mL^{-1}). Aliquots (100 μL) of the bacterial suspensions were added to KB monolayers and incubated at 37°C for 1 h in 5% CO_2 atmosphere with gentle shaking. For each strain, untreated controls were included. After incubation, cells were disrupted by adding 200 μL of

cold distilled water, and lysates were transferred to PICO-FLUOR 15 scintillation fluid (Packard Instruments Company Inc., Ill.). Radioactivity was assayed in a liquid scintillation counter and, by the use of cell labeling efficiency, the number of bacteria per monolayer was evaluated. The inhibitory activity of the test materials was gauged by comparing fraction treated samples to the respective untreated controls (100%). Controls without bacteria were always included to evaluate KB cell viability in the presence of the test materials by trypan blue exclusion. Experiments were run in triplicate and were performed at least twice.

Inhibition of Bacterial Internalization. The capability of the selected homogenates/extracts to inhibit bacterial internalization into KB22 monolayers was evaluated; before performing the described experiments, the toxicity of the tested compound at 2x concentration towards KB cells after 5 h incubation was tested by trypan blue exclusion. Only mushroom showed toxicity (even at very low concentrations) and was not used.

KB Cell-Invasion Assay. KB monolayers were prepared in 16 mm well of 24-well tissue culture plates, in Dulbecco's Modified Eagle's Medium (DMEM) high glucose prepared as above without antibiotics; before the assay, monolayers were washed twice with PBS. Bacterial suspensions (*P. intermedia* and *A. naeslundii*) were prepared in KB cell growth medium without antibiotics, containing different concentrations of the test materials (pH adjusted to 7) (final bacterial concentration, 6–8 $\times$ 10^7 cfu mL^{-1}), and added (1 mL) to monolayers. For each strain, fraction untreated controls were included. After 90 min incubation at 37°C in 5% CO_2 atmosphere with gentle shaking, monolayers were washed with PBS to remove nonadherent bacteria. To evaluate total cultivable bacteria per monolayer, cells were disrupted by adding 1 mL of cold distilled water. Suitable dilutions of the lysates were plated onto Fastidious Anaerobe Agar (FAA; Biogenetics, Italy) plus 5% (v/v) defibrinated horse blood; after 36–48 h incubation under anaerobic conditions, colony-forming units were counted. To evaluate cultivable internalized bacteria per monolayer, external bacteria were killed by covering monolayers with cell growth medium containing bactericidal concentrations of gentamicin (300 $\mu g\,mL^{-1}$), metronidazole (200 $\mu g\,mL^{-1}$), and penicillin (5 $\mu g\,mL^{-1}$). After 90 min incubation at 37°C in 5% carbon dioxide, cells were extensively washed and lysed in cold distilled water. Suitable dilutions of the lysates were plated as above and colony-forming units of internalized bacteria were counted after incubation. Cell-invasion efficiency was measured by comparing internalized cultivable bacteria per monolayer to total cultivable bacteria per monolayer (100%). The inhibitory activity of the test materials was gauged by comparing fraction treated samples to the respective untreated controls. Each strain was tested in three separate assays on different days; each assay represented the average of triplicate wells. Controls without bacteria were always included to evaluate KB cell viability by Trypan blue assay in the presence of the test materials.

2.3.6. Inhibition of Host Cell Proinflammatory Cytokine Production Induced by the Target Organisms. The ability of the test materials to inhibit cytokine production by monoMac 6 cells (a human monocytic cell line) in response to bacteria (*F. nucleatum and P. intermedia*) was evaluated.

Maintenance of Mono-Mac-6 Cells. The myelomonocytic cell line Mono-Mac-6 was maintained in RPMI-1640 medium containing 2 mM L-glutamine, 5% heat-inactivated FCS, insulin (9 mg/mL), oxaloacetic acid (1 mM), sodium pyruvate (1 mM), and nonessential amino acids (0.1 mM, Sigma). Cells were cultured in 75-cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2 in air. Weekly, cells were split at a ratio of 1 : 5 by centrifugation at 1500 $\times$ g for 5 min and resuspended in fresh medium. Cells were then fed with fresh medium once a week.

Inhibition of Bacteria-Induced Host Cell Proinflammatory Cytokine Production. Mono-Mac-6 cells were centrifuged at 1500 $\times$ g for 5 min and resuspended in media with 2% (v/v) FCS. The viable cells were dispensed into 24-well tissue culture plates at 2 $\times$ 10^6/500 μL/well. The selected test or control agent (in triplicate) was then added to cells neat and at dilutions of 1 : 10 and 1 : 100. Bacterial strains were inoculated into 10 mL of the appropriate broth and grown in appropriate conditions. Bacterial cultures were then diluted in fresh broth and grown to exponential growth stage, as determined spectrophotometrically. At this point, an aliquot of the bacterial suspension was removed to determine the number of bacteria added to Mono-Mac-6 cells retrospectively. The aliquot was serially diluted and plated onto appropriate agar. After 5-day incubation under the appropriate conditions, plates were counted to determine the CFU/mL used in the experiment.

Bacteria were pelleted by centrifugation, washed with PBS, repelleted by centrifugation, and resuspended in RPMI-1640. Bacteria were then added to wells containing Mono-Mac-6 cells to obtain a multiplicity of infection of 1, 10, and 100 bacteria to 1 Mono-Mac-6 cell (each in triplicate). The number of bacteria added to Mono-Mac-6 cells was judged on the OD of bacterial cultures and previously determined CFU/mL at a particular OD (data not shown). Mono-Mac-6 cell numbers for each experiment were determined by centrifugation of contents of tissue culture plate well and the cells counted using a haemocytometer. Bacteria were centrifuged onto the monolayer at 2000 $\times$ g for 10 min at room temperature and then plates incubated at 37°C in an atmosphere containing 5% CO_2 for 5 h.

For determination of cytokine release at the end of the culture period cytokines released into the medium were assayed by in-house ELISA for IL-6 commercially available kit for the detection of IL-6.

2.3.7. Prevention of Adhesion to, and Induction of Detachment from, Hydroxyapatite (HA). The capability of the selected homogenates/extracts to prevent bacterial adhesion to HA beads was evaluated following three experimental approaches: (a) the tested compound and the radiolabelled

bacterial suspensions were added simultaneously to saliva coated beads; (b) saliva coated beads were pretreated with the tested compounds; (c) labeled bacteria grown in THB supplemented with the test material (at 1/2 MIC) were added to the beads. In the case of green tea and cranberry, their activity was evaluated following approach "a" only.

Bacterial Growth and Labeling. See Section 2.3.5.

Preparation of Hydroxyapatite (HA) Beads. Fifty mg aliquots of spheroidal HA beads (Sigma Aldrich, UK) were washed with 1 mM PB, pH 7.0, in glass tubes and autoclaved. Beads were collected by centrifugation (100 × g, 1 min, 4°C) and equilibrated in 1 mM PB, pH 7.0 (1 h at room temperature). HA was then treated (1 h at room temperature) with 200 μL undiluted saliva, which was collected from unstimulated donors, clarified by centrifugation (15,000 × g for 30 min at 4°C), and sterilized through 0.22 μm nitrocellulose membrane filters. Beads were then collected by centrifugation as above and washed with 10 mM PB, pH 7.0.

Bacterial Adherence to HA Beads. The effect on bacterial adherence to HA beads of unfractionated whole materials was tested. Suspensions of labeled bacteria were prepared in 10 mM PB, pH 7.0, containing different concentrations of the test materials (pH adjusted to 7) (final bacterial concentration, 6–8 × 10^7 cfu mL^{-1}). Aliquots (1 mL) of the cell suspensions were added to saliva coated HA beads (50 mg) in polypropylene microfuge tubes and incubated at room temperature on Rotomix test tube rotator (TKA Technolabo ASSI, Italy). Controls (no test material added) were included in all treatments. After 1 h incubation, the beads were collected by centrifugation (100 × g, 1 min, 4°C), washed twice with 10 mM PB to remove nonadherent bacteria, and transferred to PICO-FLUOR 15 scintillation fluid (Packard Instruments Company Inc., III.). Radioactivity was assayed in a liquid scintillation counter and, on the basis of cell labeling efficiency, the number of bacteria adhering to HA beads (50 mg) was evaluated. The inhibitory activity of the test materials was gauged by comparing test material-treated samples to the respective untreated controls (100%). Controls for bacterial settling due to aggregation were also included; the amount of settled bacteria was always <1% of the inoculum. Experiments were run in triplicate and were performed at least twice.

Induction of Detachment from HA Beads. Aliquots (1 mL) of cell suspensions, prepared in 10 mM PB, pH 7.0, were added to saliva-coated HA beads (50 mg) and incubated at room temperature on a Rotomix test tube rotator (final bacterial concentration, 6–8 × 10^7 cfu mL^{-1}). After 1 h incubation, the beads were collected by centrifugation (100 × g, 1 min, 4°C) and washed twice with 10 mM PB, pH 7.0, to remove nonadherent bacteria. HA was then resuspended in the same buffer (1 mL) supplemented with different concentrations of the test compounds (pH adjusted to 7) and incubated at room temperature on a Rotomix test tube rotator. Untreated control samples were included. Other samples were included to assess total HA-bound bacteria, as described previously. Immediately after beads resuspension (time zero) and after 1 and 2 h incubation, the mixtures were centrifuged (200 × g, 5 min, 4°C), and the supernatants were transferred to PICO-FLUOR 15 scintillation fluid. Radioactivity was assayed in a liquid scintillation counter and, on the basis of cell labelling efficiency, the number of bacteria present in the supernatant, corresponding to detached cells, was evaluated. The percent of detached *versus* total HA-bound bacteria was determined. The effect of the tested compounds was evaluated by comparing treated samples with the untreated controls. Experiments were run in triplicate and were performed at least twice.

2.3.8. Effect on Signal Transduction. The effect of homogenates/extracts on *S. mutans comDE* gene expression was determined as described here in after:

S. mutans cells were grown in BHIB at 37°C, 5% CO_2 to the early log phase, OD_{600} = 0.2. The inoculum was exposed to the tested homogenates/extracts in BHI for 2 h. Total RNA was extracted from the *S. mutans* cells. After being exposed to the tested agents, the above bacterial suspension was centrifuged, washed with PBS, and resuspended in Tri-Reagent (Sigma-Aldrich) [39]. The bacteria were disrupted with the aid of glass beads (Sigma-Aldrich) in a Fast Prep cell disrupter (Bio 101, Savant Instrument Inc., NY, USA). The suspensions obtained were centrifuged and the RNA-containing supernatant was transferred to a new microcentrifuge tube. The homogenate was supplemented with BCP-phase separation reagent (Molecular Research Center, Cincinnati, OH, USA), and the upper aqueous phase, containing the RNA, was precipitated with isopropanol. The RNA pellet was washed with ethanol, centrifuged, and the purified RNA was resuspended in diethyl pyrocarbonate-treated water (Invitrogen, Carlsbad, CA, USA). Due to the sensitivity of the PCR, residual contaminating DNA was removed by RNase-free DNase [40]. The RNA concentration was determined spectrophotometrically according to the A_{260}/A_{280} ratio, using a Nanodrop. The integrity of the RNA was assessed by 1.5% agarose gel electrophoresis.

Reverse transcription and quantitative real-time PCR were performed as described previously [41]. Briefly, a reverse transcription (RT) reaction mix (20 μL) containing 50 ng of random hexamers, 10 mM dNTPs mix, and 1 μg of total RNA sample was incubated at 65°C for 5 min and then placed on ice. ×10 RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U of RNaseOUT Recombinant Ribonuclease Inhibitor, and 50 U of Super Script II RT (Invitrogen) were added to the reaction mix in each tube. After incubation at 25°C for 10 min, the tubes were transferred to 2°C for 50 min. The reaction was terminated by heating the mixture at 70°C for 15 min, and the cDNA samples were stored at −20°C until used.

Real-time qPCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with an SYBR Green PCR Master Mix (PE Applied Biosystems). The reaction mix (20 μL) contained 1 μL of the cDNA sample and 0.5 μM of the appropriate PCR primer. The cycle profile was as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 1 min, and 30 cycles at 95°C

for 15 sec and at 60°C for 1 min, following a dissociation stage: a 15 sec hold at 95°C and at 20 sec for 20 sec, and a slow ramp (20 min) from 60 to 95°C. The critical threshold cycle (C_T) was defined as the cycle at which fluorescence becomes detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with C_T values obtained from amplification of known quantities of cDNA from *S. mutans* GS5. The standard curve was used for transformation of the C_T values to the relative number of cDNA molecules.

Data were expressed as the mean plus standard deviation of triplicate experiments. Contamination of genomic DNA was measured in control reactions devoid of reverse transcriptase. The same procedure was applied to all the primers.

The *comC/D/E* primers were designed by using the algorithms provided in Primer Express (Applied Biosystems). For each set of primers a standard amplification curve was drawn. Only curves with slope ≈ -3 were accepted as reliable primers. The primer set 16S-F/R, corresponding to the 16S rRNA gene of *S. mutans* (Acc. No. X58303), was designed to correspond to the expression of the housekeeping gene. The data were presented as the effect of various agents on *comC/D/E* mRNA expression and normalized by endogenous control of 16S rRNA transcription [41]. They were presented in relative units compared with those of the control, grown in the absence of the tested agents to calculate the per cent inhibition of *comC/D/E* mRNA expression by test fraction.

2.3.9. Inhibition of Acid Production by Caries-Associated Species. Caries is a multifactorial disease with low pH as a driving force for mineral dissolution. Plaque pH is lowered by organic acids (e.g., lactate, acetate, and propionate) that are released by oral bacteria as fermentation products. The ideal anticaries therapeutic agent would inhibit fermentation activity (acid production) of oral microorganisms, especially those that are known to be involved in caries aetiology, for example, *S. mutans*.

The effects of each homogenate/extract on acid production by the target organisms were carried out using an acidogenicity assay described by Damen et al. [42]. Biofilms of *S. sanguinis, S. mutans, L. casei,* and *A. naeslundii* were grown in 96-well microtiter plates and each was exposed to each test material and a positive and negative control. After incubation in glucose solution, biofilms were resuspended and sampled for organic acid content determination. Briefly, *S. sanguinis*, *S. mutans*, *L. casei,* and *A. naeslundii* were inoculated (streaked for single colonies) onto appropriate solid agar media to ensure purity. A single bacterial colony was picked and used to inoculate 5 mL of full-strength BHIB. This was incubated overnight yielding approximately 10^9 CFU/mL. The wells of 96-well flat-bottomed polystyrene microtiter plates (bio-one; Greiner, Frickenhausen, Germany) were inoculated with 10^6 CFU/mL of the test organism in 0.2 mL of biofilm growth. Biofilms were grown in 10% H_2, 10% CO_2 in N_2 at 37°C. The medium was refreshed after 8 h and biofilms grown for another 16 h (in total for 24 h). After 24 h, the spent medium was carefully removed. 0.2 mL of the selected test or control agent (in quadruplicate) was applied to the biofilms as a neat and 1/10 solution in water for 5 min. Controls included a negative control of sterile water and a positive of 0.05% chlorhexidine solution. After careful removal of the test or control agent, the biofilms were rinsed three times with peptone-buffered water (PBW). Then the biofilms were incubated in 0.2 mL of 0.5% glucose solution at 37°C for 3 h in 10% H_2, 10% CO_2 in N_2. After the incubation period, the biofilms were suspended in the incubation fluid and sampled into precooled eppendorf tubes. The tubes were set on ice until further processing within one hour.

To release acids, all samples were heated at 80°C for 5 min and again cooled on ice [43]. Then the vials with plaque were centrifuged at $16,100 \times$ g for 15 min at 4°C. The supernatants were transferred into vials with a microspin filter (Ultrafree-MC 0.22 μm, Millipore, Bedford, Mass., USA) and centrifuged at $13,684 \times$ g for 5 min at 4°C. Filtered supernatants were stored at −80°C until further processing.

Organic acids in the incubation fluid were determined as their anions by capillary electrophoresis on the Waters Capillary Ion Analyzer (Milford, Mass., USA) [42]. Sodium salts of formic, acetic, propionic, butyric, succinic, and lactic acid were used to prepare single and mixture standard solutions in MilliQ-water. Calibration curves were constructed for each acid separately. As an internal standard, $NaNO_3$ was included in all samples. Formic, butyric, succinic, propionic, acetic, and lactic acids were determined in duplicate samples and expressed as nmol acid/sample in 0.2 mL of incubation fluid.

3. Results and Discussion

All test materials were tested for microbiological contamination before and after freeze drying or after juicing. No contamination was recorded.

3.1. Prevention of Biofilm Formation by the Target Organisms. Six species *(V. dispar, F. nucleatum, A. naeslundii, P. intermedia, S. mutans, S. mutans* (+ sucrose), and *L. casei*) were used to test 4 concentrations of extract (2x, 1x, 0.5x, and 0.25x. Only 1x data shown) from cranberry, green tea, black tea, beer, raspberry, chicory, and mushroom. Even at the lowest concentration tested, all homogenates/extracts were able to inhibit biofilm formation by the target organisms to some extent although chicory was ineffective at inhibiting biofilm formation of *S. mutans* in the absence of sucrose (Table 1).

With regard to the gingivitis-associated species, F. nucleatum, A. naeslundii, and P. intermedia, the most active materials were Raspberry = Chicory = Mushroom = Beer > Black Tea = Green tea = Cranberry.

With regard to the caries-associated species, *S. mutans S. mutans* (+ sucrose) and *L. Casei,* the most active substances were Raspberry > Green tea = Black Tea > Mushroom > Beer > Cranberry = Chicory.

3.2. Antibacterial Activity of Homogenates/Extracts. All the extracts were assayed for their antibacterial activities in a standard Minimum Inhibitory Concentration (MIC) assay.

With regard to the gingivitis-associated species, *A. naeslundii* and *P. intermedia*, all extracts showed some inhibitory

Table 1: Results from 9 high-throughput assays used to evaluate the extracts for their potential antigingivitis activities and anticaries activities.

Assay	Beer	Black tea	Chicory	Cranberry	Green tea	Shiitake	Raspberry
1. Inhibition of biofilm formation. ± = 25%, 1 = 25–50%, 2 = 51–80%, 3 = 81–100% compared to untreated control.							
Sm	2	2	0	1	2	1	3
Sm (+sucrose)	2	1	1	1	2	3	3
Lc	±	3	1	±	2	2	3
Vd	3	3	3	3	3	2	3
Fn	3	3	3	3	3	3	3
An	3	3	3	3	3	3	3
Pi	3	2	3	2	2	3	3
2. Antibacterial activity (MIC)							
Pi	>50	>50	50	3.13	>50	12.5	50
An	12.5	25	>50	3.13	25	6.25	12.5
Lc	50	50	>50	>50	12.5	12.5	12.5
Sm	50	50	>50	>50	3.13	12.5	12.5
3. Prevention of coaggregation. 0 = no inhibition, 1 = partial inhibition, 2 = complete inhibition							
Pi/Ss	0	2	0	2	1	1	1
Fn/Ss	0	1	0	2	0	0	1
Fn/Ns	0	2	1	1	1	2	0
Sa/Vp	0	1	0	1	0	0	1
Sa/Ns	1	0	1	2	0	1	0
Sm/Ss	1	0	1	1	0	2	0
Sm/Lc	0	0	0	2	0	0	0
4. Disruption of pre-existing biofilms. % disrupted (% dead)							
An	22 (12)	11 (1)	38 (4)	nt	nt	52 (4)	32 (6)
Pi	67 (6)	45 (10)	27 (13)	ny	nt	31 (19)	55 (19)
Lc	51 (7)	50 (6)	59 (3)	15 (7)	53 (3)	31 (2)	31 (8)
Sm	33 (9)	31 (27)	38 (5)	26 (9)	6.5 (1)	75 (58)	44 (4)
5a. Prevention of the adhesion to gingival epithelial cells							
An	53	53	81	76	91	Toxic	66
5b. Prevention of the invasion into gingival epithelial cells							
An	93	95	98	94	100	Toxic	69
7a. Prevention of adhesion to hydroxyapatite							
Sm	32	65	75	98	96	44	81
Ss	27	79	55	91	21	70	92
Lc	24	88	89	98	52	80	93
7b. Prevention of induction of detachment from hydroxyapatite							
Sm	6	27	16	21	22	21	10
Ss	4	NT	5	21	2	14	6
Lc	5	15	22	5	8	28	7
8. Disruption of signal transduction. % of control							
Sm	38	87	39	39	61	21	51

Sm: *S. mutans*; Lc: *L. casei*; Vd: *V. dispar*; Fn: *F. nucleatum*; An: *A. naeslundii*; Pi: *P. intermedia*; Ns: *N. subflava*; Ss: *S. sanguinis*.

characteristics exhibiting MIC values ranging from >50% to 3.13% (Table 1). The relative activities of the extracts against gingivitis-associated species were Cranberry > Mushroom > Raspberry > beer > Green Tea = Black tea > Chicory.

With regard to the caries-associated species *L. casei* and *S. mutans*, mushroom, raspberry, beer, and green tea exhibited some inhibitory activity. The relative activities of the extracts were Green Tea > Raspberry = Mushroom > Beer > Black Tea > Chicory = Cranberry.

3.3. Prevention of Coaggregation of Target Organisms. Coaggregation is an important factor when complex biofilm communities are being studied. Important relationships exist between certain strains which allow aggregation and biofilm formation. Inhibition of this may be an important factor in preventing biofilms forming. All homogenates/extracts showed some ability to inhibit coaggregation of at least one pair of target organisms (Table 1). With respect to gingivitis-associated organisms, the relative activities were Cranberry > Black Tea > Mushroom > Raspberry = Green Tea > Chicory > Beer.

With regard to the caries-associated pairings the most effective at inhibiting coaggregation of these organisms was Cranberry > Mushroom > Chicory = Beer.

3.4. Disruption of Preexisting Biofilms of the Target Organisms. Biofilms which build-up in low-shear environments such as those in interproximal regions and plaque within gingival margins are able to become well-established climax communities. These mature biofilms are more resistant to antimicrobials and antibiotics than biofilms forming in high-shear systems.

The proportion (%) of cells disrupted from the biofilm after 1 min incubation with the extracts and % of dead cells were determined for the homogenates/extracts.

With respect to gingivitis-associated organisms (*A. naeslundii* and *P. intermedia*), all homogenates/extracts showed some ability to disrupt biofilms of the target organisms (Table 1). In many cases, a high proportion of the disrupted organisms were killed by the homogenates/extracts, the most active being beer which disrupted 67% of *P. intermedia* cells (6% were dead) and the least active being black tea which disrupted 12% *A. naeslundii* cells (with 1% dead). Raspberry disrupted 55% of *P. intermedia* cells with 19% killed. The relative activities were Beer > Raspberry > Mushroom > Chicory > Black Tea.

With respect to caries-associated organisms (*L. casei* and *S. mutans*), all homogenates/extracts were able to disrupt biofilms of both target organisms to some extent. Chicory, mushroom, beer, Black tea, and Raspberry were most active with between 30% and 75% disruption of biofilms (<10% dead cells). Green tea and Cranberry were much less active.

3.5. Prevention of Adhesion to, and Invasion of, Gingival Epithelial Cells. The tested substances did not show cytotoxicity with the exception of mushroom. This was therefore used at 0.5x concentration (subcytotoxic). Species used in the prevention of adhesion assay was *A. naeslundii*. The most active substance was shown to be Green Tea > Chicory > Raspberry > Cranberry Beer = Black Tea.

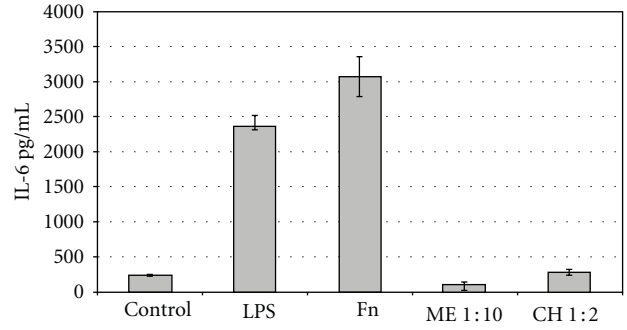

Figure 1: Graph showing the inhibition of IL-6 release from Mono-Mac-6 cells when challenged by LPS or *F. nucleatum* (Fn) supernatant and by dilutions of Chicory (CH) and Mushroom (ME) homogenates.

Most of the homogenates/extracts (mushroom was an exception) were able to inhibit *A. naeslundii* internalization.

The *P. intermedia* strain, in control tests, presented a low internalization capability or no internalization at all making it impossible to evaluate the effect of substances.

3.6. Inhibition of Host Cell Proinflammatory Cytokine Production Induced by the Target Organisms. The proinflammatory cytokine, IL-6, released by host cells in response to subgingival bacteria is considered to be a mediator of the inflammation accompanying gingivitis. Compounds able to prevent such cytokine production will, therefore, help to maintain the gingival tissues in a healthy state. The most effective bacterial inducer of IL-6 from the Mono-Mac-6 cells was determined. The cells were exposed to *F. nucleatum* cells, or *P. intermedia* cells, or LPS and the quantity of IL-6 released into the supernatant was assayed. The supernatant from *F. nucleatum* displayed the greatest IL-6 inducing activity and therefore was used in subsequent experiments. The results of chicory and mushroom homogenate are shown in Figure 1. Both Chicory and mushroom extracts inhibited the release of IL-6 by the *F. nucleatum* supernatant. No other extract showed any activity.

3.7. Prevention of Adhesion to, and Induction of Detachment from, Hydroxyapatite (HA). All homogenates/extracts inhibited adhesion of the target bacteria to hydroxyapatite to some extent. The most active substance was shown to be Raspberry = Cranberry > Black Tea > Mushroom > Chicory > Beer > Green Tea.

Induction of detachment of caries-associated species (*S. mutans* and *L. casei*) and *S. sanguinis* from HA beads by the Homogenates/extracts was determined. All tested substances induced a higher detachment from the hydroxyapatite beads in comparison to the respective controls.

The most effective material at inducing detachment was mushroom which detached 21% of *S. mutans* cells, 14% *S. sanguinis* cells, and 28% of *L. casei* cells. Overall ranked

results were Mushroom > Cranberry > Chicory> Green Tea > Raspberry > Beer.

3.8. Disruption of Signal Transduction. Some materials may affect the triggering of signal transduction suppression systems by bacteria and this may have an effect on colonization of teeth and the induction/progression of disease. The effect of 0.1% (v/v) of the homogenates/extracts on *S. mutans comDE* gene expression was determined. All homogenates/extracts inhibited *S. mutans* comDE gene expression to some extent. The range of inhibition of gene expression was 87% to 21% of control. The overall ranked results were Mushroom > Beer > Chicory + Cranberry > Raspberry > Green Tea > Black Tea.

3.9. Inhibition of Acid Production by Caries-Associated Species. Acid production was either unaffected by all the homogenates/extracts or increased.

4. Most Appropriate Food and Beverages to Further Fractionate and Test

The 9 assays were used to form a comprehensive set of tests aimed at easily assessing the anti-caries or anti-gingivitis nature of the homogenates/extracts. A large amount of data was generated and the collation and interpretation of this was challenging. In addition, aspects of intellectual property rights with some of the other foods and beverages (Cranberry, Black Tea, and Green tea) became apparent and this also therefore influenced the decision. Therefore based on all these aspects, raspberry, chicory, and mushroom extracts were taken forward for further fractionation and testing as potential anti-gingivitis agents, and mushroom extracts were taken forward for further fractionation and testing as a potential anti-caries agent.

Due to the labour intensive nature of performing all the assays with all the species, the utilization of the full set of assays for subsequent testing was reassessed. A decision was made to reduce both the number of assays and the number of species tested as appropriate for anti-caries or anti-gingivitis testing.

5. Determination of Anticaries Activities of the HMM and LMM Fractions of Shiitake Mushroom

The effects of the HMM and LMM fractions of the shiitake mushroom on organisms associated with caries and health in assays specifically relevant to this disease were carried out. The assays aimed to assess the ability of each test material to

(i) prevent adhesion of the target organisms to hydroxyapatite,

(ii) prevent biofilm formation by the target organisms,

(iii) elicit an antibacterial effect against the target organisms,

(iv) prevent coaggregation by the target organisms,

(v) disrupt preexisting biofilms of the target organisms.

The prevention of adhesion to hydroxyapatite assay showed that the LMM fraction of mushroom inhibited adherence of the target organisms to hydroxyapatite by c50% while the HMM fraction only caused c15% inhibition. The LMM fraction of mushroom was able to inhibit biofilm formation of *S. mutans* by 99% and *S. sanguinis* by 87% while the HMM did not inhibit any formation with *S. mutans* and only 14% with *S. sanguinis*. The LMM fraction of mushroom showed a greater inhibition of bacterial growth (up to 1 : 8 dilution) against the target organisms than the HMM fraction which did not inhibit bacterial growth. The LMM fractions completely inhibited coaggregation of *F. nucleatum* plus *S. mutans* and *F. nucleatum* plus *N. subflava* while the HMM fractions did not show any inhibition with any pairing. The LMM disrupted *L. casei* and *S. mutans* biofilms by 60% and 58% (7% and 30% dead cells), respectively, while the HMM disrupted *L. casei* and *S. mutans* biofilms by 45% and 31% (2% and 17% dead cells), respectively.

Based on the results from the assays of mushroom LMM and HMM fractions, the conclusion was that the LMM fraction was the most active and would be further fractionated.

6. Determination of Antigingivitis and Anticaries Activities of the HMM and LMM Fractions of Mushroom, Chicory, and Raspberry

The effects of the HMM and LMM fractions on organisms associated with gingivitis (*A. naeslundii* and *P. intermedia)* and caries (*S. mutans, S. sanguinis, and L. casei)* in assays specifically relevant to these diseases were carried out.

The assays aimed to assess the ability of each test material to

(i) prevent biofilm formation by target organism,

(ii) disrupt preexisting biofilms of the target organisms,

(iii) inhibit adhesion of organisms to epithelial cells,

(iv) inhibit adhesion to hydroxyapatite.

The LMM fractions of mushroom, raspberry, and chicory were more effective at preventing biofilm formation by the target gingivitis organisms (Chicory, 94%; Mushroom, 97%, and Raspberry, 100% inhibition) than the HMM fractions (Chicory, 7%; Mushroom, 1%, and Raspberry, 74% inhibition). The LMM fractions of mushroom and raspberry, but not chicory, were able to inhibit biofilm formation by *S. mutans* (99% and 32%, resp.) Determination of the disruption of preexisting biofilms by the fractions is shown in Table 2. This shows that the LMM fractions of shiitake mushroom and chicory were most effective at disrupting biofilms of the target organisms (both caries associated and gingivitis associated) than the HMM fractions. In the case of raspberry, the HMM fraction was the most effective. The mushroom and raspberry also showed most antibacterial effect with up to 53% of cells killed (LMM Mushroom).

Both the LMM and HMM fractions of raspberry and chicory were able to inhibit adhesion of the target organisms

Table 2: Percentage disruption of biofilms by HMM and LMM fractions of chicory, mushroom, and raspberry. Numbers in parenthesis are percentage of dead cells.

Target organism	Homogenate/extract					
	Chicory		Mushroom		Raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
S. mutans	33.5 (18.7)	56.7 (14.9)	31.3 (16.6)	58.1 (29.9)	nt	nt
L. casei	33.7 (2.7)	63.2 (2.9)	45.3 (1.7)	60.0 (7.2)	nt	nt
A. naeslundii	4.3 (1)	40.4 (8)	47.9 (38)	51.1 (39)	40.3 (31)	37.5 (30)
P. intermedia	8.2 (2)	28.7 (7)	11.6 (9)	63.1 (53)	58.1 (29.9)	33.9 (24)

to epithelial cells. Neither the LMM nor HMM fractions of shiitake mushroom displayed inhibitory activity. The LMM fractions of mushroom, chicory, and raspberry had a greater inhibition of the growth of *A. naeslundii* and *P. intermedia* (up to 1 : 16 dilution for raspberry LMM) than the HMM fraction which inhibited to a max level of 1 : 2. Both the LMM and HMM fractions of mushroom, chicory, and raspberry were able to inhibit adherence of the target organisms to hydroxyapatite. LMM Chicory versus *A. naeslundii* and LMM Raspberry versus *S. mutans* are most effective (68% and 62%, resp.), while LMM Chicory versus *S. mutans* and LMM mushroom versus *A. naeslundii* are least effective (4% and 14%, resp.). It is interesting to note the difference in effectiveness of Chicory LMM versus different organisms.

Based on the results from the assays of mushroom, chicory, and raspberry LMM and HMM fractions, the conclusion was that the LMM fractions of mushroom and chicory were the most active and would be further fractionated. An additional issue was the fact that the most active fraction of raspberry also contained most of the fruit sugars.

Shiitake mushroom extract has previously been shown to have an inhibitory effect on a range of oral bacterial species [44, 45] and also on water-insoluble glucan formation by *Streptococcus mutans* and *Streptococcus sobrinus* [19]. Indeed, the same study also showed that in a rat model caries is reduced in rats fed with shiitake mushroom compared to controls. A number of different compounds from Shiitake, as aqueous extract, have been shown to have antimicrobial activity on food-borne pathogenic bacterial strains [45].

The antimicrobial nature of chicory has been evaluated against *Agrobacterium* sp, *Erwinia carotovora, Pseudomonas fluorescens,* and *P. aeruginosa* [46] and there is some evidence and folk literature supporting its use as an antimalarial. Apart from the Oligofructose being nonfermentable by *S. mutans* chicory has no known oral health benefits.

In conclusion, the homogenates and LMM fractions show promise as anti-caries and anti-gingivitis agents, and further testing and clinical trials will need to be performed to evaluate their true effectiveness in humans.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 217164, 10 pages
doi:10.1155/2012/217164

Research Article

The Anticaries Effect of a Food Extract (Shiitake) in a Short-Term Clinical Study

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Received 30 August 2011; Accepted 21 September 2011

Academic Editor: Itzhak Ofek

The main objective was to investigate whether low-molecular-weight fraction of edible mushroom shiitake extract (*Lentinus edodes*) possesses caries-preventive properties. The study was designed as a double-blind, three-leg, cross-over, randomized, controlled clinical trial carried out on two series of volunteers at the University of Gothenburg, and the Academic Centre for Dentistry Amsterdam. Volunteers rinsed twice daily with a solution containing low-molecular-weight fraction of edible mushroom, placebo (negative control without active ingredients), or Meridol (positive control, AmF-SnF_2) for two weeks, with a two-week washout period between each rinsing period. Changes in the acidogenicity of dental plaque before and after a sucrose challenge, shifts in microbial composition, and plaque scores were determined. Frequent rinses with shiitake reduced the metabolic activity of dental plaque. No reduction of plaque scores and no inhibition of the production of organic acids in plaque was found. Minor differences in microbial composition between test sessions were found. To conclude, the results indicate that shiitake extract has anticariogenic potential, but not to the same extent as the positive control.

1. Introduction

Dental caries constitutes a multifactorial disease with a complex origin where the acidogenicity of dental plaque as a consequence may affect dental hard tissues [1–3]. The dental plaque is a complex multispecies biofilm. A reduction in plaque pH occurs following the release of organic acids, primarily lactate, acetate, and propionate, by oral microorganisms as fermentation products.

Over the years, different approaches have been designed intended to prevent this disease from occurring. Apart from strengthening the tooth mineral using fluoride, pronounced changes in environmental factors such as diet, oral hygiene measures, and the use of antimicrobials have been suggested in order to induce ecological shifts in biofilm composition [3, 4]. The latter are designed to inhibit the fermentation activity of cariogenic microorganisms, particularly those harboured in the oral biofilm, which will in turn determine a shift from a diseased to a healthy state [5].

Several possible mechanisms by agents of this kind have been suggested. They include the prevention of bacterial adhesion, a reduction in plaque formation, and interference

with the bacterial metabolism. The most commonly used antimicrobial agent is chlorhexidine, a bisbiguanid, with known strong antimicrobial activity [6], but xylitol, fluoride, and essential oils are also known to possess similar, albeit weaker, activity [7, 8]. Chemical components able to produce one or more of the actions on different kinds of biological activity have also been shown to be present in a number of foods [9, 10]. The presence of compounds with antibacterial activity on different pathogens, as well as antiadhesive activity and inhibitory activity on matrix formation, has been demonstrated by different food products [11].

When it comes to different food products and their constituents, interest has recently focused on an edible mushroom, shiitake (*Lentinus edodes*). The extract from *L. edodes* has been studied in rats and an inhibitory effect on one of the virulence factors of *Streptococcus mutans* has been demonstrated [12]. There are few reports related to the general antimicrobial effects of different compounds obtained from shiitake. An aqueous extract from *L. edodes* displayed high antimicrobial activity on food-borne pathogenic bacterial strains [13]. A diet containing 5% of dried *L. edodes* has been found to reduce the viable counts of the total number of microorganisms, streptococci, *Escherichia coli,* and lactic acid bacteria in the intestinal flora of piglets [14]. In a series of *in vitro* studies, a number of biological activities relevant to caries prevention have been identified, the most prominent of which are the induction of the detachment of cariogenic microorganisms from hydroxyapatite, changes in cell surface hydrophobicity, bactericidal activity against cariogenic microorganisms, the prevention of the coaggregation of microorganisms, and the disruption of signal transduction in *Streptococcus mutans* [15, 16].

The hypothesis was that frequent mouth rinses with low-molecular-weight fraction of edible mushroom shiitake extract may reduce plaque metabolic activity, change plaque cariogenic microflora towards a healthier oral flora, and reduce plaque amount. Thus, the aim of the present study was to conduct a short-term clinical trial to determine the *in vivo* potential of rinsing with a low molecular weight extract (<5.000 Da) of shiitake (*Lentinula edodes*) on the acidogenicity of dental plaque, microbial composition, and plaque index score.

2. Materials and Methods

2.1. Study Design. This study was carried out as a double-blind, randomized, placebo-controlled, three-leg, cross-over clinical trial. Two sets of data were obtained—one at the Department of Cariology, University of Gothenburg (GOT) and one at the Department of Preventive Dentistry in collaboration with the Department of Periodontology, Academic Centre for Dentistry Amsterdam (ACTA). Despite being very similar, the two substudies were not identical in study design and the data that were obtained and, for this reason, the protocol cannot be regarded as a multicentre approach. The study was performed within an International EU Sixth Framework Programme Consortium project (NUTRIDENT, FOOD-CT-2006-036210), which was granted in order to identify beverage/food constituents that are able to reduce the risk of dental caries and gingivitis. This study focused on the opportunity to prevent dental caries and inhibit dental plaque formation. The two series were approved by the Ethical Committee at the University of Gothenburg (Dnr 102–09) and by the institutional review board at ACTA (METc VUmc, protocol number BL21480.029-08), respectively.

All volunteers made eight visits to the laboratory (GOT) respective clinic (ACTA), for a first visit, when a clinical examination was carried out and information about the study was given, and for a total of seven subsequent test visits. In all, there were four washout periods. The study started with a two (GOT) and a three-week (ACTA) preexperimental washout period followed by three two-week periods with daily mouth rinsing with the assigned product intermitted with two-week washout periods. The total duration of the study was, therefore, 14 (GOT) or 15 (ACTA) weeks.

At the screening (GOT) or at the first visit (ACTA), a medical questionnaire was completed and the oral health status of the participants was determined by an intraoral examination. Professional oral hygiene for GOT was performed at the start of each test period as well as directly after each test period (prior to washout) and for ACTA before the start of the first washout (before the baseline samples).

At each of the following visits, the subjects underwent the following data collection in the order mentioned: (1) collection of resting and fermented plaque for protein/acid analyses, (2) plaque acidogenicity (only GOT), (3) collection of plaque for microbiological analyses, and (4) assessment of plaque score. At the end of each two-week test period, the subjects were asked to fill in a questionnaire with questions related to the usage and experience of the product used. The investigators involved in plaque sampling and pH measurements were blinded with respect to the treatment allocation of the subjects.

2.2. Study Population. The research population at GOT was made up of students and staff at the Institute of Odontology, as well as individuals in the nearby vicinity recruited via advertisements on bulletin boards. A total of 65 volunteers were screened. For ACTA, recruitment was performed using the existing database (approximately 600 entries being not-dental students) at the Department of Periodontology. The inclusion criteria were healthy adults, possessing at least three premolars/molars in each quadrant, who were able to reduce their plaque pH by at least one pH unit after a mouth rinse with 10% sucrose solution for 1 min (only GOT), no metal fillings in the premolar/molar region (only GOT), and a stimulated saliva secretion rate of > 0.7 mL/min (only GOT). The exclusion criteria were subjects with untreated caries or periodontal disease, wearing partial dentures, wearing orthodontic bands, and the use of antibiotics less than three months prior to the start of the study. All the subjects were given verbal and written information about the study and signed an informed consent form prior to the start of the study.

Sample size calculations were made by using the results of plaque acidogenicity relating to the effect of Meridol mouthwash (AmF-SnF_2) on the amount of lactate in sucrose-fermenting plaque [17] where an effect size of 0.759 had been

found. Since no data were available on the effect of shiitake extract rinse on lactate production, a more conservative effect (75%) was used for calculation. An *a priori* two-tailed analysis of the required sample size with an alpha-error probability of 0.05, a power of 0.8, and an effect size of 0.569 was performed. This gave a minimum sample size of 27. In GOT, 30 subjects who fulfilled the inclusion criteria were enrolled, while at ACTA 35 subjects were enrolled compensating for potential dropouts in order to complete the study with at least 30 individuals. The subjects were randomised using a computer-generated allocation schedule, and the subjects were not informed of their allocation.

Apart from the specific instructions given to participants in GOT/ACTA for each test period, the volunteers were asked to refrain from any oral hygiene procedures during the last 72 hours (GOT) and 48 hours (ACTA), respectively, prior to each visit, as well as eating/drinking during the last two hours prior to the test. A toothpaste containing 1450 ppm F as NaF was distributed to all subjects to be used twice daily throughout the entire study: Pepsodent Super Fluor, Unilever Sverige AB, Stockholm, Sweden (GOT) and Prodent, Sara Lee, the Netherlands (ACTA), respectively.

2.3. Test Products. The following three products were tested: (1) shiitake (low-molecular-weight fraction of shiitake mushroom (*Lentinula edodes*) extract), (2) placebo (negative vehicle control without active ingredients), and (3) Meridol (AmF-SnF_2, positive control). The active product was produced and shipped by the subcontractor MicroPharm Ltd (UK) in 20 mL aliquots. The product was prepared at MicroPharm Ltd according to the GMP guidelines at the company. In addition, the placebo formulation (negative control) was distributed by MicroPharm Ltd in identical vials containing 20 mL aliquots. The active and placebo solutions contained identical flavouring and preservative agents. The positive control (Meridol, GABA International AB, Münchenstein, Switzerland) contained 125 ppm AmF + 125 ppm SnF_2. Prior to the start of the study, the solution was aseptically distributed in 20 mL aliquots into empty vials identical to those used for the active and placebo solutions. At the start of each test period, the subjects received a total of 30 vials (28 vials + 2 extra) to be used during the 14-day test period.

The volunteers were asked to rinse with the assigned solution twice daily. On each rinsing occasion, they were instructed to rinse vigorously with 10 mL (1/2 of the volume of the vial) for 30 sec, after which they expectorated the solution. A second identical rinsing procedure with the remaining 10 mL was repeated directly after the first one. The total daily exposure was, therefore, 40 mL for 120 sec. No food or drink intake was allowed for at least one hour after the rinse. To standardise the sampling procedure after two weeks' use of the mouthwash, all the volunteers were asked to rinse exactly three hours before the visit on day 14. No food or drink intake was allowed for at least one hour after the rinse.

2.4. Plaque Acidogenicity. In GOT, changes in plaque acidogenicity were measured before and after a mouth rinse with 10% sucrose using the microtouch method [18]. An iridium microelectrode (Beetrode MEPH-1, WPI Instruments, New Haven, Conn, USA) was inserted into the plaque in an interproximal area in the left and right upper premolar/molar region. The electrode was connected to an Orion SA720 pH/ISE Meter (Orion Research, Boston, Mass, USA) to which a reference electrode was also connected. The reference electrode was placed in a solution of 3 M KCl into which a finger of the volunteer was also inserted in order to create a salt bridge. Prior to and during each test session, the electrode was calibrated against a standard buffer at pH 7 [19]. After baseline registration (0 min), the subjects rinsed with the sucrose solution for 1 min, after which pH was measured at seven different time points up to 45 min.

2.5. Protein and Organic Acid Analyses. Two plaque samples were collected for the protein and acid anion profile, before (resting) and 10 min after the start of rinsing (fermented). The collection of resting plaque was carried out on the buccal surface of the right upper second molar using a sterile carver (GOT) and Teflon spatula (ACTA), respectively. The volunteers then rinsed for 2 min (ACTA) or 1 min (GOT) with 10 mL of 10% sucrose (w/v) solution. Fermented plaque, collected 10 min after the start of the sucrose rinse, was collected from the contralateral buccal surface (left second upper molar). For GOT, the fermented plaque sample was collected at the same time point as the pH measurements.

The plaque was transferred to a precooled Eppendorf tube containing 50 μL of MilliQ water. The samples were immediately spun down by centrifuging the tube for 30 sec at 16.100 $\times$g and put on ice until they were further processed within one hour. The samples were heated at 80°C for 5 min and cooled on ice. The samples from GOT were sent on dry ice to ACTA for further processing and analyses. The vials with plaque were centrifuged at 16.100 $\times$g for 15 min at 4°C. The supernatants were transferred into vials with a microspin filter (Ultrafree-MC 0.22 μm, Millipore, Bedford, Mass, USA) and centrifuged at 13.684 $\times$g for 5 min at 4°C. The supernatants and pellets were then stored at −80°C. Organic acids in resting and fermenting plaque were determined as their anions by capillary electrophoresis on a Beckman P/ACE MDQ system. Sodium salts of formic, acetic, propionic, butyric, succinic, and lactic acid were used to prepare mixture standard solutions in MilliQ water. Calibration curves were made for each acid separately. As an internal standard, oxalate was included in all samples. Formic, butyric, succinic, propionic, acetic, and lactic acid were determined in duplicate samples. Acid data were normalised by protein content of the plaque sample. Protein content was determined according to Bradford [20].

2.6. Microbiological Analyses. In GOT, a stimulated saliva sample was collected by chewing on a piece of paraffin for 5 min. The saliva sample was within one hour handled at the laboratory for microbial analyses. The samples were dispersed on a Whirlimixer, diluted in 10-fold stages in a potassium phosphate buffer and plated in duplicate on MSB agar (mutans streptococci), MS agar (total streptococci),

Rogosa SL agar (lactobacilli), blood agar (total viable count). After being incubated in its respective atmosphere, the number of colony-forming units (CFU) was counted. The number of mutans streptococci was identified by their characteristic colony morphology on the MSB agar.

At ACTA, all visible plaque was collected from a buccal surface of the upper first molar using a Teflon spatula. Plaque was put into sterile Eppendorf tubes and kept on ice until stored at −80°C. Samples were sent on dry ice to the Department of Microbial Diseases (UCL Eastman Dental Institute, University College, London, UK) for analyses of microbiological composition. The numbers of *Streptococcus sanguinis*, *Streptococcus mutans*, *Lactobacillus casei*, *Veillonella dispar*, *Neisseria subflava*, *Actinomyces naeslundii*, *Prevotella intermedia*, *Fusobacterium nucleatum,* and total bacterial 16SrDNA were determined by using multiplex quantitative PCR (qPCR) [21]. In brief, DNA was extracted from plaque biofilms using a phenol : chloroform : isoamyl alcohol (25 : 24 : 1) bead-beating extraction method [22], which involves physical cell lysis, protein removal, and finally DNA precipitation using polyethylene glycol. Three triplex qPCR assays were then carried out using 2 μL of extracted DNA to enumerate eight oral taxa as well as the total number of organisms. The assays were performed using the Rotor-Gene 6500 (QIAGEN) instrument and Sensimix Probe (Bioline) qPCR mix according to the manufacturers instructions, using previously published oligonucleotide sequences [20].

2.7. Plaque Index Amount. The plaque score was in GOT calculated using the Turesky modification of the Quigley-Hein index (TQHPI-index) [23]. The toothsurface coverage with plaque was for each tooth scored on six surfaces (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) on a scale of 0–5. At ACTA, a modification of the Silness and Löe plaque index was used [24]. All buccal and lingual areas in the lower jaw were assessed for each tooth at six sites (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual) on a scale of 0–3.

2.8. Questionnaire. At the end of each test period, the volunteers were requested to complete a questionnaire with a Visual Analogue Scale (VAS) with a total of nine questions related to their experience of using the assigned mouth rinse solution. They marked their answer on a 100 mm line with the negative extreme on the left and the positive extreme on the right.

2.9. Statistical Analyses. The mean ± SD of all clinical parameters and individuals as calculated. For plaque pH, the mean of the values for the left and right side was collected. From each pH curve, the area under the curve ($AUC_{5.7}$ and $AUC_{6.2}$), minimum-pH, and maximum-pH decrease was calculated. For the plaque score, the mean score for each tooth was first calculated, after which the mean score for the whole dentition was calculated. Protein content was expressed in μg and the amount of organic acids as μmol/mg protein. For ACTA, the total number of the different microorganisms was calculated. For GOT, all microbiological data were transformed to logarithmic values. The distribution of mutans streptococci and total streptococci in comparison to the total streptococcal flora and total oral flora (%), respectively, was also calculated. For ACTA, the Log10 CFU was calculated. For the answers on the VAS, the distance (in mm) from the left side was measured for each question and a mean score was calculated.

In GOT, two-way analysis of variance, ANOVA, was used to test the significance of differences between the seven test occasions (after each test period and the washout periods). When ANOVA rejected the multisample hypothesis of equal means, multiple comparison testing was performed using Fisher's PLSD. $P < 0.05$ was regarded as statistically significant.

At ACTA, a paired t-test was used to compare the amounts of different organic acids in resting and fermented plaque from the same visit. The General Linear Model Repeated Measures Test and the Bonferroni post-hoc test were used to compare the output parameters (amount of each acid, relative abundance of oral microorganisms, protein amount) after each of the three treatment periods and separately from the test periods, that is, between pre-experimental baseline and each consecutive washout period. The difference between the mean plaque score at the start of each test period and upon completion of each test period was calculated and used as an input variable in GLM-RM test.

3. Results

3.1. Volunteers. All 30 and 35 individuals, respectively, completed the study, apart from the final washout period for one subject in GOT. The mean age of the volunteers was 31 ± 13 years (mean ± SD) at GOT, including 19 females/11 males, and 23 ± 3 years (mean ± SD) with 32 females/3 males at ACTA.

3.2. Plaque Acidogenicity. The most pronounced metabolic activity for the sucrose rinse at the end of the three test periods was found after rinsing with the placebo, and the least attenuated pH fall was found for the positive control (AmF-SnF_2), while the active compound (shiitake) resulted in an intermediate position (Figure 1). A statistically significant difference when comparing the pH values at the different time points was found at 2 min between shiitake and placebo ($P < 0.05$). In the case of minimum pH, there was also a numerical difference between the three products, with a difference of 0.2 pH units between shiitake and placebo and the positive control, respectively (ns). Minor differences in plaque acidogenicity were found when evaluating the maximum pH decrease, as well as $AUC_{5.7}$ and $AUC_{6.2}$. Only minor numerical differences in plaque acidogenicity were found when comparing the results for the four washout periods (baseline and posttreatment; ns).

3.3. Protein and Organic Acids in Plaque. There was no difference between shiitake and placebo in plaque protein mass, while the positive control (AmF-SnF_2) resulted in significantly less plaque protein than the test or placebo rinse

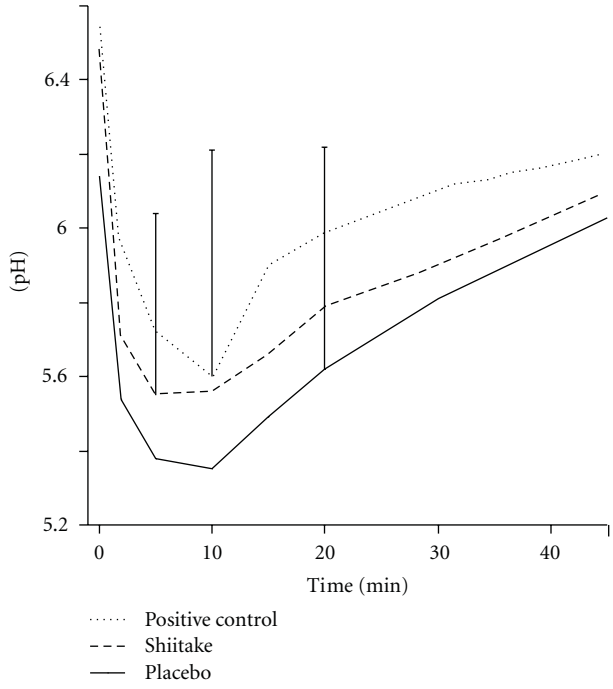

Figure 1: The changes in dental plaque pH up to 45 min after a mouth rinse with 10% sucrose for 1 min. The rinse was carried out after two weeks use of a mouth rinse with a shiitake mushroom extract, a placebo, or positive control (AmF-SnF_2). Mean values for 30 subjects. The standard deviation for some of the time points is shown.

($P < 0.001$). No significant differences in plaque protein were found between the four washout periods. In the case of ACTA, there were no differences in the amount of protein from resting versus fermented plaque, except for the positive control washout period, where less protein was found in the resting plaque ($P < 0.01$). For GOT, there was a tendency towards a generally somewhat lower protein content for the resting plaque (ns). The protein plaque content varied for the seven test periods and two conditions (resting and fermented) for GOT between 14.7 ± 0.1 and $40.3 \pm 31.4\,\mu$g and for ACTA between 30.3 ± 23.5 and $81.2 \pm 65.3\,\mu$g. Significantly less protein was found for the positive control compared with the other two test products ($P < 0.001$) (ACTA).

The profiles of acetate, lactate, and minor acids (propionate, formate, succinate, and butyrate) for the resting and fermented plaque gave similar values for GOT and ACTA (Figure 2). The highest values for all the test sessions were found for lactate for fermented plaque, with a larger variation for GOT compared with ACTA. The rinse period of the positive control (AmF-SnF_2) resulted in significantly less lactate and acetate for fermented plaque compared with shiitake and placebo for GOT ($P < 0.01$). The corresponding data for ACTA showed that the positive control period resulted in significantly less lactate and minor acids in fermented plaque compared with shiitake and placebo ($P < 0.001$). A higher amount of minor acids was found in resting plaque after the shiitake rinse compared with the positive control for ACTA ($P < 0.01$), while no such effect was seen for GOT.

3.4. Microbiological Analyses. All the microbial data are presented in Table 1. For salivary microorganisms in GOT, no statistically significant differences were found for lactobacilli or mutans streptococci in saliva between the three test periods. Rinsing with the positive control (AmF-SnF_2) resulted in a significantly lower number of oral streptococci ($P < 0.05$) and total number of microorganisms ($P < 0.001$) when compared with the shiitake rinse. The lowest proportion of mutans streptococci in comparison to the total number of streptococci was found for shiitake (ns). No significant differences were found for any of the groups of oral microorganisms or the proportion of bacteria when comparing baseline and posttreatment washout periods.

For plaque samples from ACTA analysed by PCR assays, *Neisseria subflava* was the most predominant microorganism of all the tested organisms, followed by *Veillonella dispar* and *Fusobacterium nucleatum*. There were significantly fewer microbial cells and individual organisms in the panel, apart from *S. mutans* counts in plaque samples collected after positive control than after shiitake or placebo periods ($P < 0.001$). The shiitake mouth rinse reduced the proportion of *N. subflava* significantly compared with the placebo rinse ($P < 0.05$), while *N. subflava* and *S. sanguinis* were significantly reduced by the positive control compared with shiitake ($P < 0.01$).

3.5. Plaque Index Score. The plaque scores are shown in Tables 2 and 3. For GOT, the three test periods resulted in the numerically lowest plaque scores (ns), while the positive control resulted in significantly less plaque than shiitake and placebo ($P < 0.001$) for ACTA. For the washout periods, significantly less plaque was found for GOT after the shiitake washout period compared with placebo ($P < 0.05$). For ACTA, significantly less plaque was found at the overall preexperimental baseline compared with the other three washout periods ($P < 0.001$).

3.6. Questionnaire. Taste experience was, when marked from very poor to very good, described as significantly worse by the volunteers after the shiitake test period (GOT 47.0 ± 32.7, ACTA 3.0 ± 4.6; mean $\pm$ SD) compared to both the placebo (GOT 62.4 ± 22.4, ACTA 59.2 ± 21.5) and positive control (GOT 70.6 ± 18.9, ACTA 54.9 ± 25.6); rinses for both GOT and ACTA ($P < 0.01$ or $P < 0.001$). Similar significant differences were found for duration of the taste, taste perception, and experienced rinsing time (data not shown). A significantly higher perception of sensitivity of the teeth after shiitake (36.2 ± 27.2) compared to the positive control (19.6 ± 27.7) and higher perception of staining after shiitake (24.8 ± 27.2) in comparison to the placebo (12.1 ± 18.8) rinse ($P < 0.05$) was reported for ACTA. The burning sensation of the mouth was also significantly higher after the shiitake (GOT 27.4 ± 28.6, ACTA, 43.2 ± 30.8) and placebo (GOT 29.1 ± 31.5, ACTA 38.2 ± 29.1) rinses compared to the

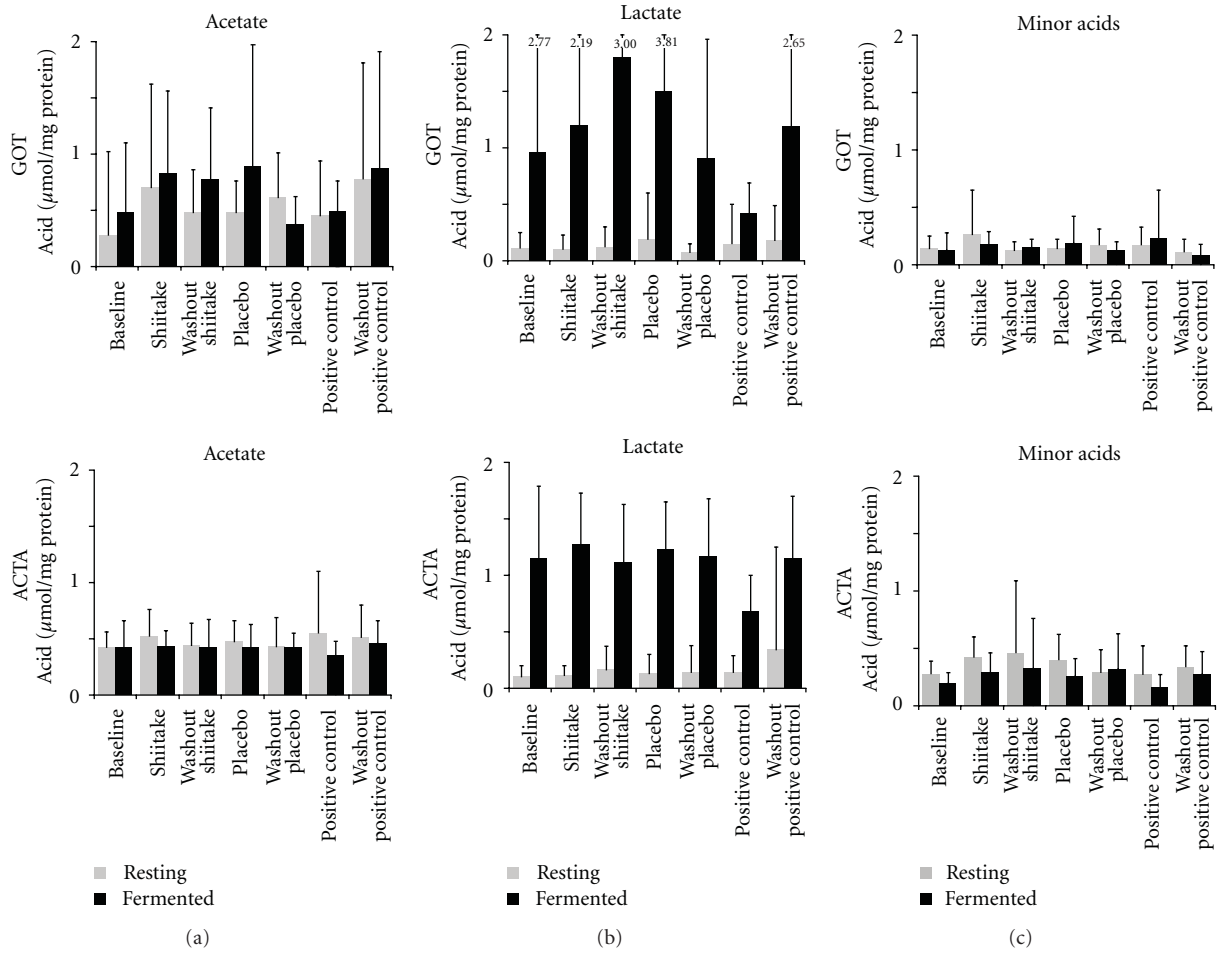

Figure 2: Amount of acetate, lactate, and minor acids (propionate, formate, succinate, and butyrate) in resting (presucrose) and fermented (postsucrose) dental plaque. After baseline, after the three legs of crossover (shiitake, placebo, positive control [AmF-SnF_2]) and after three washout periods (washout shiitake, washout placebo, and washout positive control) are all shown. Data are shown separately for volunteers in Gothenburg (GOT) and Amsterdam (ACTA). Mean values ± SD for 30 (GOT) and 35 (ACTA) subjects, respectively. Due to the high standard deviation when analysing lactate in fermented plaque for five of the test sessions in GOT, the y-axis does not correspond to the actual figure. The total amount (mean ± SD) is given above each individual column.

positive control (GOT 12.6 ± 18.8, ACTA 15.0 ± 23.4) for both test series (GOT $P < 0.01$, ACTA $P < 0.002$).

4. Discussion

The scientific approach and study design of this paper are based on the results of previous studies performed within the Nutrident project. The study was planned as a consequence of the initial chemical characterization of the shiitake mushroom [15], evaluation of the fractions and subfractions of the shiitake mushroom, and further evaluation in different *in vitro* settings [16, 25]. Due to different technical limitations at each of the two centres, the study did not follow the design of a multicentre approach. However, the fact that the conclusions are based on the results from 30 and 35 volunteers from two international centres strengthens its scientific value. No direct comparison of the subjects from the perspective of caries activity was made. However, the additional inclusion criteria in GOT, where the subjects are known to have reduced their plaque pH by at least one pH unit after a sugar rinse, indicates that these subjects may have higher caries activity. This hypothesis is also supported by their higher bacterial counts and plaque scores. The possibility that the numerical variation seen between the two substudies may be a consequence of the selection of subjects cannot, therefore, be ruled out. Due to the above-mentioned factors, the data have been handled separately for GOT and ACTA.

The main finding in this study is that rinsing twice daily with a natural food extract may reduce the metabolic activity of the dental biofilm. Although not evaluated in the present study, a reduction of this kind may result in the long term in a lower degree of demineralisation. This is supported by recent data where a subfraction of shiitake showed a strong inhibiting effect on dentine demineralisation when evaluated in an environment using saliva-derived microcosms [16].

Table 1: Number of salivary and plaque microorganisms and proportions of microorganisms after baseline, the three test periods (shiitake, placebo, positive control (AmF-SnF_2; Pos Ctrl)) and three washout periods (washout shiitake, washout placebo, and washout positive control) for GOT ($n = 30$) and ACTA ($n = 35$). Mean ± SD.

City/microorganisms	Test session						
	Baseline	Shiitake	Washout shiitake	Placebo	Washout placebo	Pos. Ctrl.	Washout Pos. Ctrl.
GOT							
Mutans streptococci (log CFU/mL)	4.8 ± 1.2	5.0 ± 1.0	4.8 ± 1.0	4.9 ± 1.1	4.9 ± 1.2	4.5 ± 1.2	4.8 ± 1.0
Lactobacilli (log CFU/mL)	3.6 ± 1.4	3.8 ± 1.3	3.8 ± 1.2	3.8 ± 1.3	3.7 ± 1.4	3.8 ± 1.4	3.5 ± 1.3
Total streptococci (log CFU/mL)	7.4 ± 0.5	7.6 ± 0.4	7.5 ± 0.4	7.5 ± 0.4	7.6 ± 0.3	7.2 ± 0.8[1]	7.5 ± 0.4
Total oral flora (log CFU/mL)	8.0 ± 0.3	8.1 ± 0.3	8.1 ± 0.3	8.0 ± 0.3	8.1 ± 0.3	7.8 ± 0.6[3]	8.0 ± 0.3
Total streptococci/total flora (%)	35.2 ± 19.4	41.0 ± 19.8	30.1 ± 16.0	32.5 ± 12.6	35.0 ± 19.0	31.9 ± 30.2	40.4 ± 23.7
Mutans streptococci/total streptococci (%)	1.1 ± 1.7	0.7 ± 0.9	1.1 ± 2.5	0.8 ± 1.1	1.1 ± 1.6	0.8 ± 1.5	0.7 ± 1.4
ACTA							
Universal probe counts (log10 CFU)	7.8 ± 0.3	7.9 ± 0.5	7.8 ± 0.5	7.8 ± 0.5	7.8 ± 0.6	7.1 ± 0.8[3]	7.9 ± 0.4
L. casei (log10 CFU)	2.1 ± 1.0	1.6 ± 1.2	1.3 ± 1.2	1.9 ± 1.0	1.5 ± 1.1	1.5 ± 1.1[3]	1.4 ± 1.2
V. dispar (log10 CFU)	6.5 ± 0.6	6.6 ± 0.9	6.5 ± 0.7	6.5 ± 0.8	6.6 ± 0.8	5.4 ± 1.1[3]	6.5 ± 0.6
N. subflava (log10 CFU)	6.7 ± 0.9	6.4 ± 1.1[2]	6.7 ± 0.9	6.7 ± 0.9	6.7 ± 0.9	5.1 ± 1.0[1,3]	6.9 ± 0.7
A. naeslundii (log10 CFU)	5.1 ± 1.2	5.1 ± 1.0	5.0 ± 1.1	5.0 ± 1.2	5.0 ± 1.3	4.0 ± 1.6[3]	4.9 ± 1.0
P. intermedia (log10 CFU)	0.7 ± 1.1	0.7 ± 1.2	0.8 ± 1.3	0.6 ± 1.0	0.8 ± 1.2	0.1 ± 0.6[3]	0.7 ± 1.3
S. sanguinis (log10 CFU)	5.9 ± 0.5	5.9 ± 0.6	5.7 ± 0.6	5.8 ± 0.6	5.9 ± 0.7	4.7 ± 1.0[1,3]	5.9 ± 0.5
S. mutans (log10 CFU)	1.3 ± 2.2	1.4 ± 2.4	1.6 ± 2.4	1.2 ± 2.2	1.6 ± 2.5	0.7 ± 1.8	1.3 ± 2.4

[1] Statistically significantly different from shiitake group (GOT $P < 0.05$ (ANOVA), ACTA $P < 0.01$ (GLM-RM test)).
[2] Statistically significantly different from placebo group (ACTA $P < 0.05$ (GLM-RM test)).
[3] Statistically significantly different from shiitake and placebo groups (GOT $P < 0.001$ respective $P < 0.01$ (ANOVA), ACTA $P < 0.001$ (GLM-RM test)).

Table 2: Quigley-Hein plaque index score (Turesky modification 1970) (mean ± SD) after baseline, the three legs of the crossover (shiitake, placebo, positive control (AmF-SnF_2; Pos Ctrl)) and three washout periods (washout shiitake, washout plaque, and washout positive control) for GOT ($n = 30$). Mean ± SD.

City	Test session						
	Baseline	Shiitake	Washout shiitake	Placebo	Washout placebo	Pos. Ctrl.	Washout Pos. Ctrl.
GOT	2.0 ± 0.9	1.7 ± 0.8	1.8 ± 0.8	1.7 ± 0.8	1.9 ± 0.8	1.6 ± 0.6	1.8 ± 0.8[1]

[1] Statistically significantly different from the placebo test period ($P < 0.001$, paired samples t-test).

Table 3: Silness & Löe plaque index score (as modified by Danser et al. [24]) before and after each of the three legs of the crossover (shiitake, placebo, positive control (AmF-SnF_2)) and three washout periods (washout shiitake, washout plaque, and washout positive control) for ACTA ($n = 35$). Mean ± SD.

	Shiitake	Placebo	Positive control
Plaque score before test period	1.5 ± 0.4	1.5 ± 0.4	1.5 ± 0.3
Plaque score after test period	1.6 ± 0.3	1.6 ± 0.3	1.2 ± 0.4[1]
Plaque score after washout	1.6 ± 0.3	1.7 ± 0.2	1.6 ± 0.3
The mean difference in plaque score before and after test period	−0.1 ± 0.4	−0.1 ± 0.3	0.3 ± 0.5[2]

[1] Statistically significantly different from the respective plaque score obtained before the test period ($P < 0.001$, paired samples t-test).
[2] Statistically significant plaque score reduction compared to other test periods ($P < 0.001$, GLM-RM; Bonferroni post-hoc test).

There may be multiple explanations for the present findings of a change in the acidogenic potential of the biofilm. Previous work focusing on shiitake mushroom extract has demonstrated biological activity relevant to caries prevention [16, 25]. This includes mechanisms such as bactericidal activity against cariogenic microorganisms, the prevention of the coaggregation of cariogenic microorganisms, the induction of the detachment of cariogenic microorganisms from hydroxyapaite, and changes in cell surface hydrophobicity.

The antimicrobial, antiadhesive, and antiplaque properties of polyphenol-rich beverages have previously been demonstrated [26, 27]. Recent studies have focused on the oral health variables of tea in particular, both when consumed naturally or when evaluating tea and cranberry in an *in vitro* or *in vivo* design [28–31]. Similar findings relating

to the plaque-lowering potential have been found both after using both different sweeteners [32] and essential oils [33].

The interpretation of the acid anion profiles of the resting and fermented plaque is complicated. Although a corresponding pattern when comparing the data with the results from the plaque-pH measurements would have seemed logical, it was difficult to obtain a clear and consistent picture from the current data. This may be related to the low "*in vivo*" activity of the food compound that was tested, a poor cooperation of the subjects, or a weak experimental design. When evaluating the same low-molecular-weight fraction of the shiitake mushroom in an *in vitro* caries model, a stronger inhibitory effect on acid production potential was observed by one of the subfractions (SF4) in comparison to the whole low-molecular-weight fraction [16].

Microbiological analyses included both the total cell count and bacteria related to periodontal diseases, dental caries, and oral health. Only minor differences in both the salivary levels (GOT) and the plaque levels (ACTA) of oral microorganisms were found between the different visits. The numerically lowest salivary number of mutans streptococci in comparison to the total number of streptococci was found for shiitake. For plaque microflora, significantly reduced proportions of microorganisms were only found for the Gram-negative organism *N. subflava* when comparing the shiitake mouth rinse with placebo. These findings are supported by a recent study in which 11 days of frequent mouth rinses with the same mushroom extract resulted in a reduced amount of plaque but a weaker effect on the decrease in total bacterial counts as well as some specific oral pathogens when compared with a placebo test period [34].

While GOT found a significant reduction in plaque score when comparing shiitake with placebo, no such difference was found for ACTA. However, a reduction in dental plaque deposition has also been found when evaluating the active compound against gingivitis- and periodontitis-related variables [34]. This finding is furthermore supported by previous studies in which inhibited plaque formation was found when using mouth rinses of oolong tea [35] and pomegranate [36]. Neither the mushroom extract nor the placebo was capable of reducing plaque formation to the same degree as chlorhexidine, an antimicrobial compound known to inhibit biofilm development and maturation [37].

The subjects reported a less favourable outcome for the different questions related to taste for the shiitake extract mouth rinse. All the subjects gave an assurance that they had followed the given instructions. However, following the reported negative reaction to the taste of the shiitake mouth rinse by a large number of the volunteers, one cannot exclude that this may have had a negative impact on compliance. As a consequence, some of the subjects may not have rinsed with the active compound according to instructions and that they may have rinsed their mouth with water shortly after using the active substance cannot be excluded. In order to secure the regular use of potential future products, it is important that this aspect is also considered seriously, as this factor alone may determine whether or not an oral health product is used. Aspects related to food safety also need to be taken into account.

Functional foods have not been introduced in order to replace traditional caries-prevention strategies but instead to add another tool to offer patients at higher risk. The positive finding of reduced plaque fermentation activity indicates that there is an opportunity to add one more strategy to the palette of preventive methods. It is not surprising that a stronger effect was found for this variable and that only a limited effect was found for several of the other variables. The metabolic activity of the dental biofilm is the end result of a large number of biological and biochemical caries-related factors. As shown by previous laboratory work [16, 25], the active compounds of shiitake mushroom may exert multiple actions on different caries-related variables. Even if the effect of each of them may appear weak, they may interfere in a positive way in the complex and diverse microbial community constituted by the biofilm.

The limited effect on several dental biofilm properties seen in the present study may indicate that frequent exposure for a longer period is needed. One important factor is believed to be the contact time between the active compound and the different oral properties. The repeated rinsing with 10 + 10 mL for 30 + 30 sec was used to diminish the dilution effect of saliva and to prolong the contact time of active compounds with the oral cavity. For this reason, both further laboratory and clinical studies are needed in order to evaluate not least the effect of a longer exposure period or variations in the concentration of these naturally derived biologically active compounds.

5. Conclusions

The main finding of this study is that frequent mouth rinses with a natural food extract (shiitake mushroom) may reduce the metabolic activity of the dental biofilm. Only a limited effect on other dental plaque properties related to the caries disease was found and not to the same extent as the positive control.

Acknowledgments

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT). Sincere thanks to Ann-Charlotte Börjesson and Ann-Britt Lundberg, Department of Cariology, University of Gothenburg, for technical support. The clinical and logistic support of Nienke Hennequin-Hoenderdos, Department of Periodontology, Academic Centre for Dentistry Amsterdam, is gratefully acknowledged.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 720692, 8 pages
doi:10.1155/2012/720692

Review Article

Good Oral Health and Diet

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Received 29 June 2011; Revised 16 September 2011; Accepted 21 October 2011

Academic Editor: David A. Spratt

An unhealthy diet has been implicated as risk factors for several chronic diseases that are known to be associated with oral diseases. Studies investigating the relationship between oral diseases and diet are limited. Therefore, this study was conducted to describe the relationship between healthy eating habits and oral health status. The dentistry has an important role in the diagnosis of oral diseases correlated with diet. Consistent nutrition guidelines are essential to improve health. A poor diet was significantly associated with increased odds of oral disease. Dietary advice for the prevention of oral diseases has to be a part of routine patient education practices. Inconsistencies in dietary advice may be linked to inadequate training of professionals. Literature suggests that the nutrition training of dentists and oral health training of dietitians and nutritionists is limited.

1. Introduction

The concept of oral health correlated to quality of life stems from the definition of health that the WHO gave in 1946. Health is understood to be "a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity". The programs for the prevention of oral diseases concern teaching about oral hygiene and healthy eating, fluoride prophylaxis, periodic check-ups, sessions of professional oral hygiene, and secondary prevention programs [1]. The term "bionutrition" refers to the important interactions which exist between diet, use of nutrients, genetics, and development. This term emphasizes the role of nutrients in maintaining health and preventing pathologies at an organic, cellular, and subcellular level [2].

There exists a biunique relationship between diet and oral health: a balanced diet is correlated to a state of oral health (periodontal tissue, dental elements, quality, and quantity of saliva).

Vice versa an incorrect nutritional intake correlates to a state of oral disease [3–6].

2. Diet and the Development of the Oral Cavity

Diet influences the development of the oral cavity: depending on whether there is an early or late nutritional imbalance, the consequences are certainly different. In fact, an early nutritional imbalance influences malformations most. Moreover, the different components of the stomatognathic apparatus undergo periods of intense growth alternated with periods of relative quiescence: it is clear that a nutritional imbalance in a very active period of growth will produce greater damage [3].

A shortage of vitamins and minerals in the phase before conception influences the development of the future embryo, influencing dental organogenesis, the growth of the maxilla, and skull/facial development [1, 2].

An insufficient supply of proteins can lead to [3, 4] the following:

(i) atrophy of the lingual papillae,

(ii) connective degeneration,

(iii) alteration in dentinogenesis,

(iv) alteration in cementogenesis,

(v) altered development of the maxilla,

(vi) malocclusion,

(vii) linear hypoplasia of the enamel.

An insufficient supply of lipids can lead to [5, 6] the following:

(i) inflammatory and degenerative pathologies,

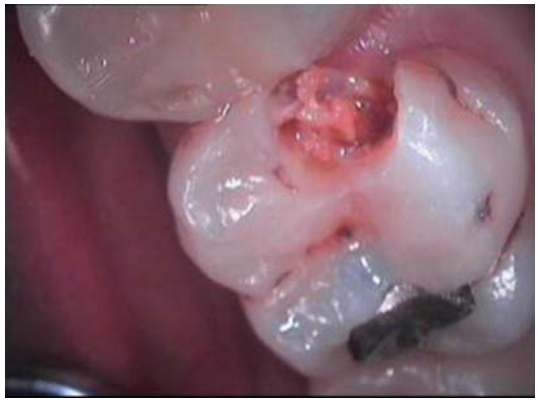

Figure 1: Caries of the teeth.

(ii) parotid swelling—hyposalivation,

(iii) degeneration of glandular parenchyma,

(iv) altered mucosal trophism.

An insufficient supply of carbohydrates can lead to the following:

(i) altered organogenesis,

(ii) influence of the metabolism on the dental plaque,

(iii) caries,

(iv) periodontal disease.

Diet influences the health of the oral cavity, conditioning the onset of caries, the development of the enamel, the onset of dental erosion, the state of periodontal health, and of the oral mucous in general.

3. Caries

Caries is a demineralization of the inorganic part of the tooth with the dissolution of the organic substance due to a multifactorial etiology. The demineralization of the enamel and of the dentine is caused by organic acids that form in the dental plaque because of bacterial activity, through the anaerobic metabolism of sugars found in the diet [7].

Demineralization occurs when the organic acids produced increase the solubility of the calcium hydroxyapatite that is present in the hard tissue of teeth (Figure 1).

The development of caries requires the presence of sugars and bacteria but it is influenced by the susceptibility of the teeth, by the type of bacteria, and by the quantity and quality of the salivary secretion.

Saliva is supersaturated with calcium and phosphate with a pH equal to 7, a level that favours remineralization. When acid stimulation is too strongdemineralization prevails until the formation of a carious lesion [8].

Very low levels of dental caries are found in isolated communities with a traditional lifestyle and low consumption of sugars [7–9]. As soon as economic conditions improve and the quantity of sugars and other fermentable carbohydrates increases in the diet, a notable increase in dental caries is noticed. This has been seen in the Inuit of Alaska and in populations in Ethiopia, Ghana, Nigeria, Sudan, and the islands of Tristan da Cunha and Sant'Elena [7–9].

A Vipeholm study in Sweden between 1945 and 1953 in an institute for the mentally ill underlined the correlation between caries and the intake of sugary food of variable viscosity. If the sugar was ingested up to a maximum of 4 times a day only during meals, it had little effect on the increase of caries, even if this occurred in great quantities; the increase in the frequency of consumption of sugar between meals was associated to an increase in caries; when they no longer ate foods rich in sugar, the incidence in the formation of caries diminished [10].

The types of sugar ingested through diet also influence the onset of illness. In fact, studies on the pH of the dental plaque have shown that lactose produces less acidity in comparison to other sugars.

A 1970 Finnish study on a supervised dietary change revealed that, in an adult population, the almost total substitution of sucrose in the diet with xylitol determines a 85% reduction in caries over a 2-year period; its mechanism of action resides in the inhibition of the growth of Streptococcus mutans, the most important microorganism responsible for the formation of caries [11].

Diet can be a good ally in the prevention of caries [12].

(i) Increase in the consumption of fibres: diminution of the absorption of sugars contained in other food.

(ii) Diets characterized by a ratio of many amides/little sugar have very low levels of caries.

(iii) Cheese has cariostatic properties.

(iv) Calcium, phosphorus and casein contained in cow milk inhibit caries.

(v) Wholemeal foods have protective properties: they require more mastication, thus stimulating salivary secretion.

(vi) Peanuts, hard cheeses, and chewing gum are good gustative/mechanical stimulators of salivary secretion.

(vii) Black tea extract increases the concentration of fluorine in the plaque and reduces the cariogenicity of a diet rich in sugars.

(viii) Fluorine.

Fluorine remains a milestone in the prevention and in the control of dental caries. It has a preeruptive mechanism of action (incorporation in the enamel during amelogenesis) and a posteruptive mechanism (topical action). Fluorine reduces caries by 20–40% in children, but it does not entirely eliminate them: even when fluorine is used, the association between the intake of sugars and caries continues to be present all the same [13].

Diet also influences the qualitative characteristics of salivary secretion. The secretive proteins (mucines) represent an important barrier against the reduction of humidity, against the physical and chemical penetration of irritants and against bacteria [14].

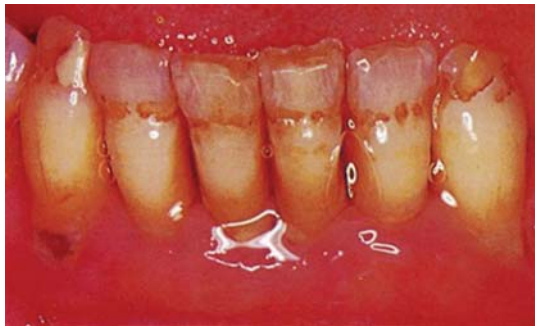

Figure 2: Hypoplasia and pits on the surface of the enamel correlate to a lack of vitamin A.

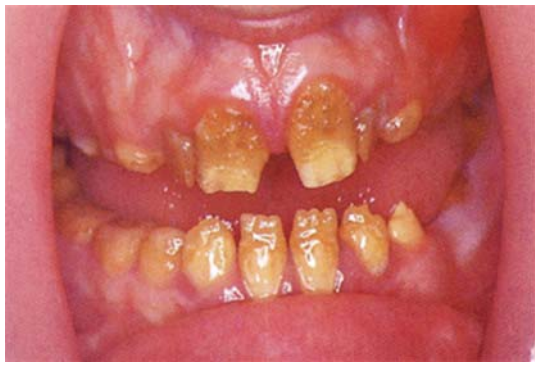

Figure 3: Hypoplasia on the surface of the enamel correlate to a lack of vitamin D.

The synthesis of glycoproteins requires vitamin A. In an imbalanced diet, there is a reduction in the content of mucines with the consequent risks for oral health (Caries!!).

4. Development of the Enamel

Teeth in a preeruptive phase are influenced by the nutritional state. A lack of vitamins D and A and protein-energy malnutrition have been associated to hypoplasia of the enamel and atrophy of the salivary glands, conditions that determine a greater susceptibility to caries. Some hypoplasia and pits on the surface of the enamel correlate to a lack of vitamin A (Figure 2); a lack of vitamin D is associated to the more diffused hypoplastic forms (Figure 3). The structural damage can testify to the period in which the lack of nutrition occurred [15].

5. Dental Erosion

"Dental erosion is the progressive irreversible loss of dental tissue that is chemically corroded by extrinsic and intrinsic acids through a process that does not involve bacteria. . .."

Extrinsic Acids Derived from Diet. They citric, phosphoric, ascorbic, malic, tartaric, and carbonic acids that are found in fruit, in fruit juices, in drinks, and in vinegar.

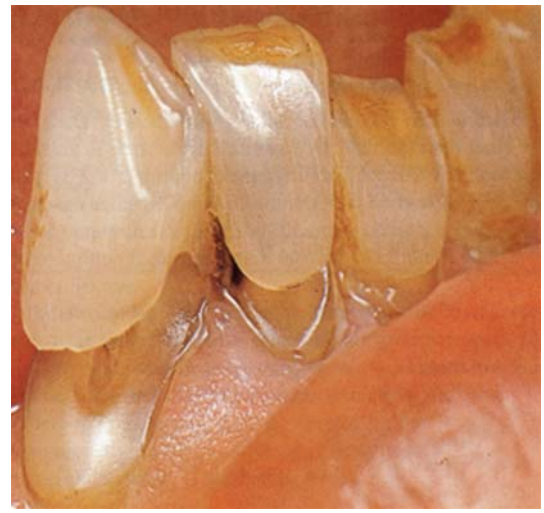

Figure 4: Dental Erosion.

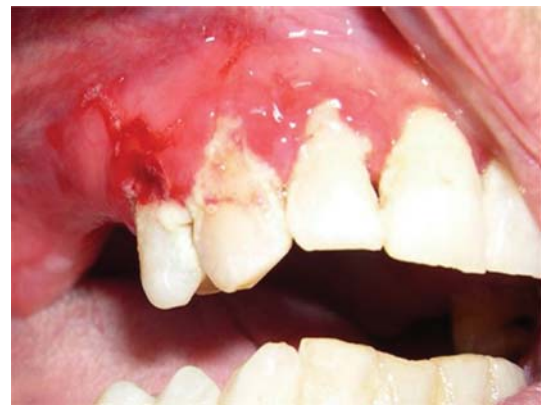

Figure 5: Periodontal disease.

Intrinsic Acids. They are derived from serious gastroesophageal reflux [16–18] (Figure 4).

6. Periodontal Disease

Periodontal disease evolves more quickly in undernourished populations: "...the pathology starts in the gum and could interest the periodontal ligament up to the alveolar bone...". The most important risk factor in the development of periodontal disease is represented by inadequate oral hygiene (Figure 5). Data supplied by the National Health and Nutrition Examination Survey 2001/02 underlined that a low level of folic acid is associated to periodontal disease. The serum level of folates is an important index of periodontal disease and can represent an objective that should be pursued in the promotion of periodontal health [19].

Malnutrition and bad oral hygiene represent two important factors that predispose for necrotizing gingivitis. Prevention programs against disease must therefore include a correct evaluation of the immune system and the promotion of nutritional programs. The aim of nutritional support in inflammatory diseases is to provide the right energy and

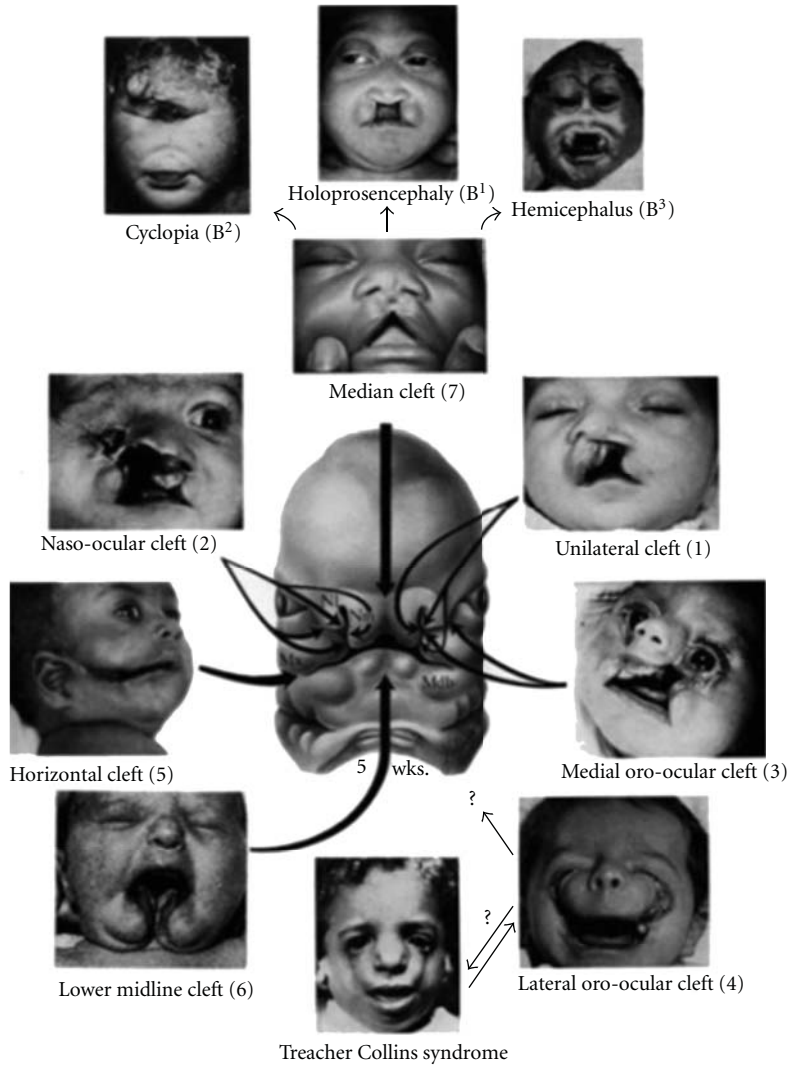

Figure 6: Cleft lip and palate.

nourishment to respond to the increased demand for protein synthesis in the acute phase, inflammatory mediators, antioxidant defence mechanisms, as well as for the promotion of tissue reparation. Some nutrients have a very important role in the resolution of the inflammatory process. These observations confirm the relationship between diet and periodontal disease [20]. In a recent interview, the president of the American Society of Periodontology, Michael P. Rethman [20], underlined the importance of diet for a healthy smile. In particular, the correlation between the income of calcium and periodontal disease can be due to the role that calcium has in the density of the alveolar bone that supports teeth. Also the intake of vitamin C is fundamental for maintenance and for the activation of reparative mechanisms thanks to its antioxidant properties [20].

Noma is an orofacial gangrene originating in the gingival-oral mucosa [21]. Although cases of noma are now rarely reported in the developed countries, it is still prevalent among children in third world countries, notably in subSahara Africa, where malnutrition and preventable childhood infections are still common [21]. Noma can be prevented through promotion of national awareness of the disease, poverty reduction, improved nutrition, promotion of exclusive breastfeeding in the first 3–6 months of life, optimum prenatal care, and timely immunisations against the common childhood diseases [21].

7. Gene Disease

Italian researchers have recently identified the genetic defect responsible for cleft lip and palate (Figure 6). The gene is a variation of the maternal gene "MTHFR" that determines the lowering of folate levels in blood. The female carriers of the discovered mutation have a greater risk of giving birth to children affected by cleft lip and palate. Folate is fundamental in the first phases of embryonic development: in fact the lack of this vitamin is able to cause defects in the embryonic development known generically as "defects of the neural tube". For this reason, in the United States B9 is

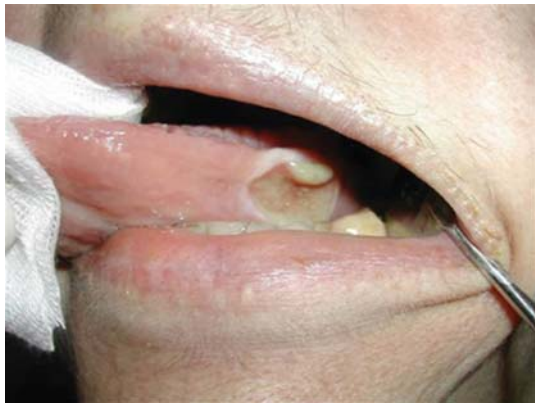

Figure 7: Oral cancer.

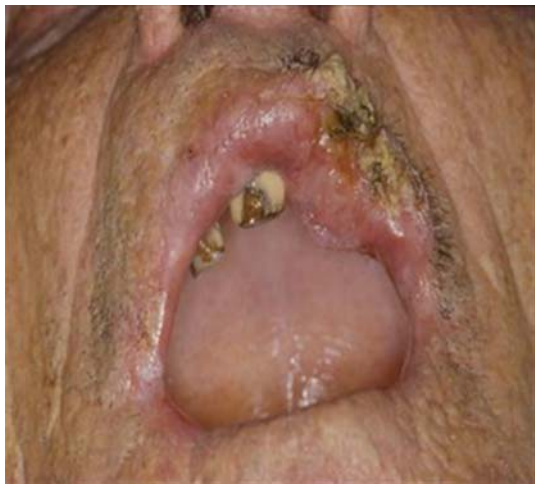

Figure 8: Oral cancer.

administered with the support of the health authorities to women who are intend to conceive and in the first months of pregnancy. Administering folate in the months preceding conception and in the first months of pregnancy, the risk of defects to the nervous system is reduced and even cleft lip and palate could be avoided with the preventive administration of the vitamin [22].

8. Neonatal Diet and Oral Health

The World Health Organization and the American Pediatric Association have shown that breast feeding influences lingual deglutition, the growth of the maxillae and the correct alignment of the teeth, as well as the modelling of the hard palate. Vice versa, bottle feeding the baby influences the formation of the ogival palate as well as the formation of "crossbite", a reduced opening of the back nasal cavity, and an increased incidence of sleep apnea. In addition, artificial feeding influences the possibility of the onset of arterial hypertension, obesity, cardiovascular illnesses, and inflammatory pathologies regarding oral mucous [23, 24].

9. Oral Cancer

The association between diet and oral cancer is extremely serious. It is a pathology that is diagnosed in three hundred thousand new cases in the world every year and presents the greatest incidence in people who smoke, chew tobacco, and consume alcohol (Figures 7, 8, and 9).

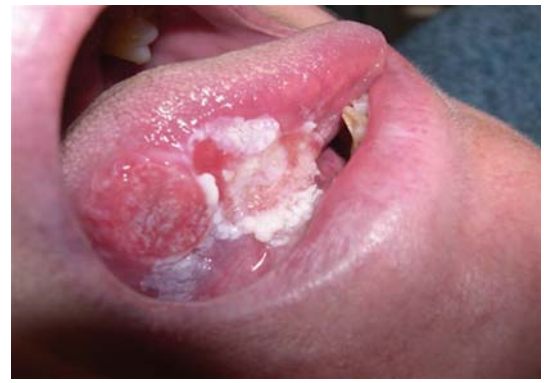

Figure 9: Oral cancer.

The use of tobacco can alter the distribution of nutrients such as antioxidants, which develop a protective action toward the cells: smokers present levels of carotenoids and vitamin E in the blood that are superior to those in the oral mucous and, in addition, have a different distribution in comparison to the norm; the levels of folates in the blood and in the cells of the oral tissues of smokers are inferior to those of nonsmokers; the inside of the cheeks of smokers presents numerous micronuclei (modifications typical of pre- and neoplastic lesions) [25, 26]. The study of the incidence of this illness has underlined the possibility that diet can represent an important etiological factor for oral carcinogenesis. Vitamins A, E, C, and Beta Carotene have antioxidant properties.

(i) They neutralize metabolic products.

(ii) They interfere with the activation of procarcinogens.

(iii) They inhibit chromosomal aberration.

(iv) They potentially inhibit the growth of malignant lesions (leukoplakia).

The mechanism that connects smoke to this disease has not been discovered but some progress has been made: smoke modifies the distribution of protective substances such as folates and some antioxidants. A rebalancing of nutrients obtained through diet can modify the altered distribution caused by the consumption of tobacco. In an imbalanced diet there is a depletion of antioxidant nutrients. Fruit and vegetables have, vice versa, important antioxidant properties. Many micronutrients (vitamins in particular) are used in chemoprevention programs formulated by the US National Cancer Institute [27].

The National Cancer Institute and the American Cancer Society have established some prudential dietary recommendations for the choice of food:

(1) maintain a desirable body weight,

(2) eat a varied diet,

(3) include a new variety of fruits and vegetables in the daily diet,

(4) consume a greater quantity of foods rich in fibre,

(5) decrease the total intake of fats (30% less than the total calories),

(6) limit the consumption of alcohol,

(7) limit the consumption of salted food or food preserved with nitrates.

In patients with an advanced tumour disease, protein-caloric malnutrition is a recurrent problem due to factors such as a form of anorexia that is established, maldigestion, malabsorption, and to a difficulty in mastication and deglutition [26]. Foods should be provided that aim to correct nutritional deficits and ponderal reduction when consumed in a large enough quantity to cover protein and caloric requirements. Malnutrition also interferes negatively with humoral and cellular immunocompetence and with tissue and reparative functions. In addition, the alteration of the liver function can change the way drugs are metabolized. Therefore, malnutrition can interfere with oncological therapy and increase the severity of the collateral effects [25].

Some studies show a small effect of dietary supplementation on cancer incidence, while others show that supplementation with antioxidant vitamins may have an adverse effect on the incidence of cancer and cardiovascular diseases or no effect [27].

Increasing attention has been given to the potential protective roles of specific antioxidant nutrients found in fruits and vegetables.

In a recent research El-Rouby showed that lycopene can exert protective effects against 4-nitroquinoline-1-oxide induced tongue carcinogenesis through reduction in cell proliferation and enhanced cellular adhesion, suggesting a new mechanism for the anti-invasive effect of lycopene [28].

In a recent report Edefonti et al. showed that diets rich in animal origin and animal fats are positively, and those rich in fruit and vegetables and vegetable fats inversely related to oral and pharyngeal cancer risk [29].

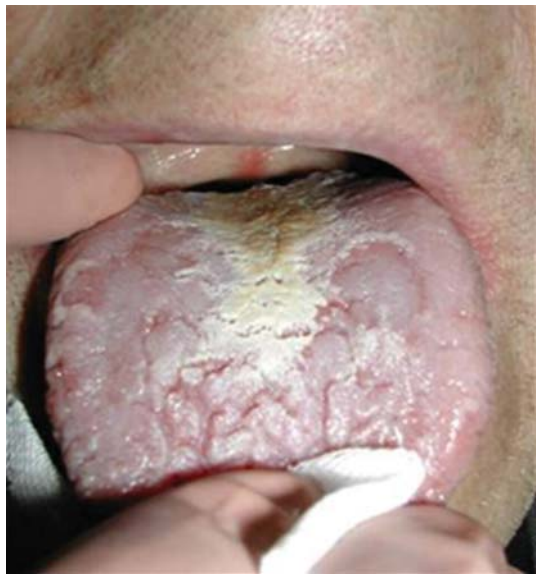

Figure 10: Oral candidosis.

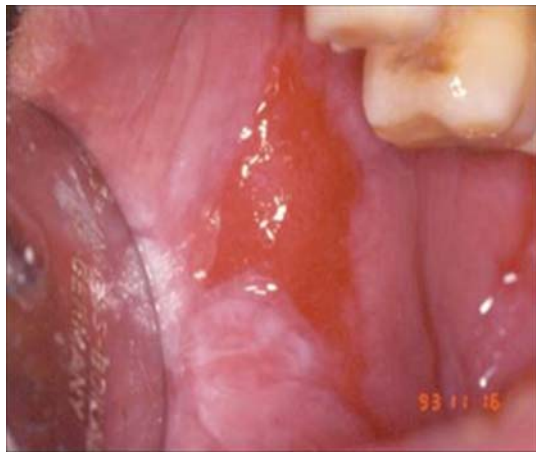

Figure 11: Oral lichen planus.

10. Oral Candidosis

A significant correlation has been evinced with a lack of iron (Figure 10). This causes alterations in the epithelium with consequent atrophy and alteration in cellular turnover, an alteration in the iron-dependent enzymatic system depression in cell-mediated immunity, phagocytosis, and in the production of antibodies. The correlation between candidiasis and the lack of folic acid, vitamins A, B1, B2, vitamins C, K, zinc, and a diet rich in carbohydrates is also significant [30].

11. Potentially Malignant Oral Lesions

These are those pathologies of the oral mucous (oral lichen planus, leukoplakia) that present a tendency for malignant degeneration if some favourable conditions persist (Figure 11). There are conflicting data in literature regarding levels of retinol and beta carotene and the onset of oral lichen planus [31]. Ramaswamy et al. affirmed that folate levels should be investigated in patients with oral lesions and symptoms especially those with risk factors of age, poor nutrition, or systemic diseases. When suspected, daily folic acid supplements should be given [32].

With regard to leukoplakia, a significant association has been found with reduced serum levels of vitamins A, C, and B12, and folic acid (Figure 12). Data in literature confirm that diets rich in fruit and vegetables, above all tomatoes and products derived from them, significantly reduce the risk of the onset of leukoplakia [33]. In a recent report Lodi et al. said that treatment with beta carotene and vitamin A or retinoids was associated with better rates of clinical remission, compared with placebo or absence of treatment. Treatments may be effective in the resolution of lesion; however, relapses and adverse effects are common [34].

12. Micronutrient Deficiencies and Mucosal Disorders

Various types of nutritional deficiencies can produce oral mucosal diseases. Changes such as swelling of the tongue,

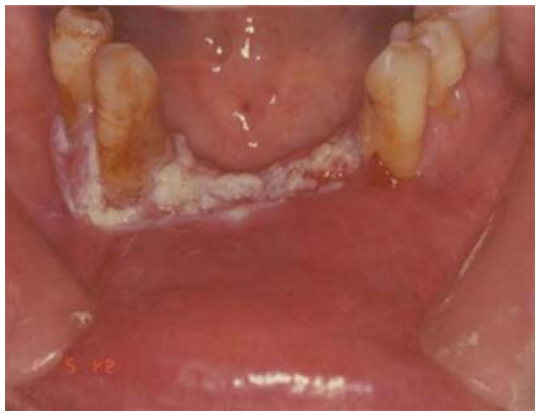

Figure 12: Oral leukoplakia.

papillary atrophy, and surface ulceration are possible in case of micronutrient deficiencies (iron, folate, vitamin B12) [35]. To establish iron, folate, or vitamin B12 deficiency, a hematologic screening that includes complete blood count, red-cell, serum iron, B12, and folate levels should be performed [35, 36]. Although they are rarely required, specific tests for suspected niacin, pyridoxine, and riboflavin deficiency are available [35]. Although glossodynia related to nutritional deficiency is statistically uncommon, it is easily curable with replacement therapy [35]. Identification of a vitamin deficiency through early oral symptoms can forestall development of serious and irreversible systemic and neurologic damage [36]. Deficiencies of vitamin B12 can produce oral signs and symptoms, including glossitis, angular cheilitis, recurrent oral ulcer, oral candidiasis, and diffuse erythematous mucositis. Plummer Vinson syndrome is associated with glossitis and angular cheilitis [35, 36].

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 590384, 7 pages
doi:10.1155/2012/590384

Research Article

Inhibition of *Streptococcus gordonii* Metabolic Activity in Biofilm by Cranberry Juice High-Molecular-Weight Component

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Received 1 July 2011; Accepted 10 October 2011

Academic Editor: Carla Pruzzo

Previous studies demonstrated that a cranberry high-molecular-mass, nondialyzable material (NDM) can inhibit adhesion of numerous species of bacteria and prevents bacterial coaggregation of bacterial pairs. Bacterial coaggregation leads to plaque formation leading to biofilm development on surfaces of oral cavity. In the present study, we evaluated the effect of low concentrations of NDM on *Streptococcus gordonii* metabolic activity and biofilm formation on restorative dental surfaces. We found that the NDM selectively inhibited metabolic activity of *S. gordonii*, without affecting bacterial viability. Inhibiting the metabolic activity of bacteria in biofilm may benefit the health of the oral cavity.

1. Introduction

For a successful bacterial colonization of the oral cavity, adherence mechanisms are essential, otherwise the bacteria get washed away and swallowed by the salivary flow. Oral bacteria have evolved several mechanisms to withstand the salivary flow and succeed in adhesion to and subsequently form biofilm on surfaces of the oral cavity. *Streptococcus gordonii* has been considered to play an important role in cariogenesis because it readily colonizes the clean tooth surfaces and is capable of forming biofilm. Among the oral bacteria, *S. gordonii* appears to have highest affinity to hard surfaces of the oral cavity [1]. *S. gordonii* biofilm forms an important component of human dental plaque by virtue of its ability to adhere to tooth surfaces [1]. Formation of dental plaque precedes cariogenesis; thus, interfering with *S. gordonii* adhesion and biofilm formation of hard tissue is likely to improve the oral health.

Dietary agents that interfere with adhesion of and biofilm formation by bacteria has been the focus of intensive research because such natural agents are likely to be nontoxic to the host [2]. Furthermore, the identified active components can be used as supplement to oral health hygiene product negating the necessity to adhere to a particular diet. Perhaps most important advantage of searching dietary agents is that approval of clinical trials would be easier to obtain, as toxicity is not an issue. In this respect, cranberry juice and isolated fractions/constituents which inhibit adhesion of bacteria to various surfaces have been studied the most [3].

Phenolic compounds of cranberry were shown to prevent adherence of uropathogen to animal cells [4, 5]. The cranberry components were also shown to reduce the risk of cardiovascular disease [6], periodontal disease [7], and inhibit host inflammatory response [8]. Earlier studies demonstrated a high-molecular-weight mass, nondialyzable material (NDM) prepared from cranberries to contain polyphenolic compounds that inhibited the secretion of proteolytic enzymes by periodontopathogens [9], adhesion of a number of bacterial species [10, 11], and were also shown to interfere with coaggregation of oral bacterial species and biofilm formation by *Streptococcus mutans* [3, 12]. The polyphenol fraction of cranberry was reported to decrease the hydrophobicity of streptococcal species [12, 13].

In the present study, we sought to determine the ability of the high-molecular-weight component from cranberry (NDM) to interfere with biofilm formation by *S. gordonii* in general and in particular on dental composites and titanium discs. We hypothesize that the cranberry NDM will have a beneficiary role by interfering with streptococcal biofilm formation on dental materials. Prevention or reduction of oral bacterial load on the surfaces of the oral cavity will have a beneficial role in improving the oral health.

2. Materials and Methods

2.1. Preparation of NDM. NDM was obtained as described previously [3] from concentrated cranberry juice made from the American cranberry, *Vaccinium macrocarpon*, and provided by Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA, USA. Briefly, NDM was obtained after lyophilization of the material retained in a dialysis bag (12,000 molecular weight cutoff) following extensive dialysis. The retentate of the bag designated NDM, is soluble in water up to 4 mg/mL, devoid of proteins, carbohydrates, and fatty acids, and was found to exhibit tannin-like properties suggests that it is rich in phenolic compounds (e.g., proanthocyanidins) [8]. Further analysis performed by Ocean Spray Inc. revealed that this fraction is devoid of sugars, acids, and nitrogen and contains 0.35% anthocyanins (0.055% cyanidin-3-galactoside, 0.003% cyanidin-3-glucoside, 0.069% cyanidin-3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside, and 0.086% peonidin-3-arabinoside) and 65.1% proanthocyanidins [9, 12].

2.2. Bacterial Strains and Culture Conditions. Streptococcus gordonii Challis (ATCC, Rockville, MD) was grown in trypticase soy broth (TSB; Difco Labs) for 48 hours at 37°C. Cells were washed in PBS and resuspended to contain 5×10^7 cells/mL.

2.3. XTT Metabolic Assay. Following the treatment of bacteria with NDM, *S. gordonii* cells were incubated with 50 μL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-{(phenylamino)carbonyl}-2H-tetrazolium hydroxide (XTT) for 4 hours at 37°C. In this assay, the tetrazolium salt XTT is cleaved to an orange-colored formazan product by mitochondrial dehydrogenase in viable cells [14]. At the end of incubation period, the absorbance of the resulting supernatant was measured at 490 nm using an ELISA reader (Bio Rad Laboratories). Prior to the measurement of number of cells in the biofilm, a standard curve was prepared with known numbers of bacteria.

2.4. Quantification of Streptococcal Metabolic Activity by XTT Assay and Biofilm Mass by Crystal Violet Staining. Following the treatment of bacteria with NDM, *S. gordonii* cells were incubated with 50 μL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-{(phenylamino)carbonyl}-2H-tetrazolium hydroxide (XTT) for 4 hours at 37°C. In this assay, the tetrazolium salt XTT is cleaved to an orange-colored formazan product by mitochondrial dehydrogenase in viable cells [14]. At the end of incubation period, the absorbance of the resulting supernatant was measured at 490 nm using an ELISA reader (Bio Rad Laboratories). Prior to the measurement of number of cells in the biofilm, a standard curve was prepared with known numbers of bacteria.

We also used the crystal violet staining method to assess the effect of NDM on the bacterial biofilm formation in a 96-well microtiter plate [15]. Adherent bacteria in the wells were fixed with methanol for 15 min, extensively washed with distilled water, and then stained with 0.4% crystal violet (100 μL) for 15 min. Wells were rinsed with distilled water and dried at 37°C for 2 h. After adding 100 μL of 95% (v/v) ethanol to each well, the plate was shaken to release the stain. The absorbance at 550 nm was recorded using a microplate reader. All assays were run in triplicate, and the means ± SD of three independent experiments were calculated.

2.5. Effect of Cranberry NDM on the Metabolic Activity of S. gordonii Growing in Culture Media Measured by XTT Assay. Freshly cultured bacterial suspension containing 5×10^7 cells/mL was prepared, from which 0.1 mL suspension was placed in each of a 96-well microtiter plate. An equal volume of serially diluted NDM (5.0 to 400 μg/mL) in phosphate-buffered saline (PBS; 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.4) was added to each well containing the bacteria and incubated for 24 hours at 37°C. After the incubation, unattached bacteria were washed off by rinsing the plate with PBS. The microtiter plate was replenished with fresh TSB media (0.2 mL) supplemented with 0.2% sucrose and incubated for an additional 24 hours. At the end of incubation, nonadherent cells were removed and the metabolic activity of the bacteria in the biofilm was assessed by XTT assay and by staining with crystal violet, as described above. For control purposes, 2.0 mL of standard bacterial suspension was immersed in a beaker of boiling water for 5 minutes and then treated with XTT reagent as the experimental cells.

2.6. Effect of Cranberry NDM Treatment of S. gordonii on Metabolic Activity of Biofilm Formed on Dental Composite, Amalgam, Hydroxyl Apatite, and Titanium Discs. ESPE composite material was molded into a 6 mm disc and cured for 10 seconds as recommended by the manufacturer. In a similar manner, amalgam discs (6 mm) were also prepared. Hydroxyl apatite discs were purchased from Berkley Advanced Biomaterials, Inc., Berkley, CA. Polished 6 mm titanium discs were supplied by Dr. Bumgardner, University of Memphis, TN. All discs were sterilized by nitrous oxide. An aliquot (0.5 mL) of standard bacterial suspension was incubated with cranberry NDM (5 to 200 μg/mL) for 60 min at 37°C. Bacteria were then added to the discs placed in a 24-well culture dish and further incubated for 24 hours at 37°C. After gentle washing to remove the nonadherent bacteria, discs were incubated with 0.5 mL of TSB supplemented with 0.2% sucrose for an additional 48 hours to facilitate biofilm formation. At the end of incubation, discs were rinsed once and then incubated with XTT reagent to assay for their metabolic activity [15].

2.7. Live/Dead BacLight Assay. Bacteria were stained using the Live/Dead *Bac*Light Kit (Molecular Probes-Invitrogen, Carlsbad, CA). This stain distinguishes live cells from dead bacteria based on membrane integrity and two nucleic acid stains. The green fluorochrome (SYTO 9) can penetrate intact membranes, while the larger red fluorochrome (propidium iodide) penetrates only compromised membranes of dead bacteria. The dye was prepared according to the manufacturer's specifications. Cells treated with NDM (25 μg/mL for 60 minutes) or not (control) were stained with the dye for 15 minutes in dark. Cells were mounted on a slide and evaluated by confocal microscope.

2.8. Flow Cytometry Analysis. Bacterial cells (5×10^6 cells) were pelleted, and the pellet was resuspended in 1.0 mL of NDM (25 μg/mL) and incubated for 60 min. The cells were stained with Live/Dead kit (Molecular Probes-Invitrogen, Carlsbad, CA) fluorescent dyes, calcein AM, and ethidium homodimer diluted according to the manufacturer's recommendation for 15 minutes in dark at room temperature. Similar numbers of bacteria were incubated with 70% isopropanol for 45 minutes to generate dead cells, which were also stained similarly (dead cell control). The effect of NDM on bacterial cells was analyzed by FACScan flow cytometry (Becton Dickinson) using 520 ± 20 nm excitation for measuring calcein green fluorescence emission and ethidium homodimer red fluorescence emission using 615 ± 30 nm. The data was processed with Flowmax software (Partec), and electronic gating with the software was used to separate positive signals from noise. Between 7,500 and 50,000 events were acquired using linear amplification for forward and side scatter and logarithmic amplification for fluorescence. Samples were measured in triplicate, and selected samples were controlled with epifluorescence microscopy to confirm the bacterial nature of stain.

2.9. Confocal Microscopy. Bacterial samples were stained with the Live/Dead *Bac*Light bacterial viability kit (Invitrogen L-13152), a rapid epifluorescence staining method as specified by the manufacturer. The bacteria were incubated for 15 minutes in dark and then examined for the difference in live and dead cells between NDM-treated and control *S. gordonii* by confocal microscope. Images were analyzed by using COMOS software (Bio Rad), and green and red images were merged and formatted on Confocal Assistant software (Bio Rad).

2.10. Statistical Analyses. Data are expressed as the means ± standard deviations of three independent experiments with triplicate samples in each experiment. Analyses of variance were performed to compare the means of the different conditions. Differences were considered significant at a P value of <0.05.

3. Results

3.1. Effect of Cranberry NDM on the Metabolic Activity of S. gordonii Grown in Culture Media. To investigate the effect of NDM on metabolic activity of *S. gordonii* in biofilm, bacteria were treated with serially diluted NDM and then measured their metabolic activity by XTT metabolic assay. Cranberry NDM inhibited *S. gordonii* metabolic activity in a dose-dependent manner (Table 1). A 50% reduction in bacterial metabolic activity was seen when treated with 7.5 μg/mL of NDM, while 50 μg/mL of NDM inhibited by 96%. Total inhibition in metabolic activity was seen with 100 μg/mL of NDM. Heat-killed bacteria did not show any metabolic activity (Table 1). Colony forming units of the NDM treatment of bacteria revealed that viability was not affected at any of the concentrations tested suggesting that the inhibition of metabolic activity was not related to NDM toxicity to bacteria (data not shown).

Table 1: Effect of cranberry NDM on the metabolic activity of *S. gordonii* growing in culture media measured by XTT assay.

NDM Concentration (μg/mL)	Metabolic activity
0	2.4 ± 0.3
2.5	1.73 ± 0.2
5.0	1.38 ± 0.11
7.5	1.19 ± 0.24
10	0.86 ± 0.12
20	0.65 ± 0.11
25	0.37 ± 0.1
50	0.23 ± 0.02
100	0
200	0
Heat-killed bacteria (control)	0

Bacterial biofilm prepared as described previously was treated with varying concentrations of NDM (2.5 μg/mL to 100 μg/mL) for 60 minutes and 4 hours. The metabolic activity was determined by XTT assay and the biofilm mass determined by the crystal violet staining.

3.2. Effect of Cranberry NDM Pretreatment of S. gordonii on the Bacterial Metabolic Function and Biofilm Formation. The results (Figure 1) show that the time of treatment and concentration of NDM affected the *S. gordonii* metabolic activity. Pretreatment of bacteria with 2.5 μg/mL of NDM for 15 minutes reduced bacterial metabolic activity as estimated by XTT assay by 20% of the bacteria when compared to the untreated bacteria (control). Bacteria treated with 25 μg/mL NDM showed metabolic reduction of 66% (Figure 1). Higher concentrations of NDM (100 μg/mL) completely abolished the metabolic activity (data not shown).

Contrary to the reduction in metabolic activity of *S. gordonii* by NDM treatment, there was no reduction in biofilm mass when stained with crystal violet (Figure 2). Biofilm mass was reduced by only 8% of the control when bacteria were treated with 100 μg/mL. There was no significant difference in biofilm mass between the groups treated with different concentrations of NDM. The data suggests that the cranberry NDM at relatively low concentrations does not appear to inhibit biofilm formation as estimated by the total mass but the metabolic activity of the formed biofilm was affected by NDM. In Figures 1 and 2, One mL aliquots of standard bacterial suspension in triplicate was incubated

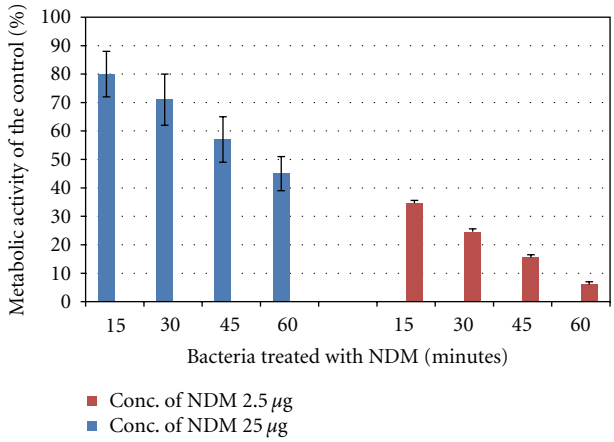


Figure 1: Effect of NDM treatment of bacteria on their metabolic activity determined by XTT assay.

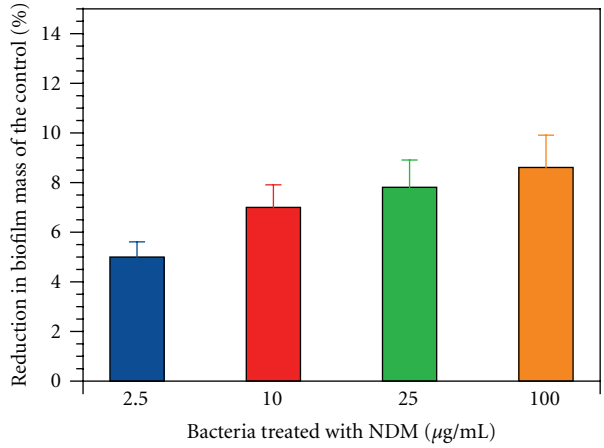


Figure 2: Effect of NDM treatment of bacteria on the biofilm mass as determined by crystal violet staining.

with varying concentrations of NDM (5.0 to 200 μg/mL) for 15, 30, 45, and 60 minutes. After the incubation, bacteria were centrifuged and the pellet was washed with PBS once and suspended in 0.2 mL of TSB culture media supplemented with 0.2% glucose for an additional 48 hours and measured their metabolic activity in the biofilm by XTT. Another parallel set of bacterial cells treated in a similar way was stained with crystal violet, and the optical density was measured at 550 nm. For control purposes, 1.0 mL of standard bacterial suspension was immersed in a beaker of boiling water for 5 minutes and then treated as above with XTT and crystal violet.

3.3. Effect of NDM on Preformed Bacterial Biofilm Formation. Next, we investigated the effect of cranberry NDM on preformed *S. gordonii* biofilm and assayed metabolic activity by XTT and biofilm mass by crystal violet method. The results (Table 2) demonstrate that the NDM failed to detach the bacteria from the preformed biofilm mass, even at a concentration of 3 mg/mL. On the other hand, it is of interest to note that the bacterial metabolic activity was reduced

Table 2: Effect of cranberry NDM on preformed *S. gordonii* biofilm.

NDM concentration (μg/mL)	Metabolic activity	Biofilm mass
0 (control)	2.21 ± 0.3	2.36 ± 0.41
2.5	2.14 ± 0.18	2.33 ± 0.33
5.0	2.24 ± 0.22	2.39 ± 0.27
7.5	2.06 ± 0.4	2.35 ± 0.44
10	1.96 ± 0.33	2.41 ± 0.31
20	1.84 ± 0.59	2.34 ± 0.41
25	1.97 ± 1.1	2.36 ± 0.29
50	1.86 ± 0.69	2.39 ± 0.3
100	1.08 ± 0.44	2.35 ± 0.28

Bacterial biofilm prepared as described previously was treated with varying concentrations of NDM (2.5 μg/mL to 100 μg/mL) for 60 minutes and 4 hours. The metabolic activity was determined by XTT assay and the biofilm mass determined by the crystal violet staining.

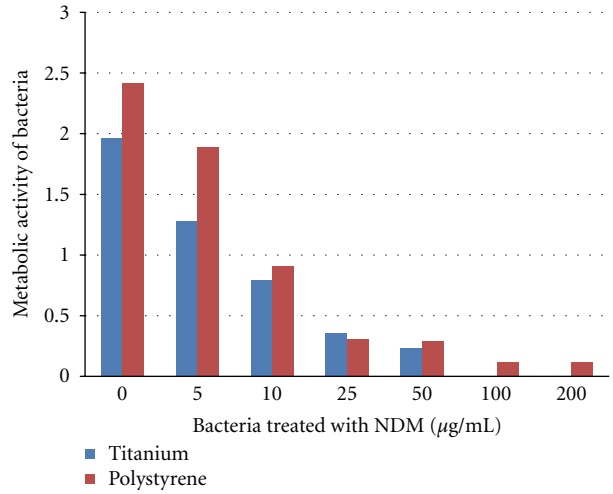


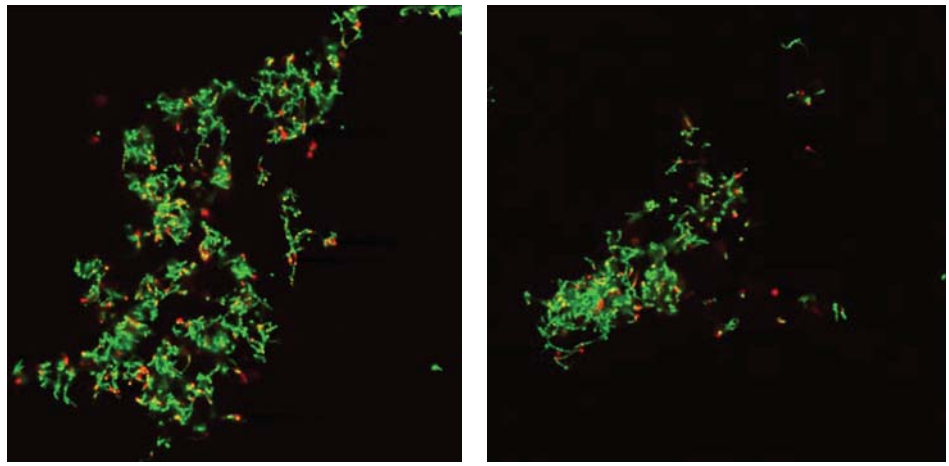
Figure 3: Effect of cranberry NDM treatment of *S. gordonii* on metabolic activity of biofilm formed on titanium and polystyrene surfaces. Bacteria treated with cranberry NDM for 4 hours were added to the titanium discs and in parallel to polystyrene 24-well culture dish. The metabolic activity of adherent bacteria was determined by XTT assay. Total biomass of biofilm bacteria was not significantly affected by none of the indicated NDM concentrations as determined by crystal violet assay (data not shown).

by NDM treatment (Table 2). NDM at a concentration of 100 μg/mL for 60 minutes reduced the metabolic activity by 50%, but the same concentration had no effect on biofilm mass. Increasing the length of treatment of preformed biofilm up to 8 hours also showed similar results (data not shown).

3.4. Metabolic Activity and Total Mass of S. gordonii Biofilm Formed on Cranberry NDM-Coated Titanium and Polystyrene Surfaces. The results (Figure 3) demonstrate that the NDM treatment of *S. gordonii* showed a reduction in metabolic activity of bacteria adherent to both titanium and polystyrene surfaces. Greater than 50% reduction in metabolic activity was seen with 10 μg/mL concentration of NDM. Higher concentration of NDM (>25 μg/mL) reduced the metabolic

Table 3: Inhibition of *S. gordonii* metabolic activity measured by XTT assay in biofilm formed on dental restorative surfaces by NDM.

NDM Conc (μg/mL)	Metabolic activity of biofilm cells on		
	Amalgam discs	Composite discs	HA discs
Zero	2.66 ± 0.44	2.51 ± 0.41	2.37 ± 0.22
10	1.84 ± 0.36	1.97 ± 0.31	2.01 ± 0.26
25	1.33 ± 0.12	1.52 ± 0.24	1.79 ± 0.19
50	0.94 ± 0.08	1.14 ± 0.18	1.21 ± 0.15
75	0.42 ± 0.05	0.56 ± 0.11	0.74 ± 0.13
100	0.0	0.0	0.0



(a) Bacteria treated with NDM (b) Control bacteria

Figure 4: Confocal microscopic analysis of *S. gordonii* biofilm stained with Live/Dead *Bac*Light.

activity of the adherent bacteria by greater than 98%. In contrast, NDM at the same concentrations reduced total bacterial mass by only 5–8 percent, when stained with crystal violet (data not shown), consistent with the data shown in Figure 2.

3.5. Inhibition of S. gordonii Metabolic Activity in Biofilm Formed on Dental Restorative Surfaces by NDM. We tested the NDM effect on metabolic activity of biofilm created on amalgam, composite, and hydroxyl apatite discs by XTT assay as described previously [14].

The results (Table 3) of the study showed that NDM treatment inhibited *S. gordonii* biofilm metabolic activity on the two dental restorative materials as well as on polystyrene surface. 25 μg/mL of NDM inhibited approximately 50% of bacterial metabolic activity on all three discs. No measurable inhibition of metabolic activity was seen when bacteria were treated with NDM concentrations greater than 100 μg/mL. In contrast, the total bacterial mass of the biofilm bacteria was reduced by only 5–8% as determined by crystal violet staining (data not shown) consistent with the data shown earlier.

3.6. Analysis of NDM-Treated S. gordonii Biofilm by Live/Dead BacLight Staining, Confocal Microscopy, and Flow Cytometry. The data obtained in this study so far suggested that the cranberry NDM inhibited the metabolic function of *S. gordonii* without being bactericidal. In order to confirm this observation, we stained the biofilm of *S. gordonii* created on polystyrene with Live/Dead *Bac*Light according to the recommended protocol by the manufacturer (Invitrogen). Stained bacteria were first viewed by a fluorescent microscope and then by confocal microscope. Figure 4 shows that NDM treatment did not appear to cause cell death, and both NDM treated (Figure 4(a)) and untreated bacteria (Figure 4(b)) appear to contain similar proportion of live and dead bacteria. This observation confirms that cranberry NDM is not bactericidal for *S. sangius*.

Further analysis of the live and dead cells was performed by flow cytometry. The results of flow cytometry (Figures 5(a) and 5(b)) indicate that NDM treatment did not change in the ratio of live to dead bacteria consistent with our microscopic observation that the NDM has no cytotoxic effect on *S. gordonii* cells.

4. Discussion

Early colonization on the tooth surface and subsequent biofilm formation by *S. gordonii* and their ability to coaggregate with several oral microorganisms result in the formation of dental plaque. The plaque and biofilm formation leads to caries and subsequently leads to periodontal disease if left untreated. Constituents of cranberry were demonstrated to decrease the hydrophobicity of streptococcal species [12, 16]. Cranberry high-molecular-weight component was shown to inhibit secretion of glucosyl and fructosyltransferases by oral

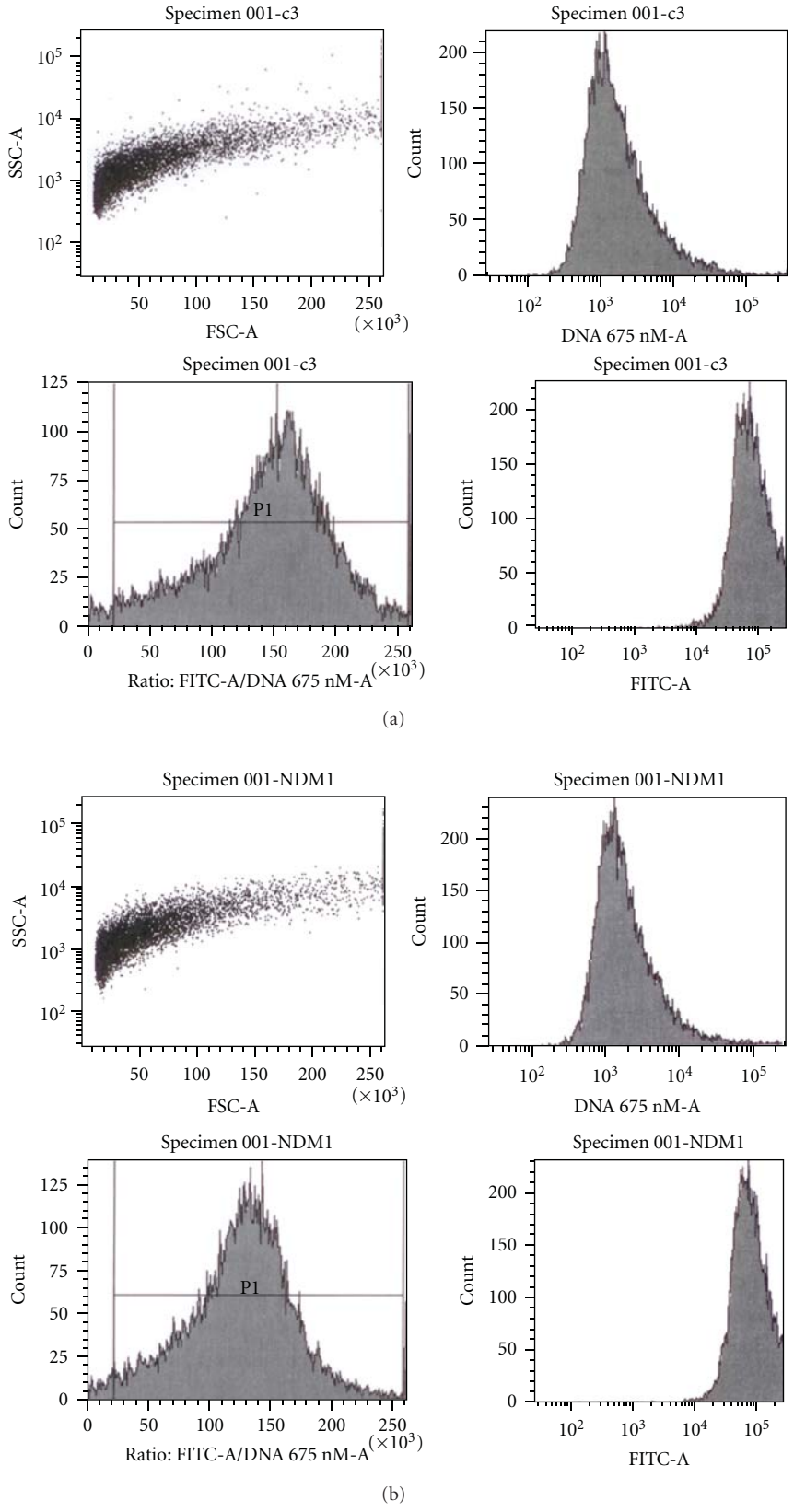


Figure 5: (a) *S. gordonii* (control) stained with Live/Dead *Bac*Light and analyzed by flow cytometry. (b) *S. gordonii* (NDM treated) stained with Live/Dead *Bac*Light and analyzed by flow cytometry.

streptococci [12] and coaggregation of bacteria [12]. The high-molecular-weight component of cranberry was shown to be highly soluble in water, lacking proteins, carbohydrates, and fatty acids [17, 18].

Previous studies have shown that relatively high concentrations of NDM (e.g., 0.5–2 mg/mL) were required to cause 80% or more reduction of biofilm formation by *S. mutans* on saliva-coated HA [16] and by *P. gingivalis* on polystyrene surfaces [7]. In the present study, we show that lower concentrations of NDM (0.05–0.1 mg/mL) selectively inhibited the metabolic activity of oral bacteria, *S. gordonii*. This conclusion is based on (i) the effect of NDM on metabolic activity of preformed biofilm that was more profound than on the total biofilm mass as measured by crystal violet stain and (ii) the confocal microscopy stain with Live/Dead stain showing no effect on the ratio of viable/dead *S. sangius*. The effect of NDM on metabolic activity may indirectly interfere with the ability of the bacteria to adhere and form biofilm onto various dental surfaces as shown in the present study and in other studies [7, 12, 16]. Previous studies have shown that NDM affects various physicochemical properties of uropathogenic bacteria [4, 9]. The present study shows an effect on metabolic activity, and further studies are needed to see how these two effects are connected. Perhaps most important is to study how these effects of relatively low concentrations of NDM on metabolic activity of *S. sangius* biofilm influences cariogenicity of the bacteria such as acid formation. Either way, NDM seems to affect *S. sangius* adhesion and biofilm formation mainly by inhibiting metabolic activity of the cariogenic bacteria. It is expected that supplementing oral health product such as mouth wash with NDM will affect not only *S. mutans* total counts as shown previously [18] but also *S. sangius* cariogenic activity.

Our study revealed the beneficial role of cranberry NDM in reducing the *S. gordonii* metabolic activity in the biofilm created on various dental surfaces such as titanium implant material, amalgam, and composite materials. The potential use of cranberry NDM in oral rinse merits further investigation, since it appears to benefit the health of the oral cavity, by reducing the metabolic activity of *S. gordonii*.

Acknowledgments

The research was partially supported by Cranberry Institute of America and The University of Tennessee College of Dentistry Alumni Research Foundation.

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 274578, 9 pages
doi:10.1155/2011/274578

Research Article

Plant and Fungal Food Components with Potential Activity on the Development of Microbial Oral Diseases

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Received 15 June 2011; Accepted 19 July 2011

Academic Editor: Carla Pruzzo

This paper reports the content in macronutrients, free sugars, polyphenols, and inorganic ions, known to exert any positive or negative action on microbial oral disease such as caries and gingivitis, of seven food/beverages (red chicory, mushroom, raspberry, green and black tea, cranberry juice, dark beer). Tea leaves resulted the richest material in all the detected ions, anyway tea beverages resulted the richest just in fluoride. The highest content in zinc was in chicory, raspberry and mushroom. Raspberry is the richest food in strontium and boron, beer in selenium, raspberry and mushroom in copper. Beer, cranberry juice and, especially green and black tea are very rich in polyphenols, confirming these beverages as important sources of such healthy substances. The fractionation, carried out on the basis of the molecular mass (MM), of the water soluble components occurring in raspberry, chicory, and mushroom extracts (which in microbiological assays revealed the highest potential action against oral pathogens), showed that both the high and low MM fractions are active, with the low MM fractions displaying the highest potential action for all the fractionated extracts. Our findings show that more compounds that can play a different active role occur in these foods.

1. Introduction

During the last decades, a close relation between diet and health have been pointed out by the findings of a large number of epidemiologic investigations. Due to these findings, today, foods are no more considered just for their nutritive value, but also for their potential positive effects in preventing and protecting against serious chronic diseases with strong socioeconomic implications in Western countries such as neoplastic, cardiovascular, neurodegenerative diseases, cataracts, diabetes, metabolic syndrome, inflammatory process, and aging. As regards cancer, for example, it is estimated that its incidence could be reduced by at least 30% adequately increasing diet vegetables and fruits.

The foods producing peculiar beneficial effects on human health are generally defined as functional foods. Up to date, there is not a generally accepted definition for functional foods. The USA and UE have no legal definition of a functional food that in these countries is strictly a marketing term, even if UE recognizes for some foods specific health claims [1]. Japan is the only country that has an established regulatory framework for functional food marketing, which dates back to the 1980s. Some organizations, such as the International Food Information Council (IFIC, founded in 1991), have attempted to establish a definition. The IFIC regards them as "foods that provide a health benefit beyond basic nutrition." The Institute of Medicine's Food and Nutrition Board (IOM/FNB, founded in 1970) defined

functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains." After this definition, foods, technological-treated food products, and their active components that can be used to prepare enriched and fortified foods or be assumed separately from foods as supplements may be considered as functional foods.

In its latest position paper, the American Dietetic Association (ADA, founded in 1917) defines a functional food as one that provides a "beneficial effect on health when consumed as part of a varied diet on a regular basis at effective levels." The organization classified functional foods into four groups: conventional foods, modified foods, medical foods, and foods for special dietary use, and called for more research into their potential health benefits.

Moreover, most of the researchers consider there is a clear difference between dietary supplements, or nutraceuticals, and functional foods. The former, which include vitamins, minerals, other substances with physiological effects, and botanicals, are taken in a dose form [2]. Anyway, other researchers consider that a nutraceutical is any food that giving nutriment helps to maintain health [3].

Among chronic diseases whose development can be influenced by the consumption of specific foods, also oral diseases such as caries and gingivitis, that are the most common and diffused infectious diseases in the world, should be counted. A consortium of microorganisms, among which *Streptococcus mutans* and *Streptococcus sobrinus* are considered the most influent, is involved in the development of such pathologies. It is well known that oral pathogens virulence can be strengthened or conversely inhibited by dietary factors. For a long time, the negative role of diet sucrose in inducing caries formation has been recognized. Recently, the emergence of pathogen resistance to conventional antibacterial agents and the need to develop new strategies for the control of infectious diseases made active the research about natural compounds able to act as antimicrobial agents. Such research led to the findings that compounds, able to act with different mechanisms, against the main infective responsible agents for oral diseases, occur in a lot of vegetable and fungal foods. First, the different kinds of tea in *in vitro* and *in vivo* both in animal and humans were studied for their protective action against oral pathologies above all by Japanese researchers. Tea polyphenols were shown to be able to inhibit caries development reducing *S. mutans* cell surface hydrophobicity and its capability to produce, starting from sucrose, the insoluble, bioadhesive polymer glucan that allows the dental plaque formation [4–8]. Then coffee and cocoa were studied. Our previous *in vitro* investigation pointed out since 1994 that coffee beverage possesses a wide spectrum antibacterial activity. Such activity was found to be relevant against a number of Gram-positive and Gram-negative microorganisms including *Streptococcus mutans* and other pathogens such as *Staphylococcus aureus* and *Escherichia coli* [9–11]. Later, it was found that also white and red wine, and barley coffee possess antimicrobial activity. Coffee components able to act against oral pathogens, in the experimental-used model system, were found to be the α-dicarbonyl compounds formed during roasting process (green coffee did not show any antibacterial activity). Interestingly, α-dicarbonyl compound activity resulted to be strongly enhanced in the presence of caffeine that alone showed no activity in the same system [12]. As regards wine, most of the antibacterial activity was due to the presence of the low-molecular organic acids naturally occurring in grape or formed during malolactic fermentation process [13].

These results, indicating a potential positive action of coffee and wine in protecting oral health due to the presence of compounds able to inhibit dangerous microorganism proliferation, prompted us to investigate the same beverages for more specific actions that have the capability to inhibit pathogen adhesion to and to induce pathogen detachment from hydroxyapatite (HA) beads in *in vitro* tests. Chlorogenic acid, trigonelline and nicotinic acid, and also high-molecular-mass melanoidin components were identified as antiadhesive compounds in coffee, whereas as regards red wine, a fraction containing anthocyanins and proanthocyanidins showed the highest activity [14]. Considering barley coffee, it was found that very high-molecular-mass brown melanoidinic components were able to remarkably inhibit *S. mutans* adhesion to and induce detachment from HA and to inhibit biofilm production [15, 16].

As regards cocoa, a number of papers reported anticariogenic effect of water soluble components and in particular of polyphenols [17–20].

Among the most studied foods in this field also propolis had to be cited [21]. Recently, a potential use of propolis as a cariostatic agent was reported [22].

The promising findings of research in this field prompted the UE to fund a systematic program of research about the ability of food/beverage constituents to protect against oral infectious diseases, that is, caries and gingivitis.

The first basic step in this contest was the selection of the food/beverages to be investigated. Due to the number of literature papers reporting polyphenols as antimicrobial and antiadhesive agents also able to interfere with biofilm and glycosyl-transferases production, plant and fungal edible materials were decided to be useful for our purposes. So, already studied materials such as green and black tea leaves, mushroom, and cranberry were selected for a systematic investigation as well as raspberry, red chicory, and beer never previously studied in relation to a potential action in protecting oral cavity from infection diseases.

The selected food/beverages had to be analysed to state their content of macronutrients, that is, protein, lipid, and total carbohydrates. Also free sugars, micronutrients such as inorganic ions that were found to exert any positive or negative influence on oral health, and the total polyphenol content of the selected food/beverages were evaluated. As the selected materials had to be tested in biological assays, useful solutions of their water soluble components had to be prepared (extracts) and their microbiological quality to be defined. Furthermore, the fractionation of the most active extracts (red chicory, mushroom, and raspberry) was performed on the basis of their molecular mass as a first step to isolate the active compound/s.

2. Materials and Methods

2.1. Chemicals and Reagents. Concentrated sulphuric acid (97%), sodium hydroxide 1 N, petroleum ether 40–70°C, mixed indicator solution (Methyl Red-Methylene Blue) were purchased from Carlo Erba Reagents (Milan, Italy).

2.2. Materials. Frozen mushrooms (shiitake, *Lentinus edodes*) were purchased from Bolem (Gorizia, Italy) and Asiago Food SpA (Veggiano, Italy). IGP "Rosso Tardivo of Treviso" red chicory (*Cichorium intybus* L., variety *Silvestre* (Bishoff) typology Tardivo) was purchased from Italian Consorzio Radicchio di Treviso (Treviso, Italy). Raspberries were purchased from Agrifrutta Soc. Coop. Agr. (Peveragno, Italy). Green and black tea (*Camellia sinensis*) dry leaves were purchased from an Italian tea importer (Berardi & C. S.n.c., Milan, Italy). Dark beer (Guinnes draught, 4.2% alcohol) was purchased from a local supermarket. Cranberry juice was acquired as concentrated juice from an Italian importer (Natex International trade spa, Pioltello, Italy) of Ocean Spray Cranberries Inc. (Lakesville-Middle-boro).

2.3. Extract Preparation. Aliquots of fresh red chicory (500 g), of frozen mushroom, and of frozen raspberries (400 g) were homogenized (for 1, 2, and 1 min, resp.) and centrifuged (for 10 min at 8000 rpm), and the juices, after separation from solid parts, were filtered on paper filter, and then submitted to sterile ultrafiltration, with the exception of raspberry juice. Green and black tea infusions were prepared from a suspension containing 30 g of dry leaves in 600 mL of water Millipore grade; after 5 minutes of infusion, the extracts were cooled at 20°C, filtered on paper filter, and then subjected to sterile ultrafiltration. Concentrated cranberry juice (5.6X) supplied by the importer was simply diluted before the analysis. Aliquots (325 mL) of Guinnes beer were submitted to elimination of CO_2 [23] and dealcoholated (bath temperature: 50°C, vacuum: 30 bar for 20 minutes).

2.4. Moisture Content Determination. Moisture was determined following the official method of analysis of AOAC International [24]. Values are means of four independent experiments.

2.5. Protein Content Determination. Kjeldahl method, the standard method of nitrogen determination, was used following the official method of analysis of AOAC International [25, 26]. Values are means of four independent experiments.

2.6. Lipid Content Determination. The Soxhlet method, as described in the official method of analysis of AOAC International [27], was applied. Values are means of four independent experiments.

2.7. Free Sugars Determination. D-glucose, D-fructose, and sucrose contents were determined using an enzymatic assay (Boehringer Mannheim, R-Biopharm, Italia srl, Cerro Al Lambro, Italy). D-glucose concentration was determined before and after the enzymatic sucrose hydrolysis, while D-fructose was determined subsequent to the determination of D-glucose. The other sugars (arabinose, galactose, mannose, rhamnose, ribose, xilose, and maltose) were revealed by thin-layer chromatography following the method of Talukder [28]. Values are means of four independent experiments.

2.8. Mineral-Content Determination. An ICP-OES Perkin Elmer Optima 3300 DV was used for the measurements of metal ions at concentration greater than 5 μg/L, and, for more diluted samples, inductively coupled plasma-mass spectrometer (ICP-MS) measurements were carried out on a Perkin Elmer Mod. ELAN DRC-e instrument. In both cases, the standard procedures suggested by the apparatus manufacturers have been followed. Linearity range between the intensity and concentration for each metal ion was obtained using standard solutions daily prepared from a 1.00 mg/mL stock solution. LODs were calculated as the amount of metal ion that gives a signal that is 3σ of the mean blank signal and LOQ as the amount of metal ion which gives a signal that is 10σ above the mean blank signal. Accuracy was checked by spikes recovery.

Fluoride ion concentration was measured by fluoride ISE on an Orion 520 potentiometer, with the standard additions method. Values are means of four independent experiments.

2.9. Total Polyphenol Content Determination. Total polyphenol contents were determined with the Folin-Ciocalteu reagent. In brief, 500 μL of Folin-Ciocalteu reagent was added to 100 μL of each extract, mixed, and added with 2000 μL of a 15% Na_2CO_3 solution and Millipore grade water to a 5 mL final volume. After mixing and waiting for 2 h, the mixtures were read spectrophotometrically at 750 nm. (±) Catechin was used as the phenolic standard compound [29]. Values are means of four independent experiments.

2.10. Microbiological Quality Control of Food/Beverage and Extracts [30, 31]. The microbiological quality controls were done through qualitative and quantitative analysis to determine the following indicator organisms:

(1) Total viable count (psychrophilic/mesophilic/bacteria) through plate count with Tryptone Soya Agar (Oxoid Ltd., Basingstoke, Hampshire, UK);

(2) Yeast and mould plate count with Malt Extract Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) and Potato Dextrose Agar (PDA) (Oxoid Ltd., Basingstoke, Hampshire, UK);

(3) Enteric indicator bacteria through plate count: determination of *Escherichia coli* with Violet Red Bile Glucose Agar (Oxoid Ltd., Basingstoke, Hampshire, UK), *Salmonella* spp with XLD Medium (Oxoid Ltd., Basingstoke, Hampshire, UK), *Streptococcus faecalis* with Slanetz and Bartley Medium (Oxoid Ltd., Basingstoke, Hampshire, UK), Sulfite reducing Clostridium spores with SPS Agar (Oxoid Ltd., Basingstoke, Hampshire, UK);

(4) Environmental indicator through plate count: determination of *Pseudomonas* spp with *Pseudomonas* Agar Base (Oxford) (Oxoid Ltd., Basingstoke, Hampshire, UK);

(5) Antrophic indicator bacteria through plate count: determination of coagulase-positive Staphylococci (*Staphylococcus aureus*), determination of coagulase-negative Staphylococci (*Staphylococcus epidermidis*) with Baird Parker Agar Base (Oxoid Ltd., Basingstoke, Hampshire, UK), and Egg Yolk Tellurite Emulsion (Oxoid Ltd., Basingstoke, Hampshire, UK);

(6) Animal indicator bacteria through plate count: determination of *Listeria monocytogenes* with *Lysteria* selective Agar Base (Oxford) (Oxoid Ltd., Basingstoke, Hampshire, UK) Lysteria selective supplement (SR 140 E) (Oxford) (Oxoid Ltd., Basingstoke, Hampshire, UK).

The possible presence of pathogen microorganisms in foods was confirmed with biochemical identification systems.

2.11. Extract Fractionation. The chicory and mushroom extracts fractionation was performed using Vivaflow 200 complete system (Vivascience) equipped with 5,000 MWCO PES membrane. The diafiltrate (MM < 5,000 Da-LMM) and the retentate (MM > 5,000 Da-HMM) fractions obtained from each extract aliquot of 250 mL, after restoring the initial volume, were submitted to sterile ultrafiltration, freeze-dried, and tested.

Raspberry extract was fractionated into low- and high-molecular-mass fractions by dialysis. Dialysis was performed using a Spectra/Por Biotech regenerated cellulose membrane (Spectrum Europe B.V., Breda, The Netherlands) with a molecular mass cutoff (MMCO) of 3,500 Da. Aliquots (60 mL) of raspberry extract were fractionated by dialysis in 5600 mL of Millipore grade water for 24 h at 4°C. The pH values (pH 3.20) of LMM and HMM fractions, reconstituted to the initial volume (60 mL), were brought to pH 4.5–5.0 (using 1.0 M NaOH) not to interfere with subsequent assays of their biological activities. Then the fractions were sterilized using ultrafiltration 0.20 μm membrane and freeze-dried.

3. Results and Discussion

In the selected raw food/beverages (red chicory, mushroom, raspberry, green and black tea, cranberry juice, and dark beer), proteins, lipids, total carbohydrates, and sugars such as glucose, fructose, and sucrose were quantitatively determined, whereas the presence of other monomeric or dimeric sugars were just pointed out. Among mineral components, the generally considered as positively influencing oral health ions such as fluoride, zinc, strontium, molybdenum, boron, and lithium and the generally indicated as negatively acting selenium, beryllium, copper, and lead were detected. The total content of polyphenols, compounds often indicated as able to interfere with different steps in caries development process, was also determined in the seven selected raw materials.

In Table 1, the content of water, macronutrients, and ash of the selected food/beverages was reported.

Carbohydrates are the most abundant macronutrients in all the selected food/beverages. Green and black tea show values higher than 60%, cranberry about 50%, and all the other materials less than 15%.

Carbohydrates are important as regards caries development when they are fermentable by oral pathogens that, embedded in dental plaque, can continuously produce organic acids (mainly lactic acid) able to demineralise the protective tooth-calcified tissues.

Among fermentable sugars, sucrose is considered as the most cariogenic sugar because the capability of microorganisms to rapidly metabolise sucrose producing both organic acids and extracellular bioadhesive polysaccharides is a relevant oral pathogen virulence trait.

As regards sugars, glucose, fructose, and sucrose determination was performed in all the food/beverages applying specific enzymatic methods (Table 2).

Black tea and beer were found not to contain any of such sugars probably because they are metabolized during fermentation process they undergo. All the other selected materials contain these sugars in very small amount (less than 1%), with the exception of cranberry in which glucose percentage is close to 3%. Thin-layer chromatographic analysis showed the presence of other monomeric and dimeric sugars as reported in Table 3.

Lipid content is very low in all the selected food/beverages with beer showing not to contain lipids at all (Table 1).

The richest in protein materials are green and black tea leaves. Due to the treatment tea leaves undergo, their moisture content is low (about 8%), determining the increase of the other component percentages; therefore, tea leaves protein content reaches remarkable values similar to that of animal foods or other dry plant foods such as dry legumes. All the other materials are very low in protein and very high in water content including raspberry, mushroom, and red chicory, although they are solid materials. It is known that fresh vegetables and fruits have a high content of water and a high activity water value so that their shelf life is quite short even if stored at refrigerate temperature because free water is abundant and available for microorganisms proliferation (Table 1).

So it is not surprising that red chicory and raspberry presented a marked microbial contamination (Table 4). In particular, red chicory presented the highest contamination values for total microbial counts (mesophilic, psychrophilic, and yeasts) and for specific indicators of contamination such as Enteric indicator (*Escherichia coli* 740 cfu/mL) and Environmental indicator (*Pseudomonas* spp 1000 cfu/mL), whereas any food showed important microbial indicators of antrophic or animal contamination such as *Staphylococcus aureus*, *Salmonella* spp, and *Listeria monocytogenes*.

Considering the elements (Table 5), green and black tea are very rich in fluoride (F) ions in comparison with the other food/beverages (about 40 ppm). F exerts a very important action in protecting from dental caries. When dental structure is forming, F ions are able to replace hydroxyl group in HA giving fluoroapatite a very protective material that more than HA resists acid attack that determines enamel demineralisation. Protective F action continues even when tooth is formed because this anion promotes enamel

Table 1: Water, macronutrients, and ash content of the selected food/beverages (g/100 g edible material).

Edible material	Red chicory	Mushroom	Raspberry	Green tea	Black tea	Cranberry	Beer
Water[(1)]	94.00	89.00	84.50	7.70	8.00	49.20	92.50
Protein[(1)]	1.85	1.43	0.84	22.86	25.11	0.36	0.33
Lipid[(1)]	0.20	1.56	0.20	1.46	0.88	0.25	0.00
Total carbohydrates[(2)]	3.35	7.99	14.90	63.95	61.01	48.99	3.00
Ash[(1)]	1.00	1.00	0.50	4.30	4.60	0.80	0.20

[(1)] Standard deviation less than 2%.
[(2)] Total carbohydrates content of food/beverages was calculated by difference, rather than analysed directly.

Table 2: Sucrose, glucose, and fructose content of the selected food/beverages (g/100 g edible material).

Edible material	Red chicory	Mushroom	Raspberry	Green tea	Black tea	Cranberry	Beer
Glucose[(1)]	0.86	0.12	0.63	0.54	trace	3.07	trace
Fructose[(1)]	0.70	trace	0.71	trace	0.00	0.78	trace
Sucrose[(1)]	trace	0.02	0.24	0.94	0.00	0.21	trace

[(1)] Standard deviation less than 3%.

Table 3: Monomeric and dimeric sugars presence in the selected food/beverages.

	Red chicory	Mushroom	Raspberry	Green tea	Black tea	Cranberry	Beer
Mannose	X	—	X	X	X	X	—
Rhamnose	—	—	—	X	—	X	—
Xilose	—	—	X	—	—	—	—
Maltose	X	—	—	X	—	—	X

X: present.
—: absent.

Table 4: Microbial contamination of selected food/beverages.

Food/beverage	Mesophilic bacteria (cfu/mL)	Psychrophilic bacteria (cfu/mL)	Moulds (cfu/mL)	Enteric indicator Bacteria (cfu/mL)	Environmental indicator bacteria (cfu/mL)	Other microbial indicators (cfu/mL)
Red chicory	2500	3560	1400	740	1000	<1
Mushroom	4	8	2	<1	<1	<1
Raspberry	400	570	160	<1	<1	<1
Green tea	2	20	10	<1	<1	<1
Black tea	4	600	52	<1	<1	<1
Cranberry	<1	<1	<1	<1	<1	<1
Beer	1	3	<1	<1	<1	<1

Table 5: Food/beverage mineral contents, expressed in ppm.

Food	F	Zn	Sr	Mo	B	Li	Se	Be	Cu	Pb
Red chicory	0.030	1.040	0.020	<0.003	0.080	<0.003	<0.001	<0.003	0.020	<0.003
Mushroom	0.010	1.030	0.150	<0.003	0.380	<0.003	<0.002	0.003	0.170	<0.003
Raspberry	<0.020	0.760	0.150	<0.002	0.025	<0.002	<0.001	<0.002	0.120	<0.002
Green tea	42.000	9.500	3.400	<0.100	4.200	<0.100	<0.060	<0.100	2.600	<0.100
Black tea	44.000	8.800	1.200	<0.100	2.640	<0.100	<0.060	<0.100	0.880	<0.100
Cranberry	<0.500	0.060	0.020	<0.010	0.050	<0.010	<0.003	<0.005	0.023	<0.005
Beer	0.160	0.040	0.060	<0.010	0.030	<0.010	<0.006	<0.005	<0.005	<0.005

Table 6: Amount of raw food needed to obtain 100 mL of extracts, the extract pH values, sugars, and total polyphenols content of the extracts.

Extract	g raw material/100 mL extract	pH value	Sucrose[(1)]	Glucose[(1)] g/100 mL	Fructose[(1)]	Total polyphenol content (mg/100 mL)
Red chicory	177 ± 2.10	6.05 ± 0.04	Trace	1.52	1.22	75.00 ± 4.16
Mushroom	140 ± 1.60	5.79 ± 0.05	0.03	0.18	trace	53.00 ± 2.27
Raspberry	224 ± 2.40	3.21 ± 0.02	0.55	1.40	1.58	107.00 ± 7.48
Green tea	5.00 ± 0.02	4.05 ± 0.02	0.05	0.03	Trace	289.00 ± 9.08
Black tea	5.00 ± 0.02	4.53 ± 0.02	0.00	Trace	0.00	272.00 ± 6.69
Cranberry		2.90 ± 0.01	0.22	3.07	0.78	207.00 ± 7.00
Beer		3.48 ± 0.02	Trace	Trace	Trace	153.00 ± 6.40

[(1)] Standard deviation less than 3%.

remineralisation and inhibits oral pathogen proliferation so helping in preventing caries lesion. It is well known that rich in F tooth-paste daily used acts consistently reducing caries incidence.

Also as regards zinc (Zn) and strontium (Sr), a positive effect is considered to be clearly demonstrated. Zn cations, as other heavy metal cations, possess antimicrobial activity and, due to this, Zn is commonly added in the products prepared for oral care, such as tooth-pastes and mouthwashes.

Because of its chemical similarity to calcium (Ca), Sr can replace Ca in HA crystals of teeth and bone imparting additional strength to these tissues. It was found that when rats are fed increased amounts of Sr, their teeth became thicker and stronger. In a 10-year study, carried out by the United States Navy Dental Service, a very low incidence of caries was registered among naval recruits coming from a small area around Rossburg, Ohio, where the water contains unusually high concentrations of Sr [32]. Again, epidemiologic studies have shown that Sr concentrations of 6 to 10 mg/L in the water supply are associated with a reduced incidence of cavities. The results of the epidemiologic investigations were confirmed by administering these levels of strontium in experimental animal studies [33].

Other trace elements in food and water have now been linked with dental caries [34]. Boron (B), vanadium (Va), and, above all, molybdenum (Mo) have been associated with reduced caries prevalence, whereas selenium (Se) and lead (Pb) appear to have adverse effects.

To be submitted to the biological screening tests, the raw selected materials required the sample preparation.

Whereas dark beer needed simply dealcoholation with restoring the volume and cranberry the dilution of the concentrated juice, the solid foods required the preparation of solutions useful for the biological assays. In this case, the water soluble components were obtained by homogenisation, centrifugation, and filtration of the raw materials as regards red chicory, mushroom, and raspberry; conversely, considering green and black tea, the leaves were treated as commonly done to prepare the infusion used as a beverage as described in Materials and Methods. In general, the solutions prepared to be submitted to the biological assays will be defined as extracts. In Table 6, the amounts of raw food needed to obtain 100 mL of extracts, extract pH values, content of sugars, and total polyphenols are reported.

The extracts were submitted to the same analysis as the raw foods/beverages and to sterile ultrafiltration that microbiological quality control showed to be efficient.

With respect to the raw materials, the extracts do not contain, but in traces, proteins and lipids, and, considering carbohydrates, they contain just the water soluble sugars. With regards raspberry, red chicory, and mushroom, as expected, glucose, fructose, and sucrose resulted to occur in a slightly higher concentration than in the whole raw materials. This is due to the fact that the determination of sugars as well as mineral ions requires the separation of these soluble analytes from the insoluble constituents of the food matrices. The separation had to be carried out in the same way used to obtain the extracts in which the water soluble components are actually determined. On the contrary, when the extraction of such water soluble analytes is carried out by infusion in a large volume of water, the extracts obtained present a very lower concentration of such components in comparison with the raw materials, and this is the case of tea leaves. So it is apparent that the analyte concentrations reported for the raw materials are often the results of calculations carried out on the basis of the analyte amounts actually determined in the extracts (Figure 1).

In general, this investigation shows that, among the selected food/beverages, tea beverages are good sources of F, Zn, Sr, B, copper (Cu), and polyphenols. Raspberry can supply us with relevant amounts of Zn, Sr, B, and Cu, whereas red chicory contains good concentrations of B. Mushroom is very rich in F, Zn, Sr, B, and Cu. Beer is rich in F, Zn, and Se. All the selected food/beverages are rich in total polyphenols, with green and black tea beverages, cranberry juice, and beer containing the highest concentration of such components. The extracts obtained from each food/beverages were submitted to microbiological assays to determine their activity against oral pathogens and their virulence factors. The results of such tests indicated the best potential against infectious oral diseases for mushroom, red chicory, and raspberry [35, 36].

To obtain preliminary indications about the molecular mass of the compounds responsible for the detected

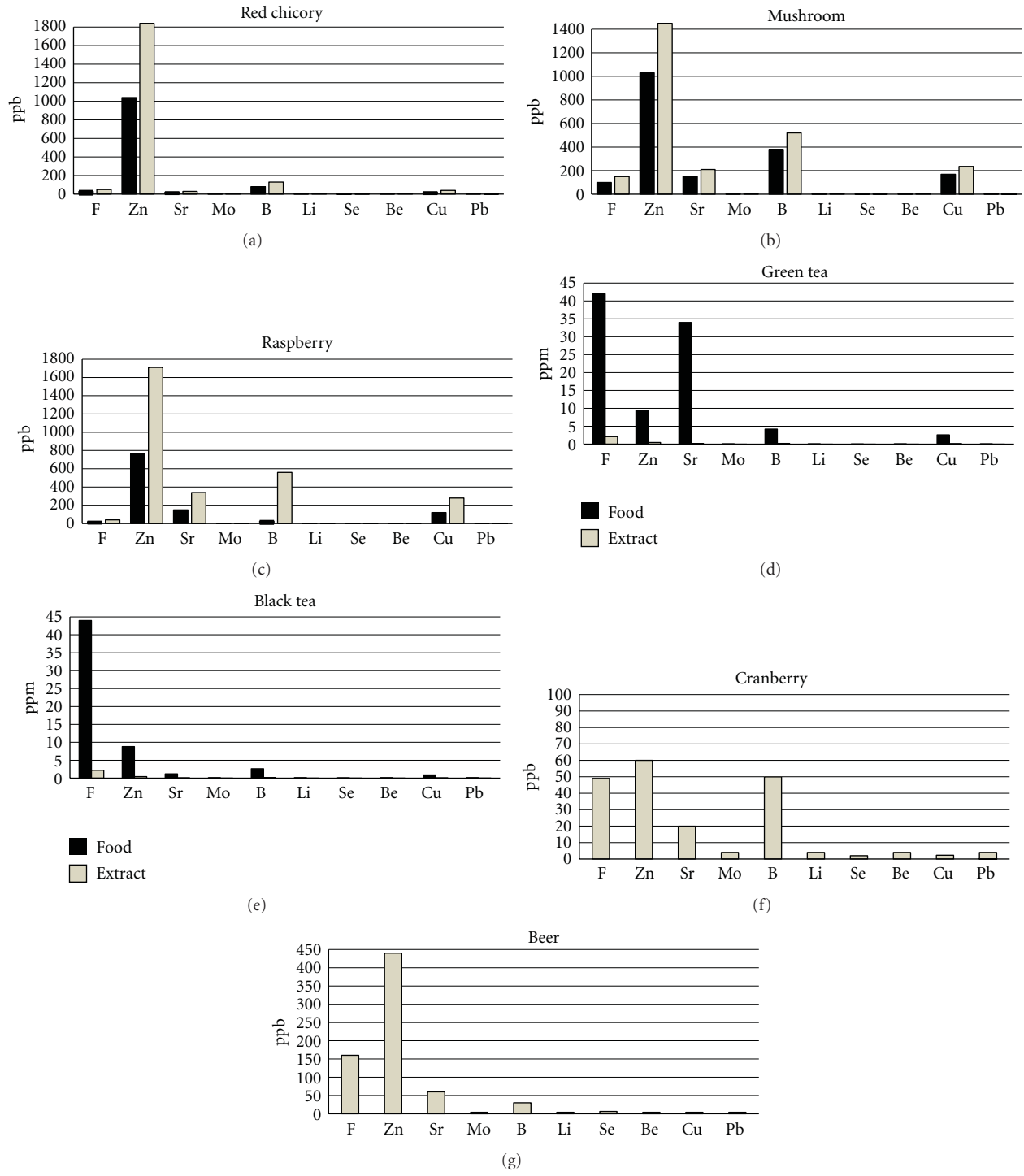

Figure 1: Mineral content of food/beverages and their extracts (espressed in ppm or ppb).

activities, raspberry extract was fractionated by dialysis due to its high viscosity and density, differently from chicory and mushroom extracts that were fractionated by ultradiafiltration. The dialysis procedure was carried out using dialysis membrane with a 3,500 Da cutoff, which allowed the separation of LMM components (mono- and disaccharides, organic acids, flavonoids and condensed and hydrolysable tannins with low degree of polymerization, and ion elements) from polymeric components, such as high degree of polymerization tannins, complex carbohydrates, and proteins. Due to the very acidic pH values of dialysis fractions (LMM fraction pH = 2.71 ± 0.12 and HMM fraction pH = 3.20 ± 0.01), which could interfere with subsequent microbiological assays, the pH value of both fractions was brought to pH 4.50. Then, dialysis fractions were sterilized using ultrafiltration and freeze-dried.

With regards to red chicory and mushroom, the extract fractionation into LMM and HMM fractions was performed using a 5,000 MWCO PES membrane. This procedure resulted in the loss of 50% and 33% diafiltrate components for red chicory and mushroom, respectively, which remained in the retentate; so the lost of such components had to be restored to obtain a diafiltrate fraction with the LMM components at the same concentration as in the raw extract. Conversely, the retentate had to be further treated to eliminate the residue LMM components. The right concentration of the LMM components for chicory in the diafiltrates was achieved submitting to diafiltration procedure a double volume of extract and a volume of extract added of 33% for mushroom. The retentate purification was performed submitting HMM fraction to dialysis process. Diafiltrate and retentate solutions (mushroom: LMM pH = 6.15 ± 0.11, HMM pH = 6.80 ± 0.13; red chicory: LMM pH = 5.90 ± 0.09, HMM pH = 6.55 ± 0.10) were sterilized using ultrafiltration 0.20 μm membrane and freeze-dried, and stored for microbiological assays.

4. Conclusions

In conclusion, the present investigation allows to know the macronutrient, mineral ions, and polyphenol content of seven food/beverages grown in defined area but exported to various countries around the world. While the macronutrient composition of such food/beverages is generally well known and tabulated as mean values, for most of these materials, very scarce literature data about their content in mineral micronutrient are available. Tea leaves resulted to be the raw materials richest in all the elements detected as mineral ions, anyway tea beverages resulted to be the richest just in fluoride ion among the selected food/beverages. The highest content in zinc ion was found in red chicory, raspberry, and mushroom. Raspberry is the richest food in strontium and boron, beer in selenium, and raspberry and mushroom in copper. As regards polyphenols, beer, cranberry juice, and, above all, green and black tea are very rich in these components confirming these beverages to be important sources of such healthy components. Among the selected food/beverage extracts, raspberry, red chicory, and mushroom extracts, considering, in the whole, the results of microbiological assays, showed the highest potential action against oral pathogens. The fractionation of the water soluble components occurring in these extracts, carried out on the basis of their molecular mass, showed that both the HMM and LMM obtained fractions of each food, present any activity in the microbiological tests indicating that more active compounds occur in these foods with the LMM fractions containing the components presenting the highest potential action against the oral pathogens.

Abbreviations

HA: Hydroxyapatite
ICP-MS: Inductively coupled plasma-mass spectrometer
LOD: Limit of detection
LOQ: Limit of quantification
MMCO: Molecular mass cutoff
LMM: Low molecular mass
HMM: High molecular mass.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 507908, 7 pages
doi:10.1155/2011/507908

Research Article

In Vitro Assessment of Shiitake Mushroom (*Lentinula edodes*) Extract for Its Antigingivitis Activity

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Received 14 June 2011; Accepted 12 July 2011

Academic Editor: Carla Pruzzo

Gingivitis is a preventable disease characterised by inflammation of the gums due to the buildup of a microbial biofilm at the gingival margin. It is implicated as a precursor to periodontitis, a much more serious problem which includes associated bone loss. Unfortunately, due to poor oral hygiene among the general population, gingivitis is prevalent and results in high treatment costs. Consequently, the option of treating gingivitis using functional foods, which promote oral health, is an attractive one. Medicinal mushrooms, including shiitake, have long been known for their immune system boosting as well as antimicrobial effects; however, they have not been employed in the treatment of oral disease. In the current study, the effectiveness of shiitake mushroom extract was compared to that of the active component in the leading gingivitis mouthwash, containing chlorhexidine, in an artificial mouth model (constant depth film fermenter). The total bacterial numbers as well as numbers of eight key taxa in the oral community were investigated over time using multiplex qPCR. The results indicated that shiitake mushroom extract lowered the numbers of some pathogenic taxa without affecting the taxa associated with health, unlike chlorhexidine which has a limited effect on all taxa.

1. Introduction

Gingivitis is one of the most prevalent infectious diseases of humans, affecting most of the population at some point during their lives [1]. It is easily preventable by the removal of the plaque biofilm but often results in high treatment costs due to poor oral hygiene among the general population. Gingivitis has long been implicated as a potential precursor to periodontitis [2, 3] and is caused by the buildup of the plaque biofilm at the gingival margin. This in turn results in a shift in the resident microbiota as a consequence of environmental changes [4, 5]. The prevalence of *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *Fusobacterium nucleatum* is known to increase during gingivitis at the expense of *Streptococcus* spp. [6–9]. This community shift causes inflammation of the gingiva as part of the immune response [3, 10, 11]. The disease can be prevented and alleviated by the removal of the plaque biofilm and by the use of oral hygiene products such as toothbrushes, toothpastes, and mouthwashes [12]. The constant depth film fermenter (CDFF) has been used previously to model the bacterial community shifts observed during gingivitis and has also been employed to assess the effects of oral hygiene products [13, 14].

Medicinal mushrooms, including *Lentinula edodes* or shiitake, have been used in Asia for centuries and have numerous health benefits. These range from their antioxidant properties, to lowering cholesterol and blood pressure, antitumor properties, and antibacterial and libido-enhancing properties [15–18]. The health benefits of shiitake mushrooms are thought to be so great that they have been

incorporated into some foods in order to be delivered to the population, creating functional foods including pork patties, cereals, and cookies [16, 19, 20]. However, shiitake has not as yet been assessed for its oral health benefits.

In recent years, high-throughput culture-independent quantitative methods have revolutionised the investigation of bacterial community structure. These methods are now being employed in the study of microbial communities involved in both oral health and disease [21–23]. In the present study, a set of assays developed previously was used to monitor the bacterial community structure changes within an *in vitro* gingivitis model and to assess the effect of shiitake mushroom extract and chlorhexidine, the leading agent used in the treatment of gum disease [24], on these communities.

2. Materials and Methods

2.1. Saliva Collection. Healthy individuals with good oral hygiene were asked to expectorate into a sterile centrifuge tube up to a volume of 2 mL. Saliva was collected from 20 individuals. The saliva samples were homogenised into pooled saliva, and glycerol was added to a final concentration of 10% v/v. The pooled saliva was dispensed into 1 mL aliquots and stored at −80°C.

2.2. CDFF Gingivitis Model. *In vitro* biofilms, representative of plaque that forms at the gingival margin, were cultured using a CDFF. The environmental conditions within the CDFF were modified in order to mimic those found during gingivitis, as described previously [14]. Briefly, the CDFF was inoculated by 1 mL of pooled saliva sample added to 500 mL artificial saliva medium [25] over 8 hours. The biofilms were cultured at 36°C for one week. The CDFF was kept under microaerophilic conditions using a gas mixture (2% O_2; 3% CO_2; 95% N at 200×10^5 Pa) pumped into the chamber through a filtered inlet at a rate of 200 cm^3 min^{-1}. Artificial saliva medium and artificial gingival crevicular fluid [26] were pumped into the chamber throughout the experiment at a flow rate of 0.72 litres day^{-1} and 0.072 litres day^{-1}, respectively.

No antimicrobials were pumped into the CDFF during the no treatment control (NTC) experiments. During the chlorhexidine (CHX) and mushroom extract (MUSH) experiments, 0.12% chlorhexidine and 2x low molecular weight shiitake mushroom extract were pumped into the CDFF from 80 h and every 12 hours thereafter to mimic the use of a mouthwash twice daily. Each pulse was pumped in at a rate of 2 mL min^{-1} for 5 minutes.

One pan, containing five disks, was removed aseptically every 24 hours. The biomass of two disks was collected as described previously [14] in duplicate. DNA extractions were then performed on the biomass collected.

2.3. Low Molecular Weight Shiitake Mushroom Extract Preparation. The 2x low molecular weight mushroom extract was prepared as described by Daglia et al. [27].

2.4. DNA Extraction Method. Total nucleic acids were extracted from all samples according to a previously described protocol [28] using a bead-beating phenol: chloroform: isoamyl alcohol (25 : 24 : 1) extraction followed by a 30% PEG 6000 precipitation and 70% ethanol wash. This method was found to be the least biased towards the extraction of nucleic acids from Gram-negative organisms.

2.5. qPCR Method. Three triplex qPCR assays were designed to enumerate four organisms associated with gingivitis (*Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Lactobacillus casei*, and *Prevotella intermedia*), three organisms associated with oral health (*Streptococcus sanguinis*, *Neisseria subflava*, and *Veillonella dispar*), one organism strongly implicated in dental caries (*Streptococcus mutans*), and all organisms as described previously [23]. The detection limits for each of the single taxa were 20 cells and the number rose to 600 cells for the universal assay.

2.6. Statistics. Data were normalised by transformation using $\log_{10}$. ANOVA analysis was used to test whether changes between the treatments were significant (significant $P \leq 0.005$; and slightly significant $P < 0.01$).

3. Results

3.1. Saliva Community. The microbial community present in the pooled saliva used as the inoculum for the CDFF was analysed using qPCR. The numbers of each of the taxa analysed are shown in Figure 1. The mean ($n = 3$) total number of organisms per millilitre of pooled saliva was found to be $1.01\ (\pm 0.41) \times 10^9$ (standard deviation is shown in brackets). The specific taxa being investigated made up 3.75×10^8 of the organisms or 37.24% of the total. Of these taxa, the most numerous were *V. dispar* (22.8%), followed by *N. subflava* (12.1%) and *F. nucleatum* (1.8%), and the least is *L. casei* (0.05%). Very low variation was observed between the three saliva samples which were profiled.

3.2. Gingivitis CDFF Plaque Biofilm Communities. The data regarding the numbers of individual taxa analysed and the total number of bacteria present over the course of the treatment experiments is shown in Table 1.

3.2.1. No Treatment Control (NTC). There was little change in total numbers of organisms present over time, the numbers increased from around 10^7 at the start to around 10^8 cells per disk up to 72 hours and remaining at this level throughout the experiment. Numbers of *L. casei, P. intermedia,* and *A. naeslundii* were very low throughout. Other taxa increased over time (mainly between the 72 h sampling point and the 96 h point) by 3 $\log_{10}$, for example, *F. nucleatum* (from 0.007% to 5.399%), *V. dispar* (from 0.072% to 13.093%), and *N. subflava* (from 0.022% to 78.446%). *S. sanguinis* increased by 0.5 $\log_{10}$ (0.139% to 0.379%). *S. mutans* was not detected at any time points.

3.2.2. Chlorhexidine Treatment (CHX). As with the NTC exper-iment, the total numbers of organisms remained broadly steady over the experiment. Numbers of *L. casei,* and *P. intermedia*, and *A. naeslundii* were found in similar levels

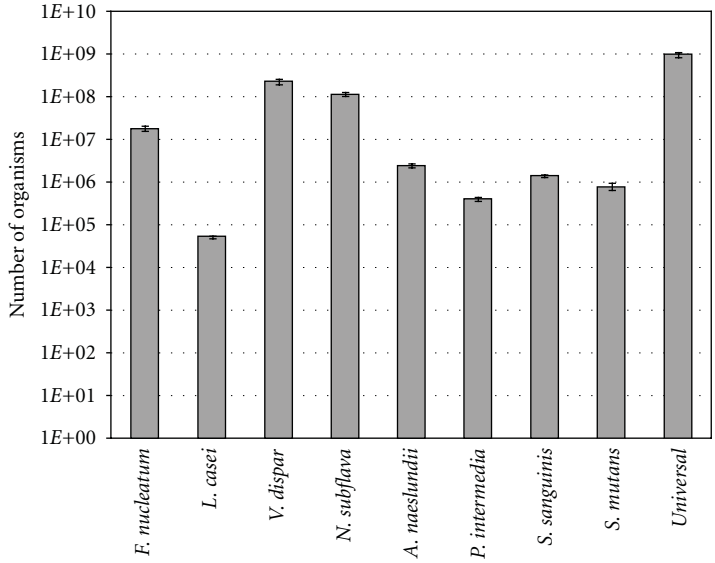

Figure 1: Numbers of each of the taxa investigated in pooled saliva. Error bars represent the standard deviation ($n = 3$).

as with the NTC experiment. However, numbers of *N. subflava* only rose by 1 $\log_{10}$ throughout the experiment (from 3.838% to 10.919%), number of *V. dispar* and *S. sanguinis* remained similar (1.590% to 0.774%; 0.360% to 0.275%, resp.), and *F. nucleatum* deceased by around 1 $\log_{10}$ (from 0.026% to 0.001%). *S. mutans* was not detected at any time points.

3.2.3. Mushroom Treatment (MUSH). Total numbers of organisms were found to be around 10^8 cells per disk for the duration of the experiment. Numbers of *L. casei, P. intermedia,* and *A. naeslundii* were very low throughout. However, numbers of *N. subflava* rose by 3 $\log_{10}$ (from 0.011% to 54.374%) throughout the experiment, number of *V. dispar* rose by 6 $\log_{10}$ (0.000002% to 8.556%) throughout the experiment, and *F. nucleatum* remained steady (0.00001% to 0.00009%). *S. sanguinis* numbers rose by 2 $\log_{10}$ (0.001% to 1.841%) throughout the experiment. *S. mutans* was not detected at any time points.

3.3. Comparison of Taxa Numbers between Treatments. P. intermedia, L. casei, and *A. naeslundii* numbers were found in low numbers during all three of the treatments with no significant differences between treatments at any time points. The numbers of *N. subflava* cells appeared to be lower during the CHX treatment from 72 h; however, no significant difference between treatments was found until the 168 h time point (NTC, $P = 0.010$; MUSH, $P = 0.004$) (Figure 2). *V. dispar* cell numbers were found to be significantly lower during the CHX treatment at 96 h, 120 h, and 168 h ($P \leq 0.003$, $P \leq 0.021$, and $P = 0.001$, resp.) (Figure 2).

S. sanguinis numbers were significantly higher during the MUSH treatment than during the CHX treatment at time points 48, 96, 120, and 168 hours ($P = 0.047$, $P = 0.052$, $P = 0.032$, and $P = 0.021$, resp.) (Figure 2). Numbers of *F. nucleatum* were found to be significantly lowered by the MUSH and CHX treatments from 96 hours onwards ($P \leq 0.030$) (Figure 2). Finally, examining the universal assay cell numbers, the CHX experiment counts are significantly lower than those in the MUSH experiment at 48, 96, 120, and 168 hours ($P = 0.010$, $P = 0.019$, $P = 0.044$, and $P = 0.022$, resp.) (Figure 2).

4. Discussion

4.1. Saliva Community. The bacterial community found in salivary fluid is composed of the amalgamation of the communities found around the mouth. The predominant taxa were found to be *V. dispar*, *N. subflava*, *F. nucleatum, A. naeslundii,* and *S. sanguinis*. These taxa have all been associated with healthy dental plaque biofilms in previous culture independent studies [29, 30]. The tongue community in healthy subjects has previously been found to comprise mostly *Streptococcus* spp., *Veillonella* spp., and *Actinomyces* spp. [31, 32]. A recent study looking into the unculturable microbiota of the tongue has also identified the above genera, along with a *Lysobacter*-type species as the predominant organism found on the tongue [33].

Two studies using culture-independent molecular methods have shown that the dominant phyla most commonly found in saliva were Firmicutes, Bacteriodetes, Proteobacteria, Actinobacteria, and Fusobacteria, respectively [22, 34]. The multitriplex qPCR method showed a similar picture: the Firmicutes were the dominant organisms, followed by Proteobacteria, Fusobacteria, Actinobacteria, and Bacteriodetes.

4.2. CDFF Plaque Biofilm Communities. Whilst the universal assay confirmed total cells numbers in the biofilms to be high in all of the CDFF experiments, some of the taxa investigated were only detected in low levels including *P. intermedia*, *L. casei*, *S. mutans,* and *A. naeslundii*. While *Actinomyces* spp. are known to be one of the early colonizers in the formation

Table 1: Numbers of each of the taxa investigated in biofilms grown in the gingivitis CDFF over one week under various treatments: Control ($n = 2$), Chlorhexidine ($n = 4$), and LMW mushroom ($n = 2$) pulsing.

Control							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
F. nucleatum	4.68 (±0.71) × 10^3	1.38 (±0.11) × 10^3	5.39 (±0.41) × 10^3	5.07 (±0.05) × 10^5	2.99 (±1.82) × 10^6	3.31 (±0.87) × 10^6	6.26 (±0.30) × 10^6
L. casei	6.20 (±4.81) × 10^2	7.80 (±4.53) × 10^2	7.80 (±4.53) × 10^2	8.20 (±9.05) × 10^2	9.10 (±9.76) × 10^2	9.80 (±9.33) × 10^2	9.00 (±10.7) × 10^2
V. dispar	4.91 (±4.13) × 10^4	2.29 (±2.18) × 10^5	2.65 (±1.41) × 10^6	7.83 (±3.74) × 10^6	8.94 (±7.24) × 10^6	8.28 (±3.96) × 10^6	1.52 (±0.26) × 10^7
N. subflava	1.52 (±0.72) × 10^4	2.43 (±1.80) × 10^5	2.49 (±1.70) × 10^7	3.69 (±1.79) × 10^7	6.49 (±5.97) × 10^7	5.51 (±4.14) × 10^7	9.10 (±3.74) × 10^7
A. naeslundii	6.00 (±8.49) × 10^1	4.00 (±5.66) × 10^1	1.00 (±1.41) × 10^1	2.80 (±1.70) × 10^2	1.60 (±0.28) × 10^2	1.40 (±0.57) × 10^2	2.40 (±3.11) × 10^2
P. intermedia	5.00 (±1.41) × 10^1	5.00 (±1.41) × 10^1	6.00 (±0.00) × 10^1	4.00 (±2.83) × 10^1	4.00 (±2.83) × 10^1	3.00 (±1.41) × 10^1	5.00 (±1.41) × 10^1
S. sanguinis	9.49 (±1.65) × 10^4	8.05 (±1.41) × 10^4	1.39 (±0.59) × 10^6	5.63 (±0.99) × 10^5	3.15 (±1.63) × 10^5	5.18 (±3.05) × 10^5	4.40 (±1.71) × 10^5
Universal	6.81 (±2.59) × 10^7	3.48 (±2.22) × 10^7	1.56 (±1.02) × 10^8	1.72 (±1.08) × 10^8	1.51 (±1.28) × 10^8	1.30 (±0.83) × 10^8	1.61 (±0.01) × 10^8
Chlorhexidine							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
F. nucleatum	5.28 (±8.28) × 10^3	8.25 (±9.77) × 10^2	2.05 (±4.10) × 10^2	6.40 (±7.18) × 10^2	6.00 (±6.37) × 10^2	2.80 (±5.20) × 10^2	5.05 (±4.71) × 10^2
L. casei	3.60 (±5.80) × 10^2	2.60 (±3.81) × 10^2	6.50 (±13.0) × 10^1	3.20 (±5.13) × 10^2	2.60 (±3.59) × 10^2	6.00 (±10.7) × 10^1	3.15 (±3.00) × 10^2
V. dispar	3.28 (±5.02) × 10^5	4.05 (±5.69) × 10^5	3.53 (±3.35) × 10^5	4.86 (±2.24) × 10^5	2.08 (±1.93) × 10^5	7.02 (±7.24) × 10^5	5.65 (±2.77) × 10^5
N. subflava	7.91 (±10.0) × 10^5	5.98 (±8.67) × 10^5	4.80 (±4.91) × 10^6	9.34 (±12.1) × 10^6	6.95 (±7.91) × 10^6	1.08 (±1.21) × 10^7	7.97 (±4.94) × 10^6
A. naeslundii	3.88 (±3.47) × 10^1	1.15 (±1.92) × 10^1	0.00 (±0.00) × 10^0	1.50 (±3.00) × 10^1	1.00 (±2.00) × 10^1	1.50 (±3.00) × 10^1	9.75 (±12.1) × 10^0
P. intermedia	4.00 (±4.90) × 10^1	4.00 (±4.32) × 10^1	2.50 (±3.79) × 10^1	5.50 (±5.26) × 10^1	4.50 (±4.43) × 10^1	2.00 (±2.83) × 10^1	3.50 (±3.00) × 10^1
S. sanguinis	7.41 (±8.43) × 10^4	2.25 (±2.39) × 10^4	5.75 (±6.38) × 10^4	1.39 (±1.52) × 10^5	7.38 (±7.62) × 10^4	9.35 (±10.3) × 10^4	2.01 (±1.69) × 10^5
Universal	2.06 (±2.57) × 10^7	1.49 (±0.47) × 10^7	2.99 (±2.57) × 10^7	5.01 (±1.91) × 10^7	4.33 (±2.17) × 10^7	6.75 (±4.92) × 10^7	7.30 (±2.85) × 10^7
LMW mushroom							
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
F. nucleatum	3.30 (±3.82) × 10^2	5.40 (±2.26) × 10^2	3.40 (±3.68) × 10^2	3.10 (±2.12) × 10^2	6.60 (±9.33) × 10^2	3.40 (±3.96) × 10^2	2.40 (±3.11) × 10^2
L. casei	1.05 (±1.34) × 10^1	5.10 (±3.54) × 10^2	1.90 (±1.27) × 10^2	5.00 (±7.07) × 10^1	5.00 (±7.07) × 10^1	7.00 (±9.90) × 10^1	5.00 (±7.07) × 10^{-1}
V. dispar	8.05 (±11.2) × 10^1	1.32 (±0.29) × 10^4	6.73 (±0.05) × 10^6	6.92 (±2.18) × 10^6	1.46 (±0.65) × 10^7	2.43 (±1.19) × 10^7	2.35 (±1.09) × 10^7
N. subflava	4.86 (±1.78) × 10^5	2.20 (±1.40) × 10^7	5.39 (±2.25) × 10^7	7.10 (±3.29) × 10^7	1.26 (±0.65) × 10^8	1.40 (±0.83) × 10^8	1.50 (±0.50) × 10^8
A. naeslundii	0.00 (±0.00) × 10^0	2.00 (±2.83) × 10^1	2.20 (±0.57) × 10^2	3.00 (±1.41) × 10^1	0.00 (±0.00) × 10^0	0.00 (±0.00) × 10^0	0.00 (±0.00) × 10^0
P. intermedia	1.00 (±1.41) × 10^1	1.00 (±1.41) × 10^1	1.00 (±1.41) × 10^1	2.00 (±2.83) × 10^1	3.00 (±4.24) × 10^1	1.00 (±1.41) × 10^1	2.00 (±2.83) × 10^1
S. sanguinis	5.99 (±4.29) × 10^4	8.67 (±3.87) × 10^5	3.72 (±0.76) × 10^6	6.36 (±2.07) × 10^6	6.55 (±1.97) × 10^6	5.99 (±2.97) × 10^6	5.06 (±1.58) × 10^6
Universal	4.60 (±2.56) × 10^7	8.96 (±1.11) × 10^7	1.98 (±0.69) × 10^8	2.88 (±0.39) × 10^8	3.80 (±1.04) × 10^8	3.25 (±0.12) × 10^8	2.75 (±0.59) × 10^8

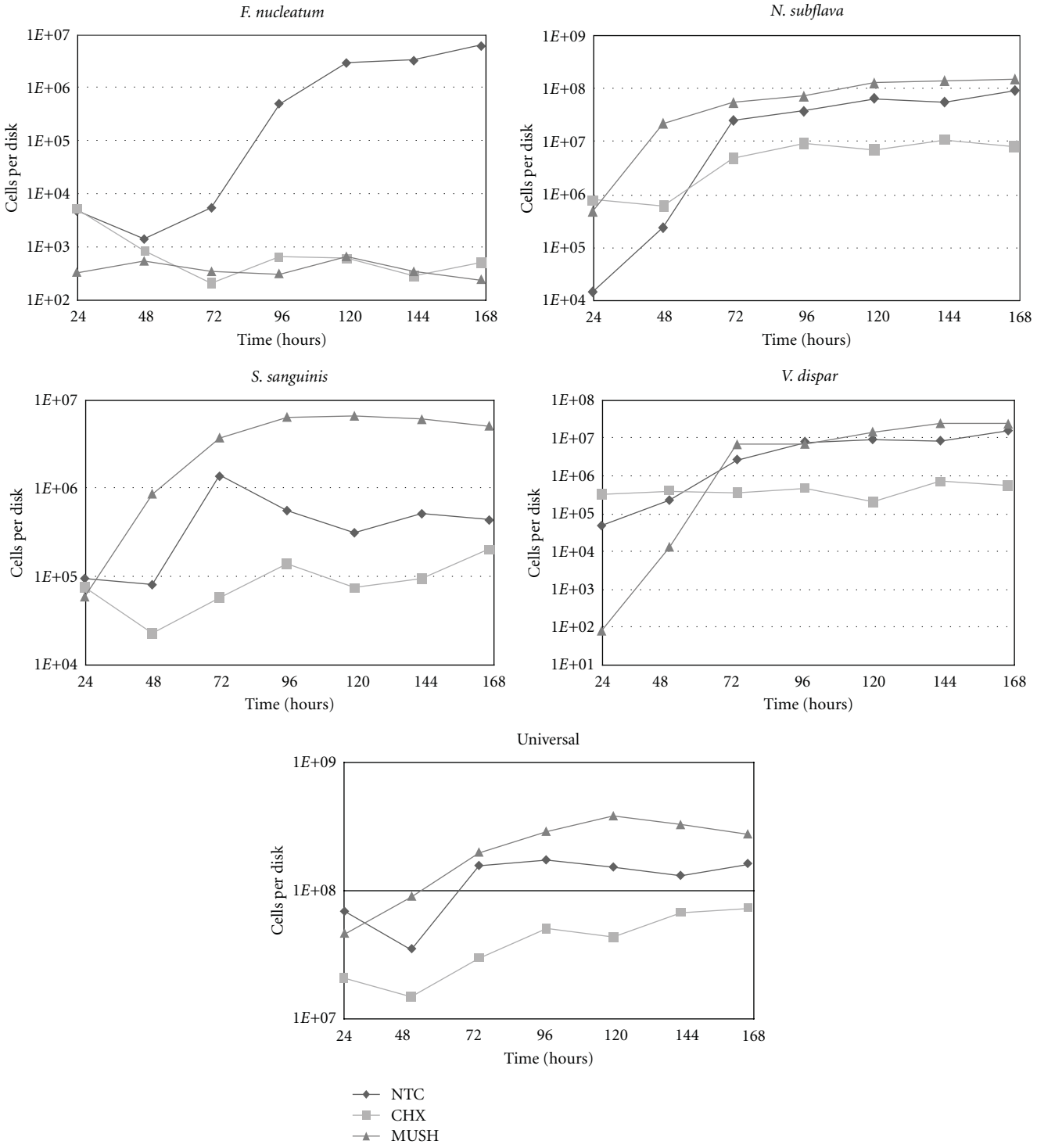

Figure 2: *F. nucleatum*, *N. subflava*, *S. sanguinis*, *V. dispar*, and total bacterial cell numbers which displayed significant differences between the different treatments.

of dental plaque [35], *A. naeslundii* is only one species representative of this genus. It is likely that the environmental conditions within the CDFF experiments were not optimal for the above taxon, but other members of the genus may have been present. Previous studies have found that *L. casei*, *S. mutans*, and *A. naeslundii* all grow well in biofilms cultured using saliva and the addition of a carbohydrate such as glucose or sucrose [22, 36]. The lack of glucose or sucrose in the culture media in the present study could account for the low detection rates of these organisms. A previous study has shown that *Prevotella* spp. were detectable in the CDFF inoculum but not during the duration of the experiment

using molecular methods [37], supporting the data from the current study where the pathogen was detected at very low levels throughout.

The organisms found in consistently high numbers from the beginning of all of the experiments were *N. subflava*, *S. sanguinis,* and *V. dispar*. All of these organisms have been shown to be early colonizers during the formation of dental plaque as well as being among the most abundant taxa in the oral cavity [34, 35, 38]. *F. nucleatum* numbers increased at a slightly later stage of plaque biofilm formation once the environmental conditions were optimal [39], as seen in the NTC experiment designed to mimic conditions during gingivitis.

Gingivitis is caused by the buildup of the plaque biofilm at the gingival margin, which in turn results in a shift in the resident microbiota as a consequence of environmental changes [4, 5]. The prevalence of *Actinomyces* spp., *Lactobacillus* spp., *Prevotella* spp., and *F. nucleatum* is known to increase during gingivitis at the expense of *Streptococcus* spp. [6, 7, 9]. It was apparent that numbers of *F. nucleatum* rose over time in the NTC experiment and that *S. sanguinis* numbers declined after an initial peak at 72 h coinciding with the *F. nucleatum* increase.

Looking at the treatment effects, the application of chlorhexidine significantly lowered the numbers of *N. subflava*, *V. dispar*, and *F. nucleatum* compared to NTC The total cell numbers were also lower during the CHX treatment, no doubt in part due to the lower numbers of the above taxa. Chlorhexidine is considered the gold standard [24] in the treatment of gum disease, and its action has been well studied. Previous studies looking at the effects of chlorhexidine on plaque biofilms *in vitro* have shown an effect on *Veillonella* sp., *Fusobacterium* sp., and *Streptococcus* sp. numbers [22, 25], supported by the current study. The MUSH treatment significantly lowered the numbers of *F. nucleatum*, an oral pathogen, but also resulted in significantly higher numbers of *S. sanguinis*, normally associated with oral health, when compared to the CHX treatment. This increase in *S. sanguinis* numbers despite the gingivitis conditions in the CDFF is an important effect. Furthermore, the MUSH treatment did not have a negative effect on *N. subflava* and *V. dispar*, both organisms associated with oral health [34, 38]. The data presented in the current study are supported by previous research which demonstrated the antimicrobial effects of shiitake mushroom products on a number of Gram-positive and negative organisms including some oral pathogens [17, 40, 41].

In conclusion, the comparison of the different treatments using the CDFF has given a valuable insight into the community dynamics of dental plaque as well as an indication of the efficacy of the treatments. Chlorhexidine was found to be effective at lowering a number of taxa, associated with both health and disease; however, shiitake mushroom extract was shown to be effective at reducing the numbers of the oral pathogen *F. nucleatum*, while having little effect on some of the taxa associated with health. The results imply that the action of shiitake mushroom extract should be investigated further for its beneficial effects on oral health.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the Contract no. FOOD-CT-2006-036210 (Project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 230630, 8 pages
doi:10.1155/2011/230630

Research Article

Effects of Fruit and Vegetable Low Molecular Mass Fractions on Gene Expression in Gingival Cells Challenged with *Prevotella intermedia* and *Actinomyces naeslundii*

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Received 14 June 2011; Accepted 12 July 2011

Academic Editor: Itzhak Ofek

Low molecular mass (LMM) fractions obtained from extracts of raspberry, red chicory, and Shiitake mushrooms have been shown to be an useful source of specific antibacterial, antiadhesion/coaggregation, and antibiofilm agent(s) that might be used for protection towards caries and gingivitis. In this paper, the effects of such LMM fractions on human gingival KB cells exposed to the periodontal pathogens *Prevotella intermedia* and *Actinomyces naeslundii* were evaluated. Expression of cytokeratin 18 (CK18) and β4 integrin (β4INT) genes, that are involved in cell proliferation/differentiation and adhesion, and of the antimicrobial peptide β2 defensin (HβD2) in KB cells was increased upon exposure to either live or heat-killed bacteria. All LMM fractions tested prevented or reduced the induction of gene expression by *P. intermedia* and *A. naeslundii* depending on the experimental conditions. Overall, the results suggested that LMM fractions could modulate the effects of bacteria associated with periodontal disease in gingival cells.

1. Introduction

Periodontal diseases are a heterogeneous group of inflammatory conditions that involve the supporting tissues of the teeth and include gingivitis, in which only the gingivae are involved, and the various forms of periodontitis, chronic inflammatory conditions initiated by a polymicrobial infection that leads to gingival tissue destruction and alveolar bone resorption [1]. Gingivitis is the most prevalent form of periodontal disease that can be defined as "a nonspecific inflammatory process of the gingivae (gums) without destruction of the supporting tissues". This is a reversible condition as a return to meticulous dental hygiene practices will restore gingival health [2]. Several bacterial species have been implicated as aetiological agents of this disease: these include *Actinomyces israelii*, *Actinomyces naeslundii*, *Actinomyces odontolyticus*, *Lactobacillus* spp., *Prevotella intermedia*, *Treponema* spp., and *Fusobacterium nucleatum* [3].

Bacteria and their products can directly damage periodontal tissues and/or initiate inflammation locally. The clinical outcomes of these events are determined by the host response to the infections [1]. Different experimental systems can be utilized to evaluate cellular responses to different bacteria or antibacterial agents, from fibroblasts

derived from human periodontal ligaments to epithelial cells and fibroblasts derived from human gingivae [4].

Foodstuffs as a source to obtain agents/fractions that can improve oral health have been the focus of intensive research because such natural agents are likely to be nontoxic and edible; for example, they can be used to supplement various oral hygiene products. In studies described elsewhere in this issue, it has been shown that low molecular mass (LMM) fractions obtained from extracts of raspberry, chicory, and mushrooms inhibit coaggregation, biofilm formation, and adhesion to hydroxyapatite and/or cultured gingival cells of oral bacteria involved in caries and/or gingivitis [5]. To further evaluate the beneficiary effect on oral health of these dietary fractions, the present study was designed to determine the effect of the raspberry, chicory, and mushroom LMM fractions on the ability of gingivitis-associated bacteria to induce deleterious gene expression in the gingival KB cell line.

2. Materials and Methods

2.1. Bacterial Cultures. *A. naeslundii* ATCC 19039 and *P. intermedia* ATCC 25611 were employed. Bacteria were grown in Brain Heart Infusion Broth (BHIB, Difco Laboratories, Detroit, Mich.) supplemented with haemin (final concentration, $5\,\text{mg}\,\text{mL}^{-1}$) and vitamin K (final concentration, $1\,\text{mg}\,\text{mL}^{-1}$) and incubated at 37°C under anaerobic conditions. Cells were harvested at stationary phase by centrifugation ($5,000 \times g$ for 10 min at 4°C) and washed twice with 10 mM phosphate buffered saline, pH 7.0. Bacterial suspensions (final concentration, $2 - -5 \times 10^8$ cfu mL^{-1}) were prepared in PBS (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.2 to 7.4) alone or suspensions containing different concentrations of test LMM fractions (pH adjusted to 7). Aliquots (10–100 μL) of the bacterial suspensions were suitably added to KB cell monolayers in order to reach a nominal bacteria: KB cell ratio of 50 and incubated at 37°C for different periods of time in 5% CO_2 atmosphere with gentle shaking. For each strain, untreated controls were included. In experiments with killed bacteria, bacteria were heat inactivated at 70°C for 15 min, centrifuged at $10,000 \times g$, and washed twice in PBS, before being adjusted to the appropriate density in the same buffer.

2.2. LMM Fraction Preparation. Food and vegetable extracts and fractions were prepared as described elsewhere in this issue [6]. Briefly, aliquots of frozen mushroom (*Lentinus edodes*) (400 g) and fresh chicory (*Cichorium intybus* var. *silvestre*) (500 g) and fresh raspberry fruit (*Rubus idaeus* L. var. *tulameen*) (200 g) were homogenized and centrifuged (10 min, 8000 rpm). The juice, after separation from the solid part, was filtered on paper filter. Mushroom and chicory extracts were then fractionated into low and high molecular weight (LMM and HMM) fractions using an ultradiafiltration system. The obtained LMM and HMM fractions contained all the compounds with molecular mass lower and higher than 5000 Da, respectively. Raspberry extract was fractionated by dialysis with cellulose ester membrane (Spectrum Europe B.V.) with a 3500 Da MWCO. The obtained LMM and HMM fractions contained all the compounds with molecular mass lower and higher than 3500 Da, respectively. All LMM fractions were subjected to sterile ultrafiltration and freeze dried.

2.3. Gingival Cell Culture and Treatments. The gingival fibroblast KB cell line (accession number ICLC HTL96014) obtained from Cell bank Interlab Cell Line Collection (ICLC) of IST-Istituto dei Tumori di Genova (Genoa, Italy) was cultured in a complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM)-high glucose, with $4.5\,\text{g}\,\text{L}^{-1}$ glucose and sodium bicarbonate supplemented with 10% fetal calf serum, penicillin ($100\,\text{U}\,\text{mL}^{-1}$), streptomycin ($100\,\mu\text{g}\,\text{mL}^{-1}$), and 2 mM L-glutamine. Cells were incubated at 37°C in a 5% CO_2 atmosphere to about 90% confluence and used after 5–10 passages.

For MTT assay, cells were seeded in flat-bottom 96-well plates, at a density of 2×10^4 cells per well, in 0.2 mL of complete medium without antibiotics in triplicate and incubated for 24 h at 37°C.

For RT-Q-PCR analysis cells were plated (3×10^5 cells) in to 25 cm^2 flasks in 5 mL of complete medium and incubated for 48 hrs at 37°C.

After 24 hrs of starvation in DMEM without serum, cell monolayers were washed twice with PBS and then exposed to bacterial suspensions alone or suspensions containing LMM test fractions at the indicated concentrations. A parallel set of untreated cells in triplicate was utilised as a control. All experiments were performed in quadruplicate.

2.4. Viability Assay. KB cell viability was evaluated by the MTT assay [7]. After each treatment MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide)] solution in phosphate buffered saline (PBS, pH 7.4) (final concentration of 1 mg/mL) was added to each well and incubated for 3 h at 37°C. Following incubation, 100 μL of 0.04 N HCl isopropanol was added to each sample and the plates incubated for 10 minutes at room temperature. The absorbance at 550 nm was determined by a Varian Cary 50-bio UV-visible spectrophotometer. Data are expressed as percent of control values (mean ± SD).

2.5. RT-Q-PCR. The bacteria-induced changes in mRNA expression of keratinocyte growth factor receptor (KFGR), cytokeratin 18 (CK18), β4 integrin (B4ITG), and β defensin 2 (HβD2) were evaluated by quantitative RT-PCR analysis. After harvesting, total cellular RNA was isolated by the procedure of Chomczynski and Sacchi [8] using Tri-Reagent (SIGMA, Milan, Italy). RNA purity was evaluated by measuring the 260/280 nm absorbance ratio, and only samples with $OD_{260/280} > 1.8$ were processed, resolved on a 1.5% agarose gel, and stained with ethidium bromide, to check for purity. cDNA synthesis was performed from 1.5 μg of total DNaseI (Fermentas, M-Medical, Italy) treated RNA using 200 units RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas Italy, M-Medical, Milan) in presence of 201 pmoles oligo (dT)18 (TIB MOLBIOL,

Table 1: Oligonucleotide primers used for quantitative RT-PCR analysis.

Gene	Primer forward (5′–3′)	Primer reverse (5′–3′)	GenBank
KGFR	GATTACAGCTTCCCCAGACTACC	GAAGAGAGGCGTGTTGTTATCC	M80634
CK18	TCAGAGACTGGAGCCATTACTTC	CAGTCGTGTGATATTGGTGTCAT	NM199187
ITGB 4	GCCGCTACGAGGGTCAGTT	TCCATTACAGATGCCCCCATT	NM000213
HβD2	GGGTCTTGTATCTCCTCTTCTCG	ACAGGTGCCAATTTGTTTATACC	NM004942
GAPDH	GTCAGTGGTGGACCTGACCT	AGGGGTCTACATGGCAACTG	M17851

Genova, Italy), 40 units RiboLock (ribonuclease inhibitor), and 1 mM dNTPs (Fermentas Italy, M-Medical, Milan) at 42°C for 60 min in a reaction volume of 20 μL according to the manufacturer's protocol. 5 μL of cDNA was then used to amplify the genes of interest using a Chromo 4 System real-time PCR apparatus (Biorad Italy, Segrate, Milan) in a final volume of 20 μL containing 1x iTaq SYBR Green Supermix with Rox (Biorad, Milan, Italy) and 0.25 μM of each primer (TIB MolBiol, Genoa, Italy). The primer pairs were used, and their accession numbers are shown in Table 1. The primers for KFGR, CK18, HβD2, and GAPDH were designed with the *Primer3* (v.0.4.0) software [9], those for B4ITG were from Wang et al. [10].

The thermal protocol consisted of 3 min initial denaturation at 95°C, followed by 40 cycles: 15 s at 95°C; 30 s at 54°C; 20 s at 72°C. A melting curve of PCR products 55–94°C) was also performed to ensure the absence of artefacts. Gene expression was determined relative to the expression of the gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by the comparative C_T threshold method [11] using the Biorad software tool Genex-Gene Expression Macro [12]. The normalized expression obtained was expressed as relative quantity of mRNA with respect to control samples.

2.6. Data Analysis. Data representing the mean ± SD of at least 4 experiments in triplicate were analysed by the Mann-Whitney U test ($P \leq 0.05$).

3. Results

3.1. Effects of Live Bacteria and LMM Fractions, Alone and in Combination, on KB Cell Viability and Gene Expression. The effects of the gingivitis-associated *P. intermedia* and *A. naeslundii* and LMM fractions (Raspberry, Chicory, Mushroom) alone on KB cell viability were first evaluated by the MTT assay. LMMs were tested at different concentrations (0.2x, 0.5x, 1x) and times of incubation with monolayers (4 and 6 h).

The viability of KB cells treated with live bacteria alone (at the nominal bacteria: KB cell ratio of 50) was not affected at 4 h; the number of viable bacteria remained the same during the assay as evaluated by cfu counting. KB cell incubation for longer than 4 h or in the presence of 0.5x and 1x concentrations of LMM fractions alone decreased by 20–30% (data not shown). Therefore, all subsequent experiments with live bacteria were performed by treating KB cells for 4 h with bacteria and 0.2x LMM fractions, both alone and in combination. In these conditions, no effects on KB cell viability were observed (Figure 1).

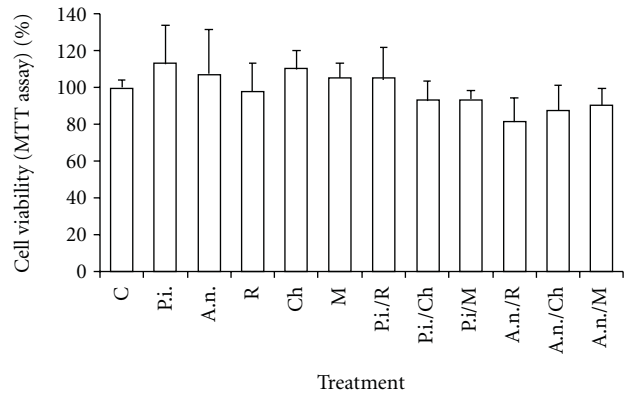
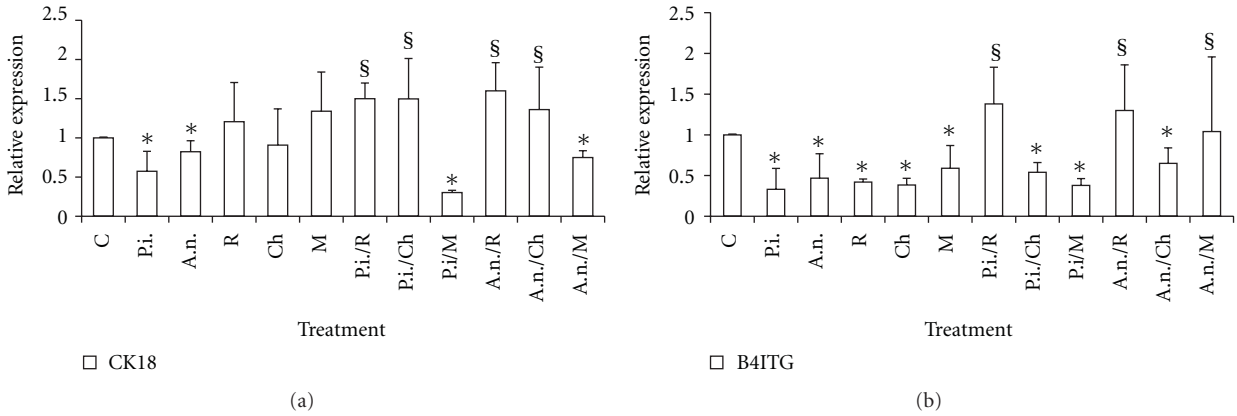


Figure 1: Effects of live *P. intermedia* and *A. naeslundii,* and of Raspberry, Chicory, and Mushroom LMM fractions on KB cell viability. Cells were exposed to bacteria (at the nominal bacteria: KB cell ratio of 50) and fractions (0.2x), alone and in combination, for 4 h. Cell viability was evaluated by the MTT assay as described in Methods. Data, expressed as percent cell viability with respect to controls, represent the mean ± SD of four experiments in triplicate. P.i.: *P. intermedia*; A.n.: *A. naeslundii*; R: Raspberry; Ch: Chicory; M: Mushroom; P.i./R: *P. intermedia*/R; P.i./Ch: *P. intermedia*/Ch; P.i./M: *P. intermedia*/M; A.n./R: *A. naeslundii*/R; A.n./Ch: *A. naeslundii*/Ch; A.n./M: *A. naeslundii*/M.

The effects of KB cell treatment for 4 h with *P. intermedia* and *A. naeslundii* and LMM fractions, alone and in combination, on the expression of different genes (KGFR, CK18, β4INT and HβD2) were evaluated. No appreciable changes could be detected in the expression of KGFR by KB cells in response to different treatments (not shown). In contrast, *P. intermedia* and *A. naeslundii* alone decreased significantly the transcription of CK18 by 40% and 18%, respectively ($P \leq 0.05$) (Figure 2(a)). No significant changes in CK 18 expression were noted in KB cells exposed to LMM fractions. Nevertheless, raspberry and chicory LMM fractions prevented the downregulation of CK18 induced by both bacteria, whereas the LMM mushroom fraction did not.

All bacteria and LMM fractions, when tested alone, induced a decrease in transcription of β4ITG with respect to controls (Figure 2(b)). The effect was significant for all treatments, with decreases in β4ITG mRNA level ranging from about −60% *(P. intermedia)* to −40% (mushroom fraction) ($P \leq 0.05$). However, when KB cells were exposed to a mixture of bacteria and LMM fractions, raspberry,



(a) (b)

Figure 2: Effects of live *P. intermedia* and *A. naeslundii,* and of Raspberry, Chicory, and Mushroom LMM fractions on expression of Cyt18 (a) and 4βINT (b) in KB cells. Cells were exposed to bacteria (at the nominal bacteria: KB cell ratio of 50) and fractions (0.2x), alone and in combination, for 4 h. Gene expression was evaluated by RT-Q-PCR as described in Methods. Data, expressed as relative expression with respect to controls, represent the mean ± SD of four experiments in triplicate. Abbreviations as in legend to Figure 1. $^*= P \leq 0.05$: all treatment versus controls; § = $P \leq 0.05$: bacteria/LMM fractions versus bacteria alone. Mann-Whitney U test.

the decrease in β4ITG expression was attenuated; moreover, mushroom LMM fraction prevented the effect induced by *A. naeslundii.* On the other hand, no effect was observed with chicory fractions.

3.2. Effects of Heat-Killed Bacteria and LMM Fractions, Alone and in Combination, on KB Cell Viability and Gene Expression. The same experiments as above were carried out with heat-killed *P. intermedia* and *A. naeslundii* alone and in combination with LMM extracts. Longer times of incubation and lower concentrations of LMM extracts (0.1x) were tested: however, from 8 to 20 hr incubation, decreases in cell viability were observed in different experimental conditions with both bacteria and test substances, with raspberry LMM extracts in particular. At 6 hr, stable cell viability was observed in all experimental conditions, and therefore this time of exposure was chosen for subsequent experiments; however, since raspberry LMM still induced significant loss in cell viability, it was no longer utilized for subsequent experiments. In Figure 3, data on viability of KB cells exposed for 6 h to *P. intermedia* and *A. naeslundii*, alone and in combination with chicory and mushroom LMM fractions, are reported.

Possible changes in gene expression induced by KB cell exposure for 6 h to inactivated bacteria and LMM fractions, alone and in combination, were evaluated, and the results are reported in Figure 4. Heat-killed *P. intermedia* and *A. naeslundii* alone did not affect CK18 expression. On the other hand, in these experimental conditions, chicory fraction caused a significant decrease (−27%) and shiitake mushroom fraction an increase (+43%) in CK18 expression ($P \leq 0.05$) (Figure 4(a)). In cells incubated with bacteria and LMM fractions, upregulations of CK18 were observed: in the presence of *P. intermedia*, both chicory and mushroom fractions induced an increase in CK18 mRNA levels with respect to control cells (+58% and +133%, resp.; $P \leq 0.05$); a larger increase was observed in the presence of *A. naeslundii*

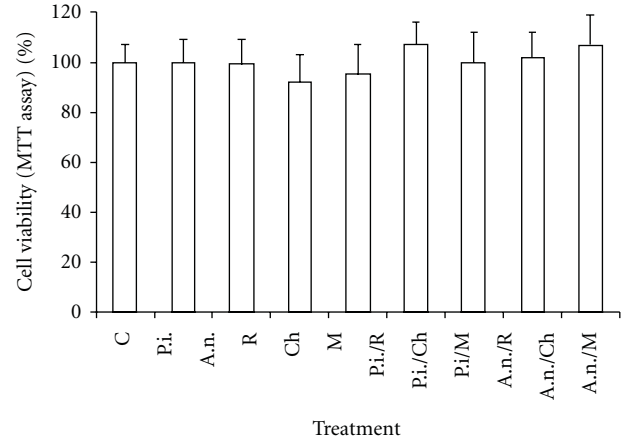


Figure 3: Effects of heat killed *P. intermedia* and *A. naeslundii,* and of Chicory and Mushroom LMM fractions on KB cell viability. Cells were exposed to bacteria (at the nominal bacteria: KB cell ratio of 50) and fractions (0.1x), alone and in combination, for 6 h. Cell viability was evaluated by the MTT assay as described in Methods. Data, expressed as percent cell viability with respect to controls, represent the mean ± SD of four experiments in triplicate. P.i.: *P. intermedia*; A.n.: *A. naeslundii*; Ch: Chicory; M: Mushroom; P.i./Ch: *P. intermedia*/Ch; P.i./M: *P. intermedia*/M; A.n./Ch: *A. naeslundii*/Ch; A.n./M: *A. naeslundii*/M.

which was induced by both chicory and mushroom (+269% and +247%, resp., $P \leq 0.05$) (Figure 4(a)).

Changes in β4ITG expression were observed with heat-killed bacteria alone, with *P. intermedia* inducing an increase (+119%; $P \leq 0.05$), and *A. naeslundii* a decrease (−30%; $P \leq 0.05$) in the level of β4ITG mRNA (Figure 4(b)). Both chicory and mushroom LMM fractions alone caused a decrease in β4ITG expression (−39% and −54%, resp.; $P \leq 0.05$) (Figure 4(b)). However, both fractions prevented the upregulation of β4ITG induced by *P. intermedia* and the downregulation of β4ITG induced by *A. naeslundii*.

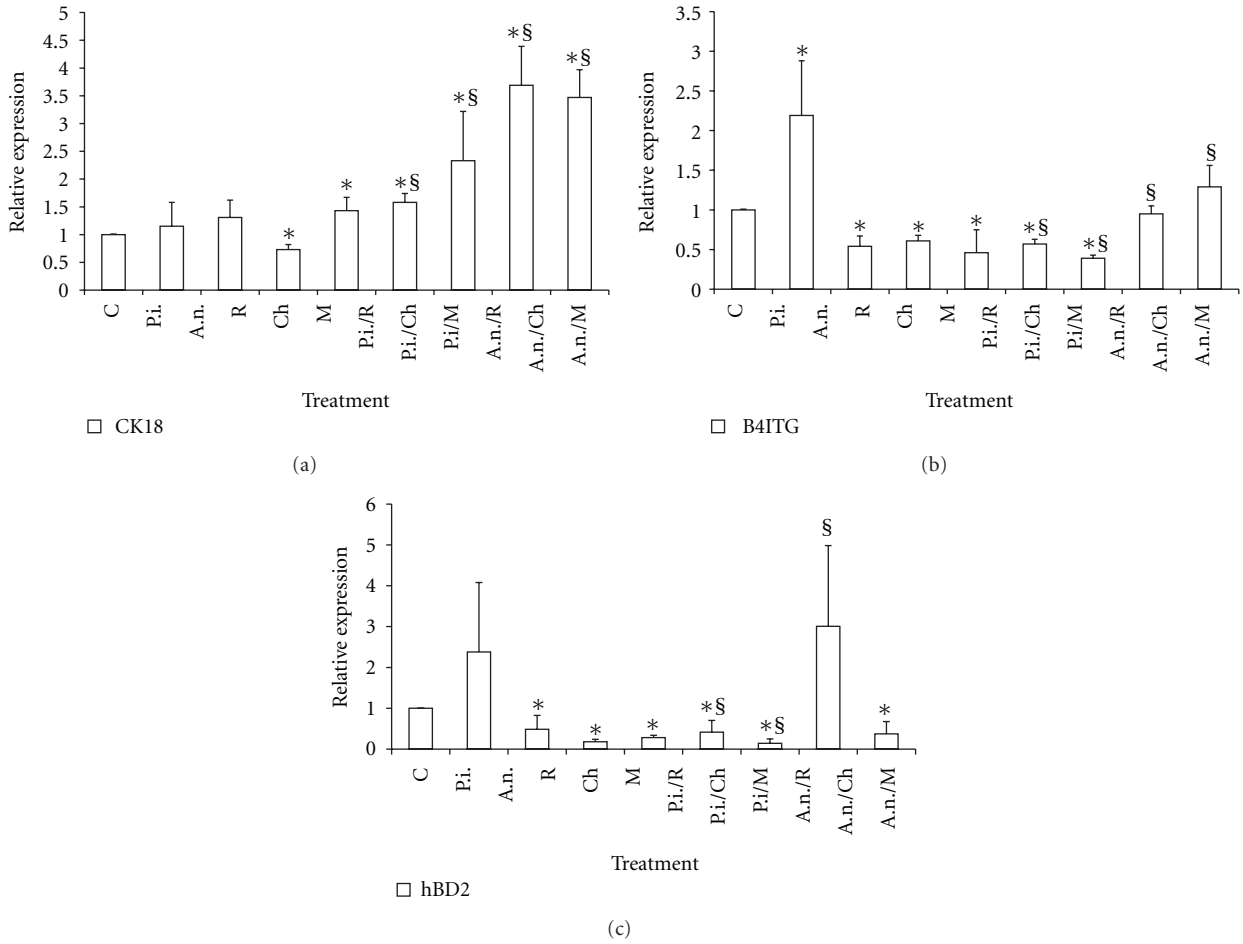


Figure 4: Effects of heat killed *P. intermedia* and *A. naeslundii,* and of Chicory and Mushroom LMM fractions on expression of Cyt18 (a), 4βINT (b), and HβD2 (c) in KB cells. Cells were exposed to bacteria (at the nominal bacteria: KB cell ratio of 50) and fractions (0.2x), alone and in combination, for 4 h. Gene expression was evaluated by RT-Q-PCR as described in Methods. Data, expressed as relative expression with respect to controls, represent the mean ± SD of four experiments in triplicate. Abbreviations as in legend to Figure 4. $^*= P \leq 0.05$: all treatment versus controls; § = $P \leq 0.05$: bacteria/LMM fractions versus bacteria alone. Mann-Whitney *U* test.

In these experimental conditions, changes in expression of the defensin gene HβD2 were also observed (Figure 4(c)). *P. intermedia* alone induced a 2-fold increase in the level of HβD2 mRNA; however, the effect was not significant due to large sample variability. In contrast, *A. naeslundii* alone induced a decrease in transcription of HβD2 (−52% with respect to controls; $P \leq 0.05$). Even larger decreases were observed with chicory and shiitake mushroom LMM fractions alone (−80% and −70%; $P \leq 0.05$). When tested in combination with *P. intermedia*, both fractions prevented the increase in HβD2 expression induced by bacteria. Moreover, chicory fractions prevented the decrease in HβD2 expression induced by *A. naeslundii*, whereas mushroom was ineffective.

4. Discussion

In this work, the effects of different fruit and vegetable LMM fractions, alone or in combination with the gingivitis-associated bacteria *P. intermedia* and *A. naeslundii*, on cell viability and gene expression by human KB gingival cells were evaluated. In preliminary experiments, different concentrations of LMM fractions and times of exposure with bacteria and fractions were tested; however, since loss in cell viability was observed in different experimental conditions, assays with live bacteria were carried out at 4 h of exposure and at a concentration of LMM fractions of 0.2x, whereas assays with heat-killed bacteria were carried out for longer times (6 h) in the presence of lower concentrations of LMM fractions (0.1x). In these conditions, no effects on cell viability were observed, as evaluated by the MTT assays, that measures the number of live and metabolically active cells.

The possible effects of bacteria and LMM fractions, alone and in combination, on the expression of selected genes associated with cell differentiation, proliferation, and adhesion, and the production of antimicrobial peptides were evaluated. Significant changes in expression of Cyt18, β4ITG, and HβD2 were observed in different experimental conditions. When expression of other genes such as KGFR (keratinocyte growth factor receptor), β1 integrin, or β3 defensin (HβD3) was evaluated, no changes were induced by different bacteria and LMM fractions, alone and in

combination (data not shown). The lack of effect could be probably due to the short duration of the experiments (4–6 h) that did not allow appreciable changes in the level of mRNAs for these genes to be observed.

Cytokeratins belong to intermediate filament proteins and are characterized by remarkable biochemical diversity, represented in epithelial tissues by at least 20 different polypeptides. Their expression varies with proliferation, differentiation, and the states of cell development and transformation [13]; Cyt 18 in particular has been utilized as a marker for healthy gingival cells in culture [4, 14]. Cytokeratins are connected through transmembrane cell-matrix junctional complexes, the hemidesmosomes, to the ECM [15]. Integrins, a component of hemidesmosomes, are heterodimeric transmembrane glycoproteins formed by the noncovalent association of α and β subunits [16]. β1 and β4 (β4ITG) are also involved in apical migration of junctional epithelium during the periodontal attachment loss [17]. β4ITG is expressed in the basal side of cells cultured from rat oral epithelium [18]. Cytokeratins and integrins are important proteins in regenerating oral epithelium [19].

Distinct changes in gene expression were observed in response to live and heat-killed bacteria, alone and in combination with LMM fractions. At 4 h incubation with live bacteria and fractions, when no changes in cell viability were observed, only changes in Cyt18 e 4βINT could be detected in different experimental conditions. Both live *P. intermedia* and *A. naeslundii* induced downregulation of Cyt18. The effect was reduced by both raspberry and chicory LMM fractions, whereas Mushroom was ineffective.

All live bacteria and fractions alone induced a decrease in expression of 4βINT. Interestingly, the effect of *P. intermedia* was prevented by Raspberry fractions and that of *A. naeslundii* by raspberry and mushroom, whereas chicory was ineffective. This indicates that simultaneous exposure to bacteria and LMM fractions in certain combinations has a synergistic protective effect on the expression of 4βINT.

Overall, these data indicate a protective effect of LMM fractions (raspberry > chicory > mushroom) on the decrease in the expression of genes involved in gingival cell proliferation and differentiation, as well as in cell attachment and signalling, induced by live *P. intermedia* and *A. naeslundii*.

Distinct effects were observed in experiments carried out with heat-killed bacteria at longer times of incubation and lower concentrations of LMM chicory and mushroom fractions. Heat-killed bacteria alone did not affect Cyt18 expression at 6 h incubation, whereas small decreases and increases were observed with chicory and mushroom fractions, respectively. Interestingly, combined exposure to bacteria and LMM fractions induced small upregulation of Cyt18 expression. This synergistic action indicates a beneficial effect of chicory and mushroom fractions on cells exposed to heat killed bacteria. *P. intermedia* alone induced an increase in expression of 4βINT, whereas all the other individual treatments resulted in downregulation. Both chicory and mushroom prevented the upregulation of 4βINT induced by *P. intermedia*. Moreover, combined exposure to *A. naeslundii* and LMM fractions abolished the downregulation induced by individual treatments, again indicating a synergistic effect between LMM fractions and heat-killed bacteria.

In these experimental conditions, changes in expression of the antimicrobial peptide HβD2 were also observed. Antimicrobial peptides (AMPs) are components of the host innate immune defence system, exerting broad-spectrum antimicrobial activity via the binding and perforation of cell membranes [20, 21], as well as exerting neutralising effects on the LPS activity of Gram negative bacteria, including periodontopathogens such as *P. intermedia* [22]. Human β-defensins are small, cationic AMPs: β-defensin 1 (HβD1) is expressed constitutively in epithelial tissues, whereas HβD2 and HβD3 are expressed in response to bacterial stimuli or inflammation [23–26]. Defensins, including HβD2, may also participate in epithelial differentiation and tissue damage repair in periodontal disease and contribute to the host defense by recruiting neutrophils to the site of inflammation and modulate the expression of cytokines, thus playing an important role in the control of oral health [22, 26–31]. Gingival epithelial cells and tissue express HβD2 mRNA and peptide in response to inflammatory mediators and challenge from commensal bacteria naturally present in the oral cavity [32]. In gingival epithelial cells, HβD2 mRNA was induced in response to the supernatant from *Porphyromonas gingivalis*, and this expression might be associated with periodontal health and disease [33]. Cell wall components of periodontal pathogens induce HβD2 expression through activation MAP kinases [34]; such effect can be mediated by activation of fibronectin-integrin components [35].

The results here obtained with heat-killed *P. intermedia* and *A. naeslundii* and with chicory and mushroom LMM fractions show that individual treatments induced changes in the level of HβD2 mRNA similar to those observed for that 4βINT, with upregulation by *P. intermedia* and downregulation by *A. naeslundii* and LMM fractions. Both chicory and mushroom prevented the upregulation of HβD2 induced by *P. intermedia* and combined exposure to *A. naeslundii* and LMM fractions abolished the downregulation induced by individual treatments. Again, the results indicate a comparable effect of Chicory and Mushroom LMM fractions on the overall changes in gene expression induced by heat-killed gingivitis-associated bacteria.

5. Conclusions

On the basis of the results of various assays, reported elsewhere in this issue, raspberry, chicory, and mushroom LMM fractions were chosen as the most active test materials towards the gingivitis-associated species *P. intermedia* and *A. naeslundii* [5]. With respect to studies of coaggregation, biofilm formation, and adhesion to hydroxyapatite and cultured gingival cells [5], in this work LMM fractions were utilised at lower concentrations and longer times of exposure in order to maintain cell viability and allow for detection in changes in mRNA transcription, respectively. Our data indicate that both chicory and mushroom LMM fractions, at concentrations that do not affect viability of KB gingival cells, can modulate the expression of different genes induced by both live and heat-killed *P. intermedia* and

A. naeslundii. This supports the hypothesis that chicory and mushroom LMM fractions can modulate the responses of gingival cells to periodontopathogens and can be used as source for obtaining agents to be included in toothpaste, mouthwashes, and other oral health care products.

Acknowledgments

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 135034, 10 pages
doi:10.1155/2011/135034

Research Article

The Effects of Fractions from Shiitake Mushroom on Composition and Cariogenicity of Dental Plaque Microcosms in an *In Vitro* Caries Model

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Received 14 June 2011; Accepted 14 July 2011

Academic Editor: Itzhak Ofek

The aim of the current study was to investigate the anticariogenic potential of the (sub)fractions obtained from the edible mushroom shiitake (*Lentinula edodes*) in *in vitro* caries model. We used a modified constant depth film fermentor (CDFF) with pooled saliva as the inoculum and bovine dentin as a substratum. The test compounds were low molecular weight fraction (MLMW) of the shiitake extract and subfractions 4 and 5 (SF4 and SF5) of this fraction. Chlorhexidine (CHX) and water served as a positive and a negative control, respectively. Dentin mineral loss was quantified (TMR), microbial shifts within the microcosms were determined (qPCR), and the acidogenicity of the microcosms was assessed (CIA). From the compounds tested, the SF4 of shiitake showed strong inhibiting effect on dentin demineralization and induced microbial shifts that could be associated with oral health. The acid producing potential was increased, suggesting uncoupling of the glycolysis of the microbiota by the exposure to SF4. In conclusion, the results suggest that SF4 of shiitake has an anticariogenic potential.

1. Introduction

Over the past decade, there has been an increasing number of food alerts creating a genuine crisis of confidence among consumers. Research on food safety and quality must therefore be a priority. To improve our understanding of the link between food and oral health, an international EU sixth framework program consortium project (NUTRIDENT, FOOD-CT-2006-36210) was granted with an overall aim to identify beverage/food constituents that are able to reduce the risk of two major dental diseases, caries and gingivitis.

Within the NUTRIDENT project, we have used the existing literature as a starting point for selecting foods or beverages that may contain such constituents. We have then built upon this knowledge by testing such materials for a range of biological activities that are relevant to the maintenance of oral health, that is, the prevention of caries and gingivitis. A number of high-throughput assays were designed and employed [1]. As a result of this work, we identified a low molecular mass extract (<5,000 Da) of shiitake mushroom (*Lentinula edodes*) which has biological activities which, if displayed *in vivo*, could protect against dental caries. The most prominent observed biological activities of this extract, relevant to caries prevention, were (1) induction of the detachment of cariogenic microorganisms from hydroxyapatite, (2) changed cell surface hydrophobicity, (3)

bactericidal activity against cariogenic microorganisms, (4) prevention of coaggregation of the microorganisms, and (5) disruption of signal transduction in *Streptococcus mutans* [1, 2]. The extract from *Lentinula edodes* has been studied in rats, and an inhibitory effect on one of the virulence factors of *S. mutans* has been demonstrated: the extract inhibited water-insoluble glucan formation by glycosyltransferases of this organism [3]. There are few reports related to general antimicrobial effects of different compounds obtained from shiitake. Aqueous extract from *L. edodes* has shown high antimicrobial activity against food-borne pathogenic bacterial strains [4]. Furthermore, a diet containing 5% of dried *L. edodes* consistently resulted in lower viable counts of total bacteria, *Escherichia coli*, streptococci, and lactic acid-producing bacteria in the intestinal microbiota of piglets [5].

Dental caries is a multifactorial disease with low pH as a driving force for mineral dissolution. We have developed an *in vitro* dental caries biofilm model [6, 7] which combines cariogenic microorganisms with dental hard tissue substratum (dentin or enamel) and allows modeling of frequent acid challenges by sucrose pulsing within a constant depth film fermentor (CDFF) [8]. The complexity of the model can be varied by selecting defined microbial consortia or saliva-derived microcosms as inocula [8, 9]. Different output parameters, related to the cariogenic potential of the biofilms, can be assessed within this model system. The most relevant output for the anticariogenicity tests is mineral loss quantification [10]. Thus, any compound with anticariogenic activity claims should result in inhibited mineral loss in *in vitro* or *in situ*-tests or ultimately, in reduction of caries, *in vivo*. Other, the so-called surrogate output parameters include reduction of acid producing potential of the biofilms and microbial shifts towards health-associated microorganisms.

The aim of the current study was to assess the effects of low molecular weight fraction of shiitake mushroom and two subfractions of this fraction on dentin demineralization, microbial composition, and acidogenic potential of saliva-derived microcosms in our CDFF caries model.

2. Materials and Methods

2.1. Preparation of Fractions and Subfractions Obtained from Shiitake Mushroom Extract. The freeze-dried test compounds were obtained as described by Daglia et al. [2], reconstituted to the original 2x concentration in ultrapure (MilliQ, Millipore) sterile water and stored at −20°C until used. Before each experiment, a frozen aliquot of the test compound was thawed and diluted 1 : 10 in Millipore grade sterile water. The treatment solutions contained 0.2x diluted low molecular weight fraction of shiitake mushroom (MLMW) extract, 0.2x diluted subfraction 4 of MLMW and 0.2x diluted subfraction 5 of MLMW.

2.2. Constant Depth Film Fermentor (CDFF) Experiments. Plaque microcosms were grown on dental hard tissue specimens in a modified CDFF model [7]. The modification of the CDFF involves operating it in a reciprocal mode: the turntable rotates 180 degrees back and forth, allowing two simultaneous growth conditions (two treatment modes) within one CDFF run. Sucrose pulses, media flow, and the treatments are provided by calibrated, computer-controlled peristaltic pumps (Type MS-4/6-100, Ismatec, Zürich, Switzerland) under software developed in LabView (National Instruments).

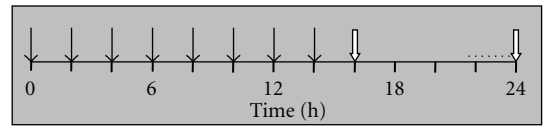

Figure 1: A diagram of daily CDFF regime for 8 days. On each day, a DMM supply (solid horizontal line) was interrupted by eight 2-hourly 5 min sucrose pulses (black arrows) and two 10 min treatments (white arrows). The treatments occurred 2 h after the last sucrose pulse and 20 min before the end of the "resting period" (DMM supply for 10 h). Dashed horizontal line indicates the sampling time on day 8.

Coronal dentin from bovine incisors was cut into 5 mm diameter discs and was recessed into PTFE pans and assembled into the turntable of the CDFF. Plaque-enriched saliva from 10 healthy individuals (no use of antibiotics in the last 3 months) was collected and pooled at equal volumes, 10% glycerol added and stored in 10 mL aliquots at −80°C. To inoculate each CDFF run, 9 mL of thawed pooled saliva was mixed with 200 mL defined mucin medium (DMM) [11]. The inoculum was pumped into the CDFF for 1.5 h (flow rate 2.3 mL/min) with the CDFF operated in the conventional mode (360° rotation). One hour elapsed between the end of inoculation and the start of DMM flow. Then, DMM was supplied through two delivery inlets at 0.3 mL/min per inlet with CDFF still operating in the conventional mode. The CDFF was operated at 37°C and under a continuous gas supply of 10% CO_2 and 10% H_2 in N_2 at a flow rate of 50 mL/min ± 0.5 mL/min.

After 24 h, the CDFF was switched to the reciprocal mode (180° oscillation) and the first 10 min treatment was started. Sterile water was used as a negative control of the treatment on one side of each CDFF run, while positive control (0.12% chlorhexidine digluconate solution) or one of the test solutions was applied on the opposite side of the CDFF. After a 10 min resting period, the LabView program with daily treatment regimen was started. The cariogenic potential of the microcosm was modelled by frequent (eight 2-hourly 5 minute pulses/day, flow rate 1.2 mL/min/inlet) pulses with 10% (w/v) sucrose solution. "Night" or remineralization phase was simulated by daily 10 h period of DMM supply alone. Summary of the daily regimen is shown in Figure 1. Two 10 min treatments were given each day at a rate of 1.0 mL/min. The first treatment commenced 2 h after the last sucrose pulse, while the second treatment started 20 min before the end of the "Night" period.

Two independent CDFF runs per compound (three test compounds and one positive control) were performed.

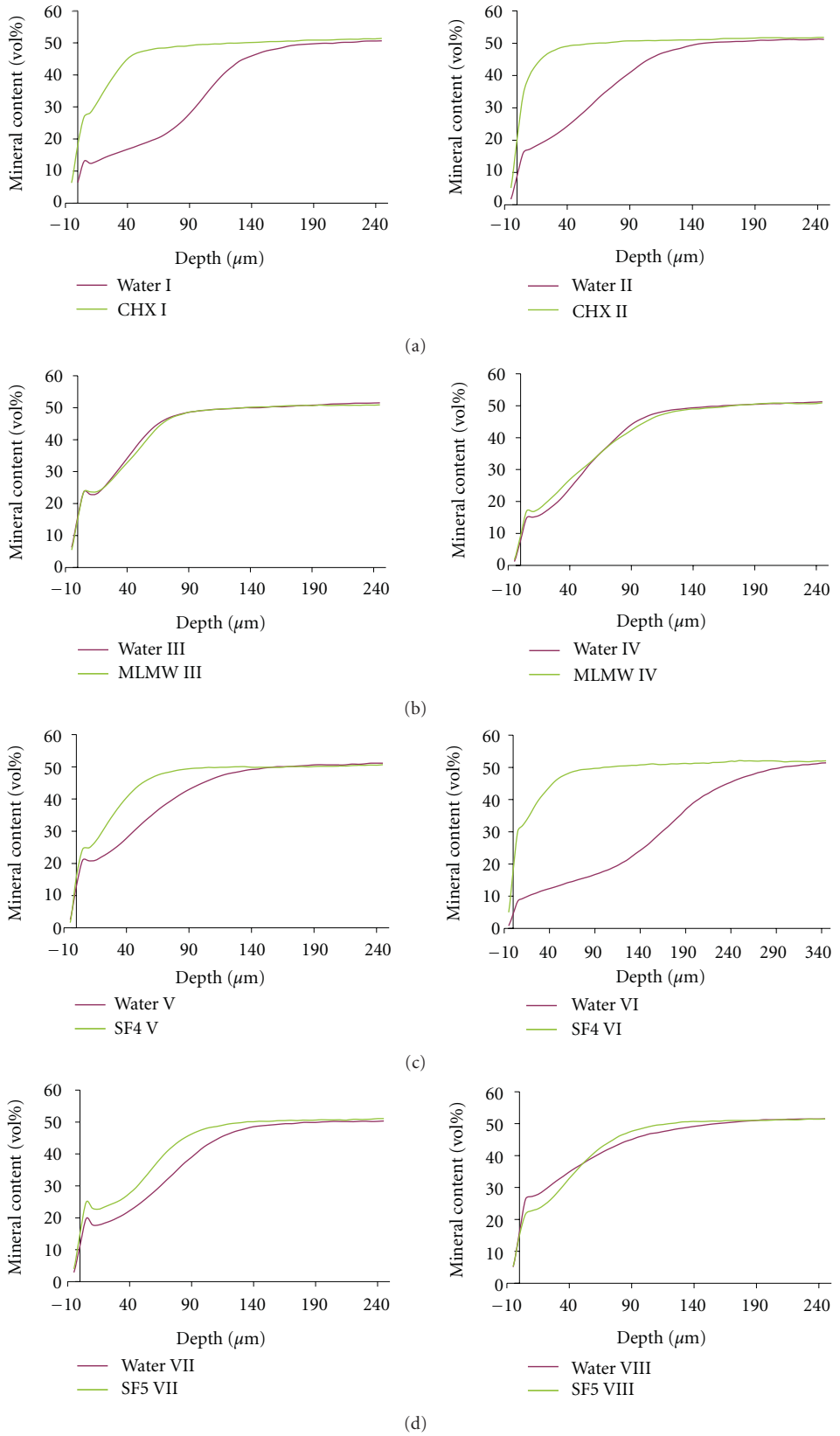

Figure 2: Average mineral content profiles showing the extent of demineralization of dentin after microcosm growth for 8 days in constant depth film fermentor (CDFF) per CDFF run (Roman numbers I–VIII—eight CDFF runs) and per treatment: (a) chlorhexidine digluconate (CHX), (b) mushroom low molecular weight fraction (MLMW), (c) subfraction nr 4 (SF4), and (d) Subfraction nr 5 (SF5) of the MLMW fraction of shiitake extract. The two graphs per treatment are the profiles obtained from the duplicate CDFF runs of the respective treatment. Each profile is an average of the transversal microradiography images obtained from 10 dentin specimens.

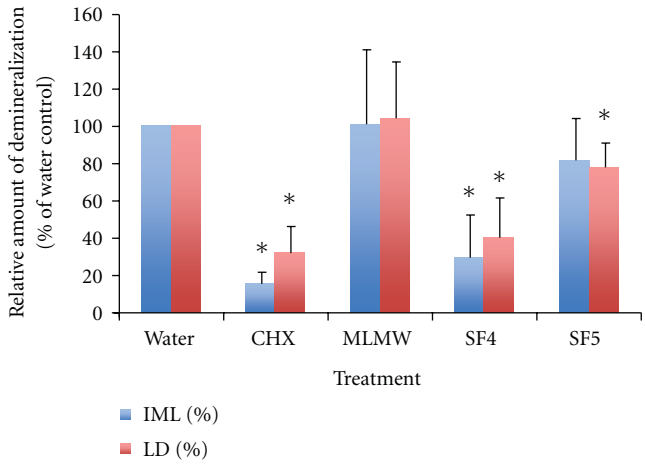

FIGURE 3: Average amount of demineralization (integrated mineral loss, IML, and lesion depth, LD) relative to the respective water control of each treatment (IML and LD of the water group were set to 100% to normalize the data among different CDFF runs). CHX—chlorhexidine digluconate; MLMW—mushroom low molecular weight fraction; SF4—subfraction nr 4 of MLMW fraction; SF5—subfraction nr 5 of MLMW fraction. $N = 20$ samples per treatment. Error bars indicate standard deviation. *One sample t-test, treatment versus 100% (water control), significantly different at $P < 0.001$.

2.3. Sampling and Sample Processing. On day 8, two CDFF sample pans per each treatment group were removed, resulting in 10 samples per treatment group. Sampling occurred during the resting period, 7.5–9.5 h after the last sucrose pulse and 5.5–7.5 h after the last treatment (dashed line in Figure 1).

Immediately after the retrieval from the CDFF, the biofilm was removed from the dentin surface by scraping against the lid of an Eppendorf tube. Subsequently, the biofilm was centrifuged for 30 s at 16,060 ×g and either 1 mL 1% glucose in buffered peptone water (BPW) solution (fermenting plaque) or BPW solution alone (resting plaque) was added to the biofilm and incubated at 37°C for 30 min. After that, the vials were cooled on ice. The samples were heated at 80°C for 5 minutes and again cooled on ice [12]. The vials were centrifuged at 16,060 ×g for 15 minutes at 4°C. From the supernatant, 200 μL were transferred into a vial with a microspin filter (Ultrafree-MC 0.22 μm, Millipore, Bedford, Mass, USA) and centrifuged at 13,684 ×g for 5 minutes at 4°C; the remaining supernatant was discarded. The filtered supernatants for organic acid determination and the pellets for protein analysis were stored at −80°C until further analyses. The dentin discs were stored at 4°C for the assessment of mineral loss by transverse microradiography (TMR).

Organic acids were determined as their anions by capillary electrophoresis on the Waters Capillary Ion Analyzer (Milford, Mass, USA) [13]. Sodium salts of formic, acetic, propionic, butyric, succinic, and lactic acids were used to prepare single and mixed standard solutions in ultrapure water (for calibration curves for each acid separately). Oxalic acid was included in all samples as an internal standard. Formic, butyric, succinic, propionic, acetic, and lactic acid were determined in duplicate samples.

The amount of acid was normalized by amount of protein/sample. The protein amount was determined by Bradford protein analysis method [14]. Results were expressed as μg protein/sample.

Dentin discs were sectioned and processed for transverse microradiography (TMR) as described elsewhere [15]. In brief, 200 μm thin dentin sections were radiographed together with an aluminium stepwedge on a high-resolution film with a nickel-filtered Cu-Kα source. The radiographic image was analyzed with a microscope-videocamera-microcomputer setup and dedicated software (TMR 2000, version 2.0.27.13, Inspektor Research Systems, Amsterdam, The Netherlands). Data obtained were the mineral content profiles of the lesions, lesion depth, and the total amount of mineral removed (integrated mineral loss).

The numbers of *Streptococcus sanguinis, Streptococcus mutans, Lactobacillus casei, Veillonella dispar, Neisseria subflava, Actinomyces naeslundii, Prevotella intermedia, Fusobacterium nucleatum,* and total bacterial 16S rDNA were determined by using multiplex quantitative PCR (qPCR) [16]. In brief, DNA was extracted from plaque biofilms using a phenol : chloroform : iso-amyl alcohol (25 : 24 : 1) bead-beating extraction method [17], which involves physical cell lysis, protein removal, and finally DNA precipitation using polyethylene glycol. Three triplex qPCR assays were then carried out using 2 μL extracted DNA to enumerate eight oral taxa as well as the total number of organisms. The assays were performed using the Rotor-Gene 6500 (QIAGEN) instrument and Sensimix Probe (Bioline) qPCR mix according to manufacturer's instructions using previously published oligonucleotide sequences [16].

2.4. Statistical Analyses. The effects of the treatments on dentin demineralization (integrated mineral loss, IML, and lesion depth, LD), biomass (protein amount), and acidogenicity of biofilms (acetate, lactate, propionate, succinate, butyrate, and formate) were assessed by independent samples t-test and by one-sample t-test, where the data in each treatment group was calculated as a relative proportion of the average value from the respective water group (negative control), set at 100%. The qPCR data were log transformed and used as absolute values and as a proportion of the total counts (universal probe counts) in statistical comparisons between the treatment groups and their respective controls, using Mann-Whitney U test. All tests were performed in SPSS, version 17.0. Significance level was set at 0.05.

3. Results and Discussion

In this study, we grew saliva-derived microcosms at the conditions that mimicked a cariogenic situation *in vivo* (eight daily sucrose pulses). We also allowed recovery of the microbial ecosystem during a daily resting period of 10 h. This in turn mimicked a night period *in vivo*. Biofilms grown in all eight constant depth film fermentor (CDFF) runs

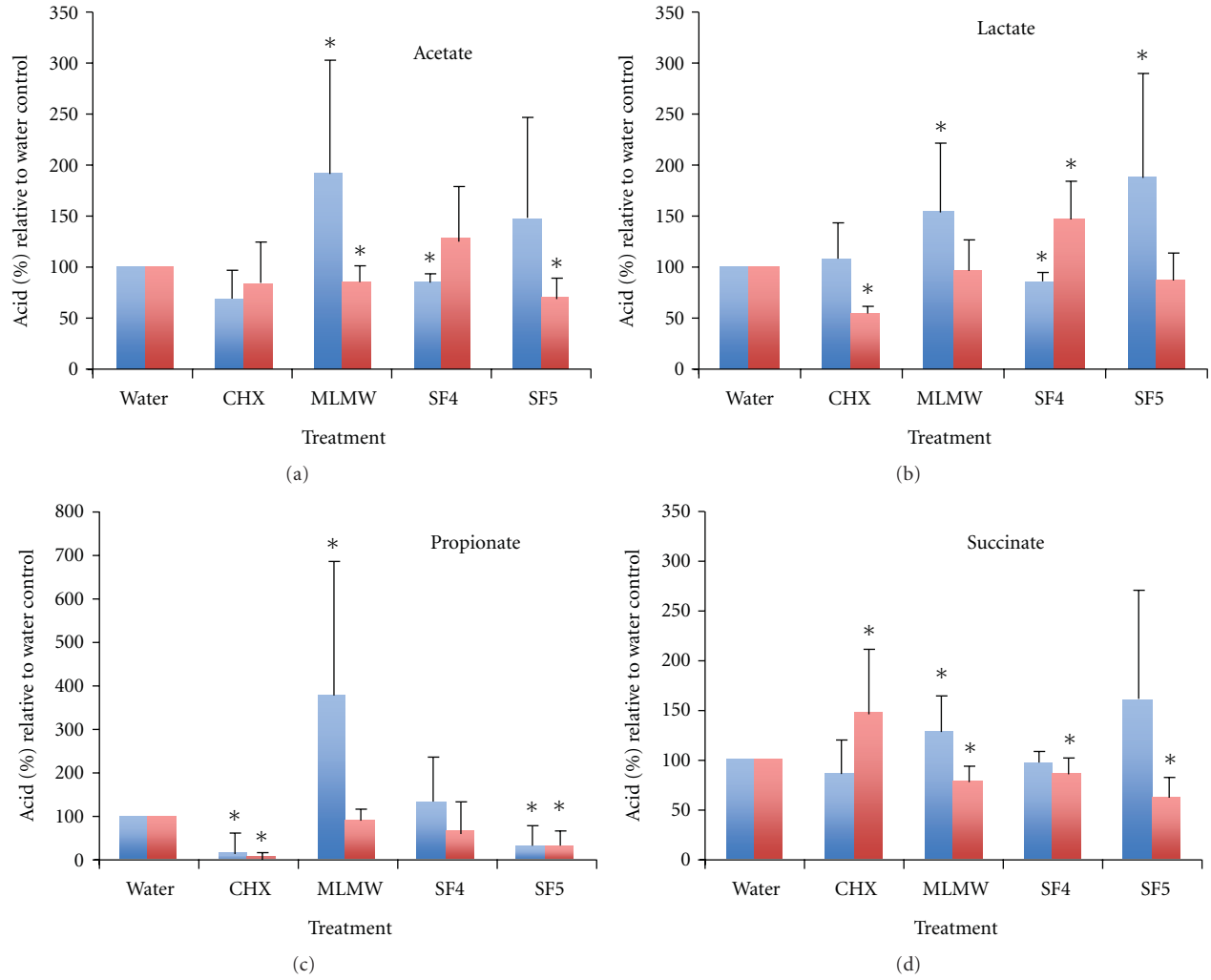

Figure 4: Amount of (a) acetate, (b) lactate, (c) propionate, and (d) succinate in 8-day microcosms at a resting state (blue bars) and at a fermenting state after 30 min incubation with 1% glucose (red bars) in the treatment group samples (CHX—chlorhexidine digluconate; MLMW—mushroom low molecular weight extract; SF4—subfraction nr 4 of MLMW extract; SF5—subfraction nr 5 of MLMW extract) relative to the samples exposed to water (negative control). Treatments were performed twice daily for 10 min (Figure 1). *One sample t-test, treatment versus 100% (water control), significantly different at $P < 0.001$.

resulted in demineralization of the underlying substratum—bovine dentin (Figure 2 and Table 1). There was a large variation among the individual CDFF runs in the amount of mineral loss and the depth of the lesions (red mineral profiles in Figure 2 represent eight individual CDFF runs (I-VIII) exposed to water). This underlines the difficulty of controlling the complex ecological systems such as microcosms derived from natural sources [18]. The reciprocal mode of the CDFF (adaptation to the CDFF with back-and-forth movement of the rotating pane [7]) allowed within each individual run the growth of both, the treated and the negative control (water) exposed biofilms. This provided the internal control to each of the treatment groups, allowing statistical comparisons between the matched test and control samples.

Twice daily exposure to the positive control, 0.12% chlorhexidine digluconate (CHX), had pronounced inhibitory effect on the cariogenic potential of the microcosms. This was seen as statistically significantly inhibited dentin demineralization (average mineral content profiles in Figure 2; IML and LD in Figure 3, Table 1), biomass (protein amount in Table 1), and main organic acids (Table 1), as well as significantly reduced absolute microbial counts (Table 2) compared to the water-exposed samples. CHX is a broad-spectrum antimicrobial agent that has been proven to have clinical antiplaque and antigingivitis effects [19] and has been shown to inhibit acids in resting and fermenting plaque [20, 21]. The previous reports on CDFF-grown biofilms treated with CHX range from little or some effects on biofilm viability and composition [22, 23] to nearly complete inhibition of the biofilm [7], depending on the type and complexity of the inoculum, growth medium, substratum, and many other parameters, such as the exposure time and the clearance of the antimicrobial from the system. Our

results with CHX showed that the conditions we have chosen were appropriate to use for the series of experiments with the test compounds that were selected in the high-throughput tests [1].

The test compounds that we have tested were derived from natural edible mushroom shiitake, *Lentinula edodes*, as described by Daglia et al. [2]. Using our CDFF model, we tested the anticariogenic potential of the low molecular weight fraction of the mushroom extract (MLMW) and the two subfractions from this fraction—subfraction 4 (SF4) and subfraction 5 (SF5). Among the three test compounds, the SF4 showed the strongest anticariogenic potential. The twice daily treatment with SF4 highly inhibited demineralization of dentin, resulting in significantly reduced IML and LD compared to the respective water control samples (Figures 2 and 3 and Table 1). The treatment with SF5 showed some, though still statistically significant, reduction of lesion depth, LD, while the treatment with MLMW did not have any significant effects on the demineralization of dentin (Figure 3 and Table 1). The average mineral content profiles (Figure 2) showed that only one of the two replicate experiments with SF5 (CDFF VII but not CDFF VIII) resulted in slight inhibition of demineralization of dentin, rendering the results of SF5 inconclusive.

Unlike CHX, none of the test compounds affected the biomass of the microcosms, that is, there were no significant differences in either protein amount (Table 1) or universal 16S rDNA probe counts (Table 2) between the treated and the respective water-control samples. However, the microbial community composition in the SF4-exposed samples was significantly changed, compared to the water-control samples. SF4 significantly increased absolute counts of *Fusobacterium nucleatum* and *Neisseria subflava* (Table 2), and the relative proportions of these two microorganisms, together with the relative proportions of *Veillonella dispar* and *Actinomyces naeslundii* (Figure 5). At the same time, the proportion of the other microorganisms that were not targeted in this study, but were quantified as the difference between the universal 16S rDNA probe counts and the sum of the targeted species, significantly decreased. Thus, the effects of the SF4 were beyond the selected targeted species for this study and have affected other microorganisms as well. The MLMW-exposed biofilms showed statistically significantly increased absolute counts of *F. nucleatum*, while there were no significant effects on the proportions of different microorganisms induced by this compound. The SF5-exposed biofilms showed shifts in microbial community composition similar to the shifts induced by SF4; however, none of these shifts reached statistical significance (Figure 5). Both, veillonellae and neisseriae are microorganisms associated with oral health [24], while fusobacteria are a part of noncariogenic resident oral flora, also known as a bridging organism in maturing dental biofilm [25]. Increase in amount and/or proportions of these noncariogenic microorganisms under highly cariogenic conditions in our CDFF microcosms indicates occurrence of health-associated microbial shifts due to the exposure to the shiitake-derived test compounds.

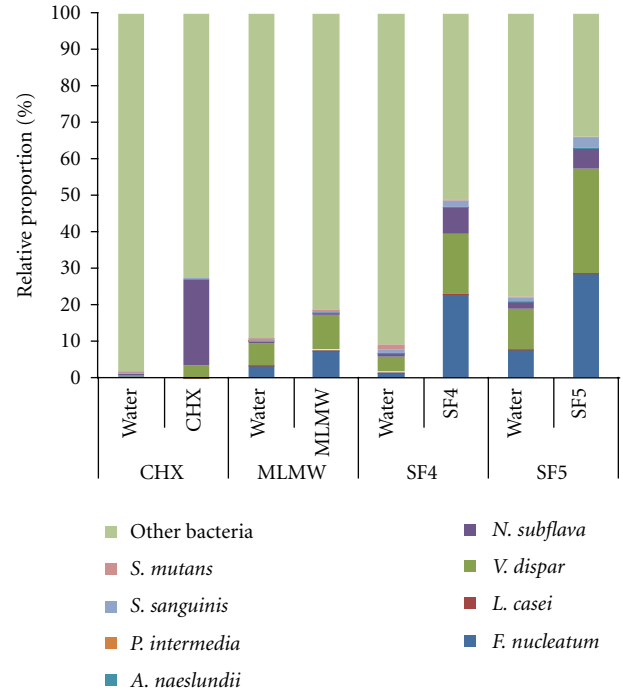

Figure 5: Relative proportions of microorganisms in microcosm samples. The other bacteria were calculated as the difference between the universal probe counts and the sum of the 8 targeted probe counts. The data are average from 6 samples obtained in 2 CDFF runs per treatment. CHX—chlorhexidine digluconate; MLMW—mushroom low molecular weight fraction; SF4—subfraction nr 4 of MLMW fraction; SF5—subfraction nr 5 of MLMW fraction.

There were no strong and conclusive inhibitory effects of the test compounds on the acidogenicity of the microcosms observed. SF4 showed some inhibitory activity on biofilms at the resting state: there was significantly less acetate (Figure 4) and lactate (Table 1, Figure 4) in the SF4-exposed resting biofilms compared to the water-exposed biofilms. In contrary, the glucose-fermenting SF4-exposed biofilms produced significantly more lactate than their respective water controls (Table 1 and Figure 4). Similar effects were observed in high-throughput acidogenicity assays with single species biofilms of *S. mutans* [1]. The SF5-exposed biofilms produced less propionate in the resting and the fermenting biofilms, and less acetate and succinate in the fermenting samples, while the resting samples showed significantly more lactate compared to the water control (Table 1 and Figure 4). The MLMW-exposed resting state biofilms had increased amounts of all major acid anions measured, including lactate, and reduced amounts of acetate and succinate in the fermenting samples. Increased lactate production during the 30 minute incubation with glucose suggests enhanced acidogenic potential of the biofilms previously exposed to the test compounds. This however did not result in increased cariogenic activity (demineralization of the dentin substratum in our CDFF model). In contrary, exposure to SF4 strongly inhibited demineralization (Figures 2 and 3). The reasons for the increased lactate production are not

Table 1: Summary of the mineral loss data (IML—integrated mineral loss, and LD—lesion depth) from the dentin specimens ($N = 20$ per treatment or control) that were used as substrata for biofilm growth in CDFF for 8 days; amount of protein (protein) and amount of main acid anions (acetate, lactate, propionate, and succinate) in the resting state (Resting) and after 30 min incubation with 1% glucose (Fermenting) of the microcosm biofilms ($N = 10$ per treatment or control). The data are average and standard deviations (SD) from samples obtained from 2 CDFF runs per treatment. CHX—chlorhexidine digluconate; MLMW—low molecular weight fraction of mushroom; SF4—subfraction nr 4 of MLMW fraction; SF5-subfraction nr 5 of MLMW fraction.

Negative control (water) and the respective treatments	IML vol% × μm (SD)	LD μm (SD)	Protein μg (SD)	Acetate nmol/sample (SD)		Lactate nmol/sample (SD)		Propionate nmol/sample (SD)		Succinate nmol/sample (SD)	
				Resting	Fermenting	Resting	Fermenting	Resting	Fermenting	Resting	Fermenting
Water (CHX)	2948 (1072)	122 (29)	104 (44)	1051 (294)	947 (163)	674 (205)	1878 (504)	198 (85)	457 (240)	905 (327)	718 (166)
CHX	508* (263)	43* (20)	53* (30)	728* (385)	789 (427)	752 (395)	1021* (387)	28* (86)	27* (87)	809 (399)	1021* (373)
Water (MLMW)	1696 (678)	83 (24)	179 (60)	940 (451)	1273 (372)	595 (183)	1986 (462)	168 (111)	506 (190)	658 (227)	826 (277)
MLMW	1717 (842)	87 (29)	194 (172)	1488* (436)	1102 (321)	851* (248)	1940 (684)	418* (144)	469 (239)	805 (103)	639 (99)
Water (SF4)	4293 (2536)	168 (69)	135 (44)	1964 (492)	1710 (157)	877 (139)	1307 (552)	117 (134)	324 (172)	1254 (235)	1292 (166)
SF4	802* (302)	57* (15)	123 (62)	1667 (145)	2189 (949)	745* (125)	1908* (316)	167 (145)	176 (178)	1214 (217)	1106* (176)
Water (SF5)	1918 (761)	113 (36)	197 (130)	2033 (500)	2733 (1438)	623 (182)	1719 (800)	7.5 (18)	407 (295)	739 (142)	1586 (967)
SF5	1537 (317)	88* (15)	124 (40)	2923* (1917)	1958 (1115)	1261 (686)	1435 (364)	75 (118)	121* (131)	1142 (720)	907* (145)

* statistically significantly different from the respective water control samples (independent samples t-test, $P < 0.05$).

Table 2: Summary of the quantitative pcr data on microcosm biofilms grown in CDFF for 8 days. The data are average log CFU-equivalents and standard deviations (SD) from 6 samples obtained from 2 CDFF runs per treatment. CHX—chlorhexidine digluconate; MLMW—mushroom low molecular weight fraction; SF4—subfraction nr 4 of MLMW fraction; SF5—subfraction nr 5 of MLMW fraction.

Negative control (water) and the respective treatments	Universal probe log CFU (SD)	*S. sanguinis* log CFU (SD)	*V. dispar* log CFU (SD)	*N. subflava* log CFU (SD)	*F. nucleatum* log CFU (SD)	*A. naeslundii* log CFU (SD)	*P. intermedia* log CFU (SD)	*L. casei* log CFU (SD)	*S. mutans* log CFU (SD)	Other bacteria[$] log CFU (SD)
Water (CHX)	6.5 (0.3)	4.3 (0.5)	4.1 (0.5)	2.4 (0.4)	3.8 (0.5)	n.d.	n.d.	0.6 (0)	3.5 (0.5)	6.5 (0.3)
CHX	5.9* (0.6)	3.3* (0.8)	3.8 (1.0)	4.3 (1.7)	2.3* (1.0)	n.d.	n.d.	0.9 (0)	n.d.*	5.8 (0.7)
Water (MLMW)	6.8 (0.2)	4.6 (0.4)	5.2 (0.8)	4.7 (0.4)	5.0 (0.8)	2.3 (1.3)	0.3 (0)	2.0 (0.8)	3.6 (0.9)	6.7 (0.3)
MLMW	7.0 (0.3)	4.5 (0.6)	5.8 (0.2)	4.5 (0.8)	5.8* (0.1)	2.2 (0.6)	0.3 (0)	2.3 (0.3)	2.7 (0)	6.9 (0.3)
Water (SF4)	6.7 (0.3)	4.4 (0.7)	5.0 (0.8)	4.6 (0.6)	4.6 (0.8)	2.7 (0)	n.d.	2.1 (0.9)	4.4 (0.8)	6.7 (0.4)
SF4	6.5 (0.3)	4.6 (0.4)	5.7 (0.3)	5.3* (0.2)	5.8* (0.3)	3.2 (0.9)	n.d.	2.0 (0.1)	3.9 (0.4)	6.2 (0.4)
Water (SF5)	6.9 (0.7)	4.3 (0.9)	5.7 (0.7)	4.9 (0.6)	5.4 (0.8)	2.7 (0.8)	n.d.	2.6 (0.3)	3.4 (1.0)	6.7 (0.8)
SF5	6.1 (0.6)	4.2 (0.7)	5.5 (0.5)	4.8 (0.4)	5.4 (0.5)	2.6 (0.9)	n.d.	2.4 (0.2)	3.6 (0.4)	5.4 (1.0)

[$]Other bacteria are calculated as a difference between universal probe counts and the sum of the 8 targeted probe counts. *Statistically significantly different at $P < 0.05$ from the respective water control (Mann-Whitney U test).

known and should be investigated further. It might be related to a phenomenon known as uncoupling [26, 27], where glycolysis is uncoupled from biomass production and enters a futile cycle. In lactic acid bacteria used in dairy industry, uncoupling has been shown to be triggered by stress factors such as subbactericidal concentrations of antimicrobials or elevated temperature [28] and leads to less efficient energy usage. Our results suggest that the subbactericidal concentrations of the SF4 of shiitake mushroom may have induced uncoupling in the biofilm cells of the plaque microcosms.

Based on the results above, we can conclude that the subfraction 4 (SF4) of the low molecular weight fraction of the shiitake mushroom has strong anticariogenic potential. This anticariogenic potential of SF4 is most likely contributable to the observed changes in microbial composition and inefficient energy usage due to uncoupling of the glycolysis.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (Project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 635348, 8 pages
doi:10.1155/2011/635348

Research Article

Effects of Mushroom and Chicory Extracts on the Physiology and Shape of *Prevotella intermedia*, a Periodontopathogenic Bacterium

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Received 15 June 2011; Accepted 11 July 2011

Academic Editor: Carla Pruzzo

Contrary to the common assumption that food has a negative impact on oral health, research has shown that several foods contain a number of components with antibacterial and antiplaque activity. These natural compounds may be useful for improving daily oral hygiene. In this study we evaluate the mode of antimicrobial action of fractions of mushroom and red chicory extracts on Prevotella intermedia, a periodontopathogenic bacterium. The minimal inhibitory concentration corresponded to 0.5x compared to the natural food concentration for both extracts. This concentration resulted in a bacteriostatic effect in mushroom extract and in a slightly bactericidal effect in chicory extract. Cell mass continued to increase even after division stopped. As regards macromolecular synthesis, DNA was almost totally inhibited upon addition of either mushroom or chicory extract, and RNA to a lesser extent, while protein synthesis continued. Cell elongation occurred after septum inhibition as documented by scanning electron microscopy and cell measurement. The morphogenetic effects are reminiscent of the mode of action of antibiotics such as quinolones or β-lactams. The discovery of an antibiotic-like mode of action suggests that these extracts can be advantageously employed for daily oral hygiene in formulations of cosmetic products such as mouthwashes and toothpastes.

1. Introduction

Periodontal diseases include a sizeable group of pathological alterations of the periodontal tissue. The commonest forms of the disease are gingivitis and periodontitis, and these figure among the most widespread human infectious diseases [1, 2]. Both are dental plaque-associated pathologies [3, 4]. Gingivitis is very common and is characterized by reversible inflammation in the marginal periodontal tissues while periodontitis is an inflammation-based infection of the support structure of the teeth with progressive destruction of them that can result in dental loss. The commonest form of periodontitis is adult chronic periodontitis [1, 2]. Although several factors including systemic pathologies such as diabetes and immunosuppression or environmental factors such as smoking are capable of contributing to the severity of periodontal

diseases, bacterial infection is considered to be the leading cause [5]. As far as adult chronic periodontitis is concerned, the role of a bacterial consortium of the dental plaque, mainly composed of gram-negative strict anaerobes has been demonstrated [5–7]. *Prevotella intermedia* is one of these potential periodontopathogenic bacterial species. Several virulence factors including fimbriae, hydrolases, hemolysins, hemagglutinins, and lipopolysaccharides have been detected in this microorganism and confer bacterial coaggregation capability, adhesion to epithelial cells, invasion of epithelial cells, and induction of inflammatory lympho-, chemo-, and cytokines [5, 8].

It is generally accepted that lowering the oral bacterial biomass is an effective method for curing and/or preventing bacterial oral pathologies. The manual dexterity of many individuals, however, is not efficient enough for them to obtain good oral hygiene, and therefore several methods have been proposed in order to assist such people. The simpler the methods are the more diffuse and effective they prove. In this context, products for regular daily oral hygiene such as mouthwashes and toothpastes which contain compounds endowed with antimicrobial and antiplaque activities are considered excellent candidates.

Contrary to the commonly held opinion that food has a negative impact on oral health, research conducted over the past three decades has shown that several foods contain a number of components endowed with antibacterial and antiplaque activity [9, 10]. Very recently, it has been shown that regular consumption of foods enriched with these active molecules is associated with modification of the oral microbial community in the direction of less periodontopathogenic microbiota [11]. It therefore seems reasonable to encourage the consumption of such foods and/or to incorporate the active compound(s) in cosmetic products for daily oral hygiene. Previous studies have shown antimicrobial and antiplaque activities in low-molecular-mass (LMM) fractions of extracts from both an edible mushroom (*Lentinus edodes,* very popular in Japan and called shiitake) or from Italian red chicory (early Treviso red chicory, an anthocyanic cultivar of *Cichorium intybus*) [12, 13].

In this work we evaluate the mode of antimicrobial action of fractions of these two natural extracts on *P. intermedia*.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions. *P. intermedia* ATCC 25611 was used throughout this study. This strain was grown in Brain Hearth Infusion (BHI, Oxoid Ltd., Basingstoke, UK) broth to which 5 μg/mL hemin and 1 μg/mL vitamin K (Sigma-Aldrich Co., St. Louis, MO, USA) were added (BHI+HK) or in Blood Agar (BA, Oxoid) plates. Cultures in both liquid and solid media were incubated at 37°C in an anaerobic chamber (Whitley DG 250 Anaerobic Workstation, Don Whitley Scientific, Shipley, UK) with an atmosphere composed of 85% nitrogen, 10% hydrogen, and 5% CO_2. In some experiments growth of *P. intermedia* in a biofilm structure was allowed on ceramic hydroxyapatite (HA) discs (Clarkson Chromatography Products Inc., South Williamsport, PA, USA). To achieve this, HA discs (2.5 mm diameter) were coated with sterile human saliva collected from a pool of donors. Each disc was then incubated in 5 mL of BHI+HK medium spread with a *P. intermedia* culture in a well of a 6-well tissue culture plate (35 mm diameter, flat bottom, Sarstedt, Verona, Italy) at 37°C in the anaerobic atmosphere. Every day (for a total of five days) culture supernatants were removed by gentile aspiration and replaced with fresh preconditioned medium. In order to analyse the effects of the test compound, on day 4 suitable dilutions of LMM fractions of either mushroom or chicory extract were added to the growth medium following incubation for an additional 15 hours. At the end of each incubation, biofilms were washed three times with sterile distilled water and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for SEM analysis.

2.2. LMM Fractions of Both Mushroom and Chicory Extracts. Aqueous extracts of both shiitake mushroom and red chicory and separation by ultrafiltration of the LMM (<5,000 Daltons) fraction were performed as described elsewhere [13]. LMM fractions were lyophilised and stored up to 3 months at −80°C. Immediately before use, a sample was rehydrated with sterile distilled water to obtain a 10x solution and kept at 4°C for no longer than a week. The 1x concentration of the LMM fraction after reconstitution represents the original concentration in the food.

2.3. Evaluation of Cell Growth and Viability. Optical density (O.D.) was measured at 640 nm wavelength with a Beckman mod. DU 530 spectrophotometer. Total bacteria were counted as cell particles with a Coulter Counter mod ZBI (Coulter Scientific) equipped with a 30 μm capillary. Viable cells were determined as colony forming units (CFU) per mL of culture. Suitable dilutions in sterile saline solution of the untreated and treated cultures were plated in BA plates and incubated at 37°C for 48 hours in the anaerobic atmosphere.

2.4. Evaluation of DNA, RNA, and Protein Synthesis. Aliquots of an exponentially growing culture (150 μL, O.D. 0.2 unit) were placed in the wells of a microtitre plate together with 5 μCi of [^{3}H] thymidine (spec. act. >10 Ci/mmol) or [^{3}H] uridine (spec. act. >20 Ci/mmol) or [^{3}H] leucine (spec. act. 50 Ci/mmol, PerkinElmer Life and Analytical Science, Boston, MA, USA). In some wells suitable dilutions of the test compounds were added. Each determination was performed in triplicate. Microtiter plates were incubated at 37°C in the anaerobic atmosphere for 60 min. At the end of this time period the culture was overlaid on a Millipore GF/C glass fibre paper soaked in advance with 10% trichloroacetic acid (TCA) and plunged in a cold 10% TCA solution. Filters were then washed three times with cold TCA and, finally, twice with acetone. Radioactivity was determined with a Beckman LS 6500 liquid scintillation counter. Three distinct identical experiments were performed.

2.5. Scanning Electron Microscopy (SEM). SEM was performed within a week of fixation of the samples. Glutaraldehyde-fixed samples from both planktonic and biofilm

Table 1: Macromolecular synthesis of *P. intermedia* treated for 60 min with different concentrations of LMM fractions of both mushroom and chicory extracts.

LMM fraction	Concentration	Macromolecular synthesis (% of untreated control)		
		DNA	RNA	Protein
Mushroom	0	100[b]	100[b]	100[b]
	0.25x[a]	19	47	83
	0.5x	9	22	48
	1x	5	5	36
Chicory	0	100[c]	100[c]	100[c]
	0.25x[a]	22	53	79
	0.5x	11	26	54
	1x	8	4	32

[a] Subinhibitory dose.
[b] Control values: DNA, 372,353 cpm; RNA, 147,367; protein, 12,682.
[c] Control values: DNA, 347,012 cpm; RNA, 129,647; protein, 11,239.

Table 2: Cell length measurement of *P. intermedia* cells treated for two hours with different concentrations of LMM fraction of both mushroom or chicory extracts in comparison with control cells at the same time.

Microorganism	Growth condition	Cell length (μm)				
		Mean	SD	Min value	Max value	Mode
P. intermedia	Control	1.09	0.41	0.54	3.01	0.75
	Mushroom 0.25x[a]	1.83	0.88	0.64	4.97	0.91
	Mushroom 0.5x	1.94	1.09	0.72	7.40	1.38
	Chicory 0.25x[a]	2.07	0.93	0.68	5.06	0.97
	Chicory 0.5x	2.37	1.36	0.76	9.09	1.32

[a] Subinhibitory dose.

cultures were washed three times with phosphate buffer followed by three rinses in distilled water. Specimens were then serially dehydrated in ethanol and subjected to critical-point drying with CO_2, mounted on metal stubs and sputter-coated with gold. Each specimen was viewed with an ESEM FEG XL30 electron microscope (Fei-Philips) at magnifications of $\times$5,000 to $\times$40,000. Cell length measurement was performed on 150 bacteria at $\times$5,000 magnification.

3. Results

3.1. Effects of LMM Fractions of Both Mushroom and Chicory Extracts on Cell Growth and Viability of P. intermedia. Different concentrations of the LMM fractions of both mushroom and chicory extracts ranging from 0.25 to 1x were tested in growing *P. intermedia*. Figure 1 shows the effects of these concentrations on increases in optical density, cell particle number, and cell viability in *P. intermedia*. As far as the LMM fraction of mushroom extract is concerned, the concentration of 0.5x was the minimum dose capable of inhibiting cell division, as evaluated by cell particle counts. At this concentration a bacteriostatic effect was observed, as well as a slight increase in O.D. in the course of the experiment. Higher concentrations (e.g., 1x) had a partial bactericidal effect with a 50% decrease in viable cells. Similar results were obtained when LMM fraction of chicory extract was tested: again the minimal dose capable of inhibiting cell division was 0.5x, but, as opposed to the same concentration of mushroom extract, this concentration reduced cell viability by 50%.

3.2. Effects of LMM Fractions of Both Mushroom and Chicory Extracts on Macromolecular Synthesis of P. intermedia. DNA, RNA and protein synthesis of *P. intermedia* were evaluated in the presence of the active concentrations reported above. Table 1 summarises the results: a strong inhibitory effect on DNA synthesis was observed during treatment with 1x and 0.5x concentrations of both mushroom and chicory extracts with a residual synthesis lower than 10% of the untreated control. The 0.25x subinhibitory concentration allowed roughly 20% DNA synthesis. RNA synthesis, albeit to a lesser extent compared with DNA synthesis, was reduced to roughly 25% and 50% of the control in the presence of 1x and 0.5x, respectively, no matter whether mushroom or chicory was tested. The 1x concentration strongly inhibited RNA synthesis. Protein synthesis was inhibited by 50% in the presence of 0.5x while 1x induced greater inhibition (70%). A reduction of only about 20% in protein synthesis was observed at 0.25x.

3.3. Morphological Examination and Cell Size Distribution of P. intermedia Treated with LMM Fractions of Mushroom and Chicory Extracts. Bacteria treated as above were also collected for morphological analysis by both optical and scanning electron microscopy (SEM). Preliminary observation by optical microscopy showed the presence of elongated cells as a result of the treatment. Thus, we resorted to SEM analysis in order to precisely evaluate cell size and distribution. Figure 2 shows the appearance of *P. intermedia* during treatment with mushroom or chicory extracts. Distinctly elongated

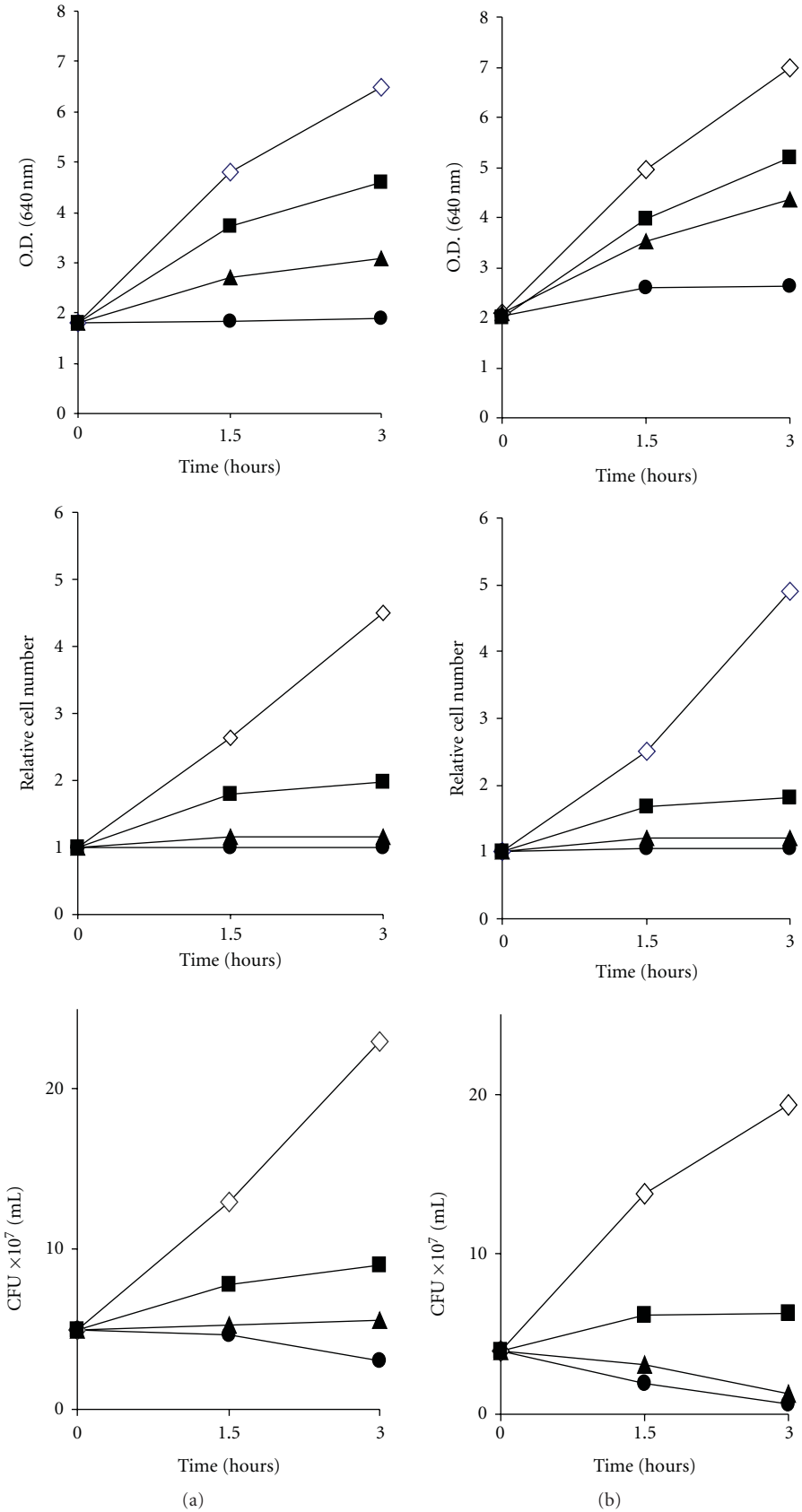

Figure 1: Effects on cell mass, cell number, and cell viability of *P. intermedia* treated with different concentrations of LMM fraction of mushroom (column (a)) and chicory (column (b)) extracts. Symbols: (◊) untreated control, (■) 0.25x, (▲) 0.5x, (●) 1x concentration.

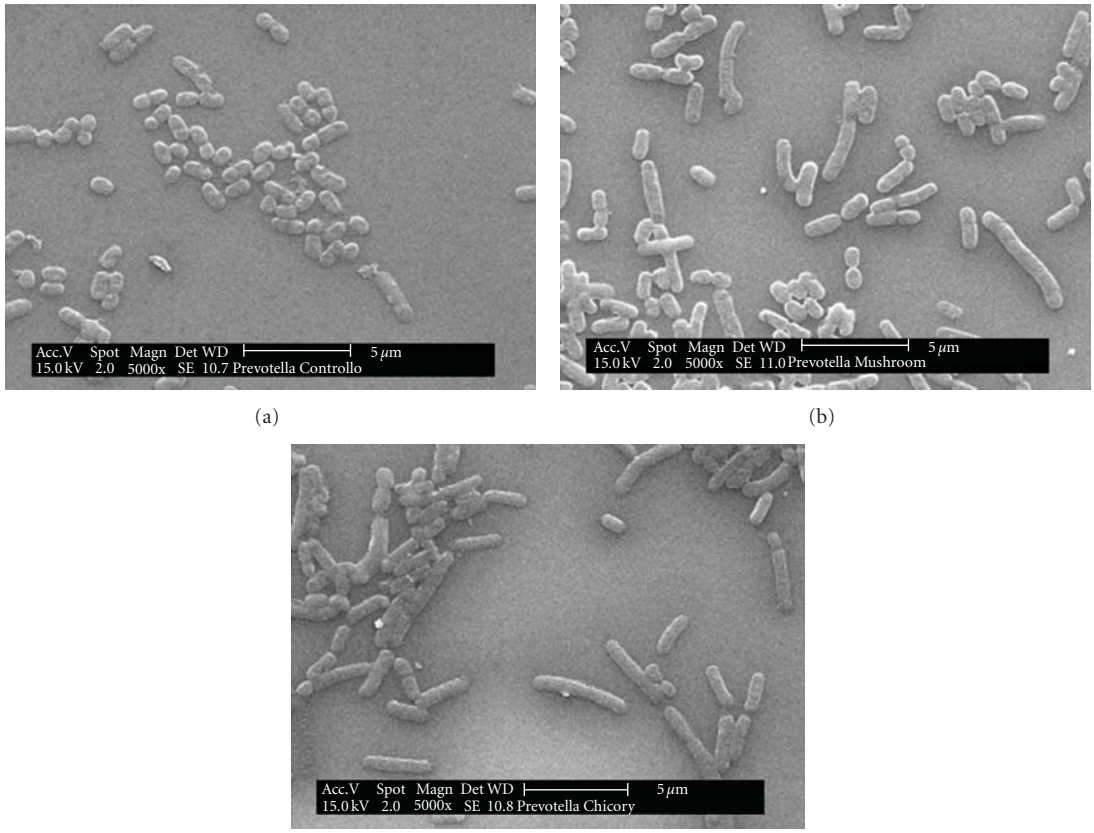

Figure 2: Scanning electron microscopy of untreated *P. intermedia* (a) and after a three-hour treatment with 0.5x of LMM fraction of mushroom (b) and chicory (c) extracts.

cells and several filaments with interrupted septa were seen after a three-hour treatment while untreated control cells prevalently presented the typical shape of short rods/coccobacilli. Table 2 shows the mean cell lengths with additional parameters of the treated cells in comparison with the untreated ones. Cell measurements confirmed the morphological observations at the inhibitory concentration. However, it is worthy of note that elongation was also observed in cells treated with a subinhibitory concentration (0.25x). Furthermore, analysis of the size distribution of untreated control and treated *P. intermedia* cells confirmed cell elongation following treatment (Figure 3). In a further set of experiments we evaluated the effects of the extracts on *P. intermedia* biofilm architecture. Figure 4 shows that untreated control biofilm appeared as a compact structure with bacteria close to one another, while in treated biofilms, both with mushroom and with chicory (the latter not shown in the figure), several gaps were observable.

4. Discussion

A substantial number of *in vitro* studies have shown that components of vegetal food exert intrinsic antibacterial, antiadhesive and antiplaque activity [9, 10]. The main family of substances endowed with such activity is that of the polyphenolic substances. These properties have been mainly detected against oral bacteria, although microorganisms responsible for infections of other human sites (e.g., lower urinary tract) may interact with them [14, 15]. These observations suggest that a diet rich in polyphenols may be useful for preventing the development of oral microbial pathologies, especially those known as plaque-dependent pathologies, such as caries and gingivitis/periodontitis. Hence, the main repercussion may be the development of so-called functional foods, that is, foods enriched with healthy substances. At the same time active compounds may be included in mouthwashes and toothpastes for daily oral hygiene. Although a large number of studies have been conducted to evaluate the efficacy of these compounds in terms of antimicrobial, antiadhesive, and antiplaque activity, very little is known about their mode of antibacterial action. The main goal of this research was precisely to try to elucidate this aspect. Throughout the study we used *P. intermedia*, one of the microorganisms included in a polymicrobial complex (mainly composed of strict anaerobe bacteria) which is involved in the etiology of adult chronic periodontitis, a pathology present worldwide and currently considered (together with dental caries) the commonest infectious disease. We evaluated the mechanism of antimicrobial action of LMM (<5,000 Da) fractions of aqueous extracts from both mushroom (shiitake) and red chicory [12, 13]. Test concentrations were referred to the original food concentrations. The minimal active concentration was 0.5x for both mushroom and chicory

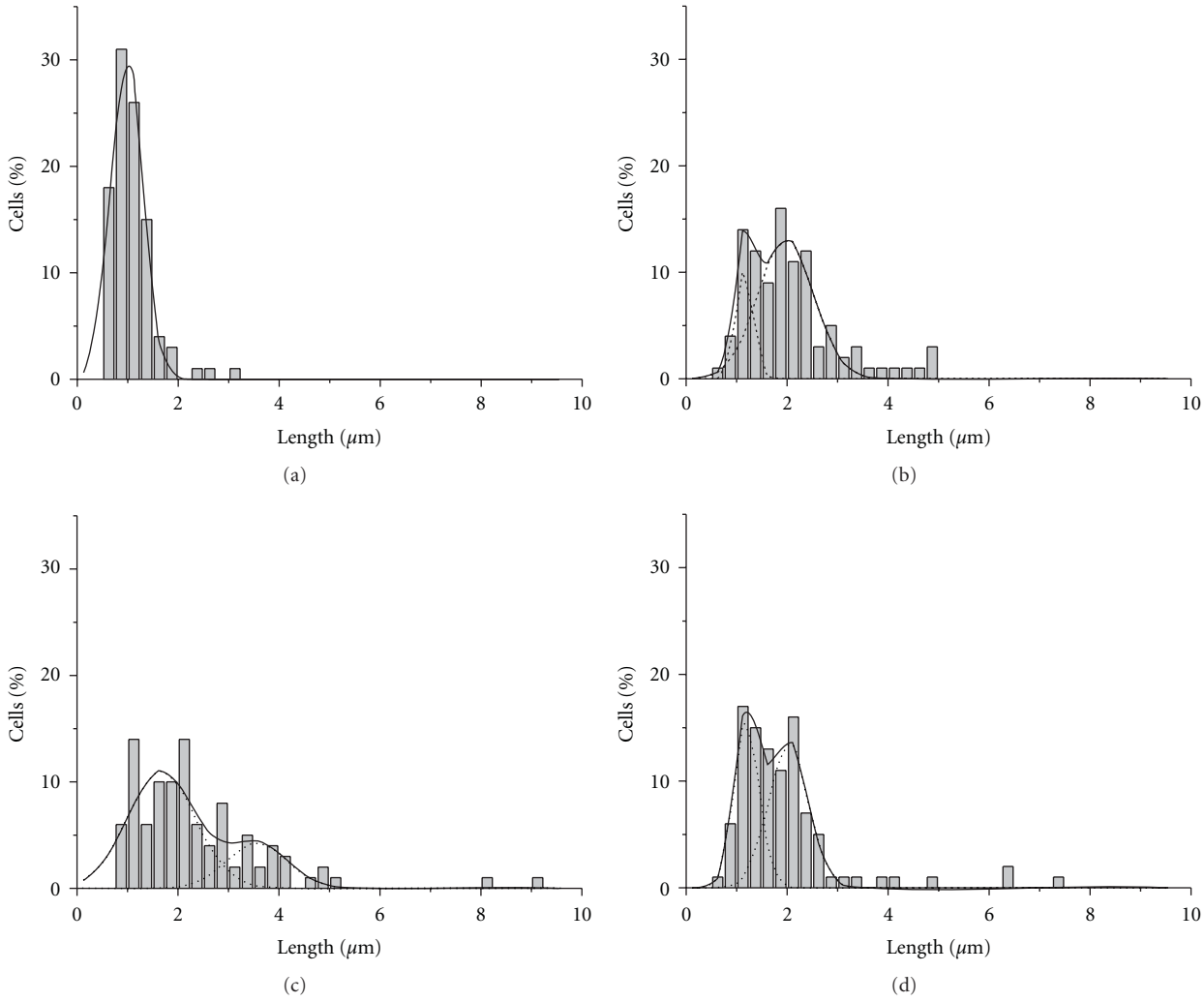

Figure 3: Cell length distribution of *P. intermedia* (a) and after a three-hour treatment with 0.5x chicory extract (b), and 0.5x (c) and 0.25x (d) mushroom extracts.

extracts. This concentration of mushroom extract acted bacteriostatically while the same concentration of chicory extract was slightly bactericidal after a three-hour treatment. At this concentration DNA synthesis was almost totally inhibited while RNA was inhibited to a lesser extent than DNA. Protein synthesis was only partially inhibited. The persistence of protein synthesis over time allows a cell mass increase, as indicated by the increase in O.D., producing cell filamentation, as revealed by SEM observation. In fact, nonseptate long rods and filaments were observed after treatment in comparison with untreated controls. These data suggest that the main target of action of both mushroom and chicory extracts is DNA synthesis. As a result of this, inhibition of septum formation occurs, but, as expected for rod-shaped bacteria, elongation still occurs. Morphological changes are compatible with the observation that protein synthesis persists (about 50% of the untreated control) and O.D. increases over time. This mode of action is reminiscent of that described for antibiotics belonging to the quinolone family (e.g., ciprofloxacin) [16]. These chemotherapeutic agents primarily block DNA synthesis followed by septum formation inhibition and cell filamentation. A similar mode of action has been described for β-lactam antibiotics (e.g., penicillins and cephalosporins). In this case septum inhibition via blockade of the "penicillin binding protein" involved in septum synthesis is the main target of action, and, in this example too, filamentation occurs after septum inhibition [17]. Thus, a mode of action comparable with that of antibiotics may be supposed for these natural extracts. This statement is further supported by the observation that sub-MICs display morphogenetic effects such as those induced by the MIC and higher doses, as previously demonstrated for both β-lactams and quinolones [16, 18]. Thus, this preliminary report indicates that a specific mode of action exists and the identification of specific target(s) is needed. This result suggests that these extracts could be used to advantage for daily oral hygiene in formulations of cosmetic products such as mouthwashes and toothpastes. The possibility of developing cosmetic products in which the active compound or compounds are natural molecules supports the tendency of consumers to prefer natural compounds to those obtained by chemical synthesis. Furthermore, foods enriched with

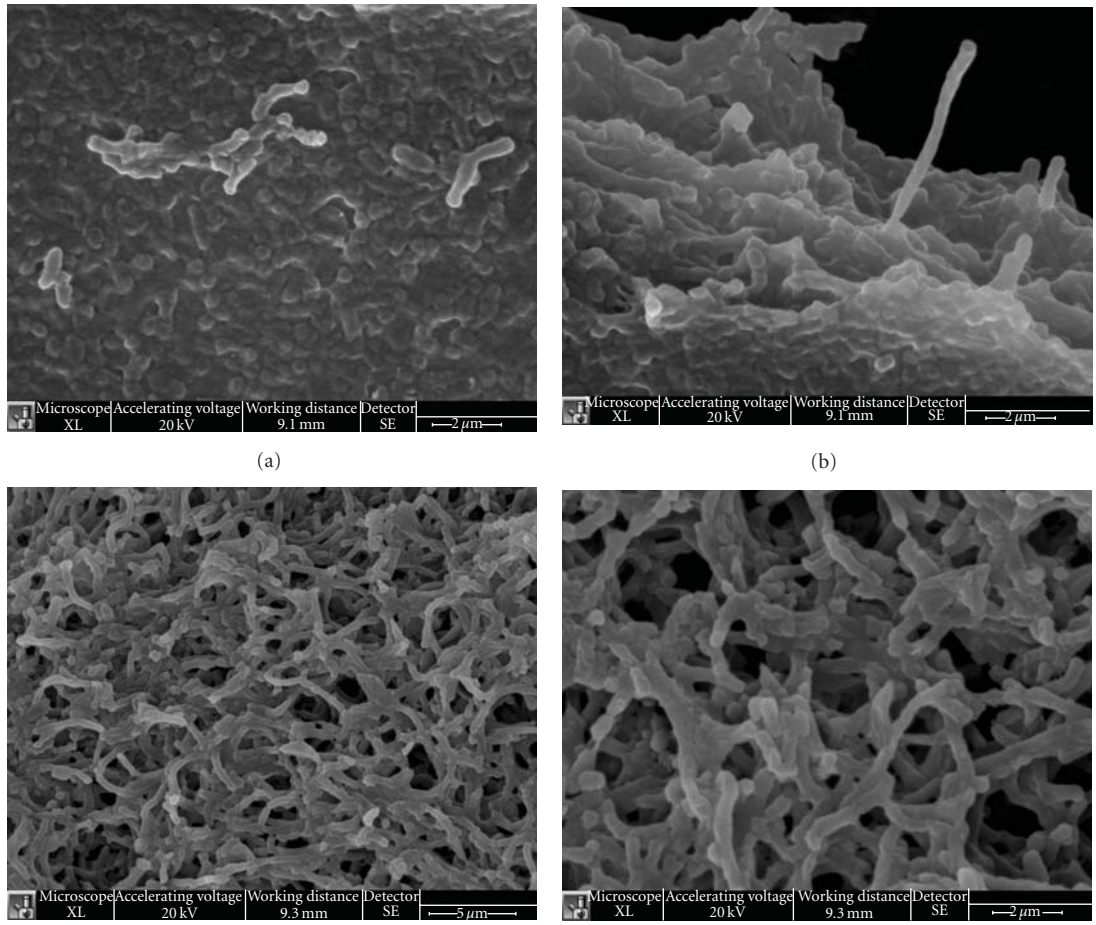

Figure 4: Scanning electron microscopy of *P. intermedia* grown in biofilm state (a and b) and after treatment with 0.5x mushroom extract (c and d).

these active compounds may be of great interest particularly in developing countries where severer economic conditions militate against the use of commercially available products for regular oral hygiene.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under the contract FOOD-CT-2006-036210 (project NUTRIDENT).

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Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 857987, 7 pages
doi:10.1155/2011/857987

Research Article

Testing a Low Molecular Mass Fraction of a Mushroom (*Lentinus edodes*) Extract Formulated as an Oral Rinse in a Cohort of Volunteers

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Received 15 June 2011; Accepted 11 July 2011

Academic Editor: Carla Pruzzo

Although foods are considered enhancing factors for dental caries and periodontitis, laboratory researches indicate that several foods and beverages contain components endowed with antimicrobial and antiplaque activities. A low molecular mass (LMM) fraction of an aqueous mushroom extract has been found to exert these activities in *in vitro* experiments against potential oral pathogens. We therefore conducted a clinical trial in which we tested an LMM fraction of shiitake mushroom extract formulated in a mouthrinse in 30 young volunteers, comparing the results with those obtained in two identical cohorts, one of which received water (placebo) and the other Listerine. Plaque index, gingival index and bacterial counts in plaque samples were determined in all volunteers over the 11 days of the clinical trial. Statistically significant differences ($P < 0.05$) were obtained for the plaque index on day 12 in subjects treated with mushroom versus placebo, while for the gingival index significant differences were found for both mushroom versus placebo and mushroom versus Listerine. Decreases in total bacterial counts and in counts of specific oral pathogens were observed for both mushroom extract and Listerine in comparison with placebo. The data suggest that a mushroom extract may prove beneficial in controlling dental caries and/or gingivitis/periodontitis.

1. Introduction

Dental plaque is a complex bacterial biofilm associated with tooth and gum surfaces [1, 2]. The so-called "plaque-dependent" oral pathologies include dental caries and periodontitis, with the former being the result of acid destruction of the tooth enamel and dentin caused mainly by *Streptococcus mutans* in the presence of sucrose [3], while the latter results from host aggression to the structure supporting the teeth by a bacterial consortium, consisting mainly of strict anaerobes [4]. Although foods, often in association with poor oral hygiene, are considered enhancing factors for these pathologies, a substantial amount of laboratory research indicates that several foods and beverages of vegetal origin contain components endowed with antimicrobial, antiadhesive, and antiplaque activities (for a recent review

see Signoretto et al. [5]). Thus, the consumption of such healthy foods and beverages could be encouraged or foods may be enriched with the active component(s). Several epidemiological studies have been conducted in distinct populations (Tibetans, Israeli Arabs, Senegalese, and Japanese, English and US schoolchildren), comparing tea drinkers versus nontea drinkers; the results showed a lower incidence of caries in tea-drinkers [6, 7]. In addition, a significant reduction in both saliva and dental plaque counts of mutans streptococci and lactobacilli has been observed, together with a reduced plaque score in adult tea, coffee, and wine drinkers versus water drinkers [8]. Very recently it has been shown that the regular consumption of foods enriched with these active molecules (wine and coffee) is associated with a modification of the oral microbial community in the direction of less periodontopathogenic microbiota [9]. In order to support the use of these substances as mouthrinses or toothpastes in daily oral hygiene, a few clinical studies have been conducted with interesting, though not conclusive, results. Tea and tea extracts [10, 11] and a high molecular mass cranberry constituent [12] in mouthrinse formulations have been tested in volunteers. A low molecular mass (LMM) fraction of an aqueous mushroom extract (*Lentinus edodes*, commonly called shiitake and very popular in Japan) has been found to exert antimicrobial, antiadhesive, and antiplaque activities in *in vitro* experiments against several potential oral pathogens [13, 14]. We therefore conducted a clinical trial in which we tested an LMM fraction of a shiitake extract formulated in a mouthrinse in 30 young volunteers, comparing the results with those obtained in two identical cohorts, one of which received coloured and aromatised water (placebo) and the other Listerine, a mouthwash used worldwide.

2. Materials and Methods

2.1. Composition of Mouthrinses. Three different mouthrinses were tested in this study. LMM fractions of the mushroom extract mouthrinse as well as a mouthrinse acting as a negative control (placebo) and composed of aromatised water alone were prepared by MicroPharm Ltd, Newcastle Emyln, UK. The aromatised water contained 12% ethanol, 6% glycerine, 0.2% PEG 40 Hydrogenated Castor Oil (Cremophor RH40), 0.2% flavour, and 0.05% saccharin. The LMM fraction of the mushroom extract mouthrinse was obtained as described elsewhere [15] and added to the aromatised water at a final concentration of 2x in comparison with the original food concentration. Twenty mL of both mouthrinses were dispensed in 25 mL tubes and maintained at 4°C in the dark for no more than 5 months. "Freshburst" Listerine (Johnson and Johnson, New Brunswick, NJ, USA) was the one we tested as a positive control. Listerine contained the following active ingredients: 0.092% eucalyptol, 0.042% mentol, 0.06% methyl salicylate and 0.064% thymol in water, alcohol (21.6%), sorbitol solution, flavoring, poloxamer 407, benzoic acid, sodium saccharin, sodium benzoate, D&C yellow no. 10, and FD&C green no. 3. Listerine was removed from the original bottle and 20 mL aliquots were poured into tubes identical to those of the other mouthrinses.

2.2. Volunteers Enrolled. A total of 90 healthy volunteers (49 males and 41 females, average age 22, range 19–27 years) from the student population of the University of Verona were included in a three-leg, double-blind, clinical trial. Exclusion criteria included antibiotic treatment at any time in the 3 months prior to the study, oral soft tissue pathologies, missing teeth, visible untreated carious lesions, smoking, and pregnancy. Subjects were randomly assigned to the experimental group in which mushroom was tested ($n = 30$), to the negative/placebo (water) control group ($n = 30$) and to the positive (Listerine) control ($n = 30$). All volunteers were asked to sign an informed consent form.

2.3. Study Design. This clinical trial was authorised by the Ethical Committee of the Verona University Hospital, reference 1653 dated March 11, 2009. A three-leg, double-blind study was carried out using the three mouthrinses twice daily for 12 days. The complete experimental period was 18 days (Figure 1). All participants were examined by a dentist and subjected to professional oral hygiene treatment at time 0. On days 1 to 6, all subjects were invited to maintain regular oral hygiene (brushing and flossing) at home. On day 6 a sample of dental plaque was collected along with clinical measurements, as described below, and each participant received the assigned mouthwash. From day 7 to day 17, they were instructed to refrain from any type of oral hygiene and to perform only two rinses with 10 mL of mouthwash for 30 sec at a 1 min interval twice daily (morning and evening). They were asked to refrain from eating and drinking 1 hour after rinsing. On days 8, 10, 12, and 17, a subgingival plaque sample was collected from each participant. Finally, on day 18 all the volunteers were subjected to professional oral hygiene to restore oral health.

2.4. Plaque Index (PI) and Gingival Index (GI) Determination. The following measurements were performed on days 6, 8, 10, 12, and 17 of the study: (i) plaque index (PI) or plaque accumulation was evaluated on the buccal surfaces following the application of disclosing solution (Butler GUM, Butler, Chicago, IL, USA), according to the Turesky modification of the Quigley-Hein plaque index [16]; (ii) gingival index or gingival inflammation was assessed on the buccal and lingual marginal gingivae and the interdental papillae, according to the Loe-Silness gingivitis index [17].

2.5. Sample Collection for Microbiological Analysis. Dental plaque samples were collected using a curette from two upper molar surfaces on the left and right sides. Plaque was dispersed in 750 μL sterile Tris-EDTA buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0), vortexed for 1 min to uniformly disperse the plaque, subdivided into three aliquots and stored at −80°C until further use.

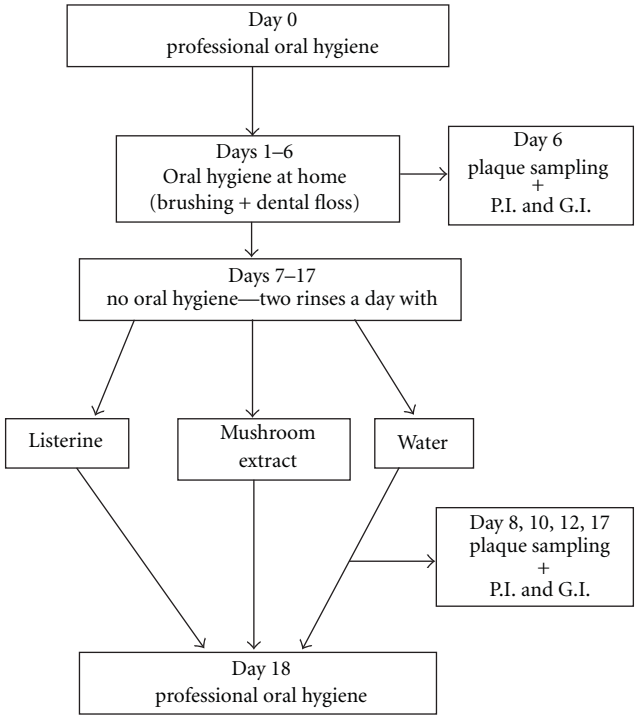

Figure 1: Outline of the study. PI, plaque index; GI, gingival index.

2.6. Quantitative PCR (qPCR). DNA was extracted from plaque biofilms using a phenol : chloroform : iso-amyl alcohol (25 : 24 : 1) bead-beating extraction method [18], which involves physical cell lysis, protein removal, and finally DNA precipitation using polyethylene glycol. Three triplex qPCR assays were then carried out using 2 μL of extracted DNA to quantify eight oral taxa as well as the total number of organisms. The taxa investigated were *Fusobacterium nucleatum*, *Lactobacillus casei*, *Veillonella dispar*, *Neisseria subflava*, *Actinomyces naeslundii*, *Prevotella intermedia*, *Streptococcus sanguinis*, and *Streptococcus mutans*. The assays were performed using the Rotor-Gene 6500 (QIAGEN) instrument and Sensimix Probe (Bioline) qPCR mix according to the manufacturers' instructions using previously published oligonucleotide sequences [19].

2.7. Statistical Evaluation. Data collected were analysed by using the Statistical Package for Social Sciences (SPSS version 16.0). For each mouthwash, the mean and standard deviation of the values was calculated. Data were analysed using one-way analysis of variance (ANOVA) followed by Turesky test for pairwise comparison. P values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical Evaluation of the Effects of the Three Mouthrinses. All 90 volunteers included in the three groups, one of which received the mushroom mouthrinse, the second coloured aromatised water (placebo), and the third Listerine, completed the study. At the beginning and on another four days of the period of mouthrinse use (Figure 1), clinical parameters such as PI and GI were determined for all volunteers. Table 1 shows the results of the PI calculation of mean values ± SD. Mean values increased over time in the three mouthrinse categories, but, as expected, the placebo group showed the greatest increase. Significant differences ($P < 0.05$) were observed on day 12 regarding mushroom treatment versus placebo. A similar pattern was observed when GI was evaluated (Table 2). GI values increased with time, but, again, higher values were observed in the placebo group in comparison with both the mushroom and Listerine mouthrinses. Statistically significant differences ($P < 0.05$) were obtained on day 12 for both mushroom versus placebo and mushroom versus Listerine treatment.

Table 1: Cumulative plaque index scores (± standard deviation) at the various test sessions during the treatment of volunteers with three different mouthwashes. Statistically significant differences ($P < 0.05$) are marked by $*$.

Test session	Mushroom	Water	Listerine
Day 6	0.24 ± 1.61	0.17 ± 0.12	0.19 ± 0.18
Day 8	0.49 ± 0.26	0.62 ± 0.36	0.63 ± 0.34
Day 10	0.84 ± 0.38	0.93 ± 0.42	1.01 ± 0.39
Day 12	0.83 ± 0.30*	1.04 ± 0.41*	0.91 ± 0.38
Day 17	0.95 ± 0.36	1.05 ± 0.38	0.93 ± 0.34

Table 2: Cumulative gingival index scores (± standard deviation) at the various test sessions during the treatment of volunteers with three different mouthwashes. Statistically significant differences ($P < 0.05$) are marked by $*$ or ‡.

Test session	Mushroom	Water	Listerine
Day 6	0.20 ± 0.20	0.15 ± 0.15	0.22 ± 0.28
Day 8	0.26 ± 0.18	0.31 ± 0.25	0.27 ± 0.20
Day 10	0.40 ± 0.26	0.42 ± 0.24	0.46 ± 0.22
Day 12	0.53 ± 0.30*‡	0.70 ± 0.34*	0.69 ± 0.32‡
Day 17	0.56 ± 0.31	0.65 ± 0.33	0.59 ± 0.31

3.2. Microbiological Measurement of the Effects of the Three Mouthrinses. At the time-points above indicated and also at the time of collection of the clinical data, plaque samples were obtained in order to determine the load of total bacteria and of selected oral microorganisms by quantitative PCR (pPCR). We quantified four microorganisms associated with gingivitis (*A. naeslundii*, *F. nucleatum*, *P. intermedia*, *L. casei*), one strongly implicated in dental caries (*S. mutans*), and three microorganisms associated with oral health (*S. sanguinis*, *N. subflava*, *V. dispar*). The eight oral microorganisms amounted to about 23 ± 6% of the total microbiota of the dental plaque. Figure 2 shows the evolution of the four microorganisms associated with gingivitis in comparison with the total count during treatment with the three distinct mouthrinses. In the placebo group, the mean total cell count values increased over time (from 1.48 (±1.63) $\times 10^7$ on day 6 to 2.27 (±0.99) $\times 10^8$ on day 17). Standard deviation is presented in brackets. Individual counts were *F. nucleatum*

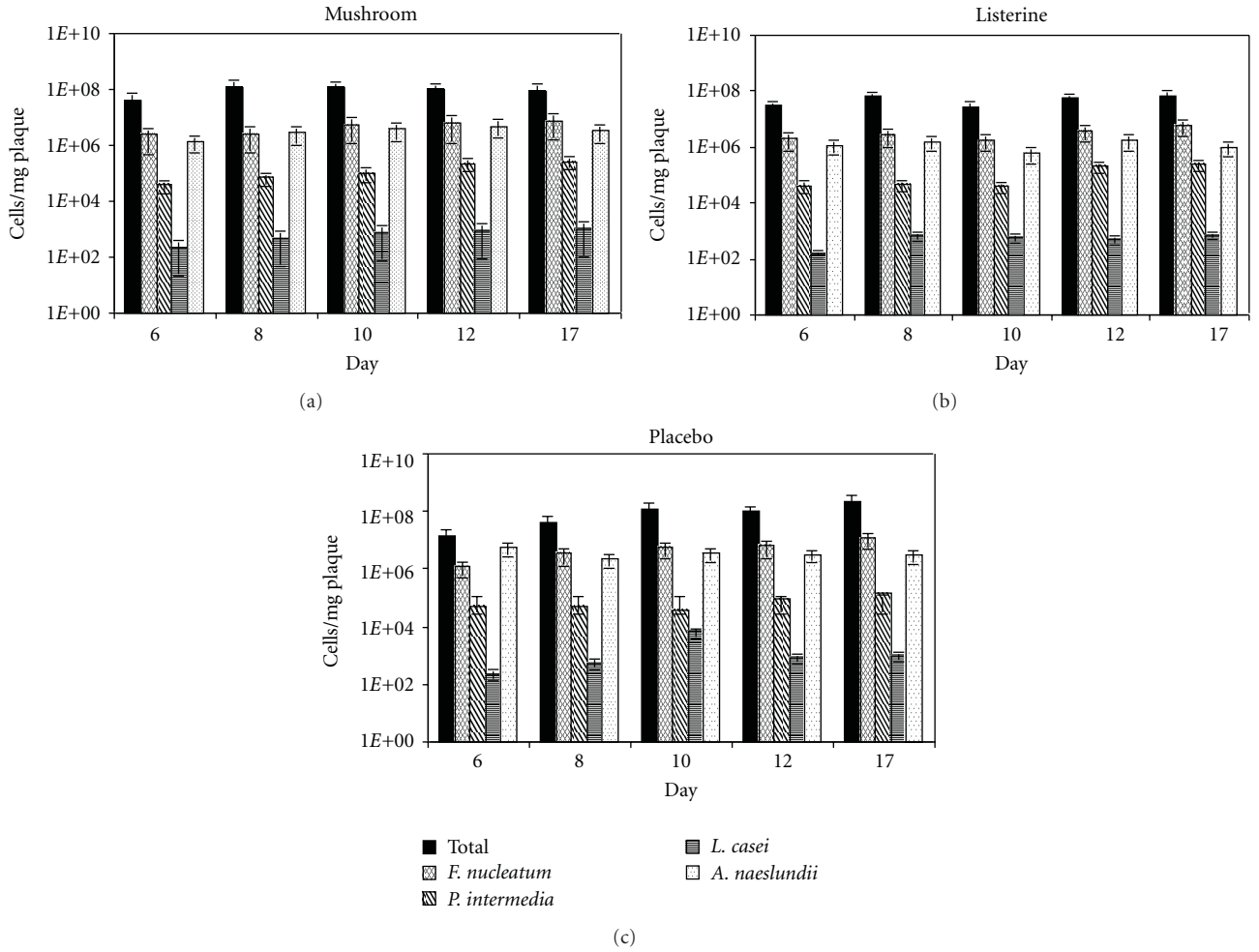

Figure 2: Numbers of each of the investigated taxa associated with gingivitis and total counts in dental plaque samples of the three cohorts enrolled in the study. Standard error bars represent the standard deviation ($n = 30$).

(from 1.28 ($\pm$1.85)$\times 10^6$ to 1.24 ($\pm$0.76)$\times 10^7$), *P. intermedia* (from 4.89 ($\pm$10.3) $\times$ 10^4 to 4.53 ($\pm$3.25) $\times$ 10^5), and *A. naeslundii* (from 5.48 ($\pm$8.35) $\times$ 10^5 to 2.99 ($\pm$3.72) $\times$ 10^6 on days 6 and 17, resp.) increased in accordance with the total count, while *L. casei,* after an initial increase on day 10, tended to decrease (from 2.20 ($\pm$2.86) $\times$ 10^2 on day 6 to 6.43 ($\pm$2.86) $\times$ 10^3 on day 10 to 9.34 ($\pm$4.09) $\times$ 10^2 on day 16). The oral rinses with mushroom or Listerine in general did not block the increase either in total counts or in those of the four gingivitis-associated microorganisms, but these increases never exceeded half the final value reported for placebo. In detail, *F. nucleatum* during treatment with mushroom increased from 2.23 ($\pm$2.95) $\times$ 10^6 on day 6 to 7.56 ($\pm$4.28) $\times$ 10^6 on day 17, and from 1.93 ($\pm$3.05) $\times$ 10^6 to 5.74 ($\pm$3.57) $\times$ 10^6 during treatment with Listerine; *P. intermedia* from 3.71 ($\pm$7.72)$\times 10^4$ to 2.60 ($\pm$6.66)$\times 10^5$ with mushroom and from 4.26 ($\pm$10.7)$\times 10^4$ to 2.46 ($\pm$2.69)$\times 10^5$ with Listerine treatment; *A. naeslundii* from 1.41 ($\pm$2.27) $\times$ 10^6 to 3.30 ($\pm$5.48) $\times$ 10^6 and from 1.15 ($\pm$2.03) $\times$ 10^6 to 9.70 ($\pm$10.4) $\times$ 10^5; and *L. casei* from 2.23 ($\pm$2.91) $\times$ 10^2 to 9.28 ($\pm$2.76) $\times$ 10^2 and from 1.57 ($\pm$1.98) $\times$ 10^2 to 5.13 ($\pm$1.75) $\times$ 10^2 after treatment with mushroom and Listerine, respectively. Figure 3 shows the effects of the three mouthrinses tested on the cariogenic *S. mutans*. In the placebo group, the *S. mutans* cell count increased over time (from 4.17 ($\pm$9.94) $\times$ 10^4 to 4.57 ($\pm$2.67) $\times$ 10^5) on day 17. Although both mushroom and Listerine induced cell number increases, the final *S. mutans* values were lower than those of the placebo group (i.e., 2.15 ($\pm$5.58) $\times$ 10^5 and 3.68 (1.15) $\times$ 10^5 for mushroom and Listerine, resp.). Finally, Figure 4 shows the effects of the three mouthrinses on three microorganisms associated with oral health. *S. sanguinis*, *N. subflava*, *V. dispar* counts increased (up to 2 log) over time from day 6 to day 17 in both the placebo and mushroom groups. The values obtained were *S. sanguinis* from 1.60 ($\pm$2.26)$\times 10^5$ to 3.92 ($\pm$1.03)$\times 10^6$ for placebo and from 4.32 ($\pm$5.62)$\times 10^5$ to 2.53 ($\pm$0.62)$\times 10^6$ for mushroom group; *N. subflava* from 7.04 ($\pm$8.52)$\times 10^5$ to 4.48 ($\pm$0.45)$\times$ 10^7 and from 7.14 ($\pm$8.48) $\times$ 10^5 to 9.49 ($\pm$2.60) $\times$ 10^6 for placebo and mushroom, respectively; *V. dispar* from 1.31 ($\pm$2.36)$\times 10^6$ to 2.10 ($\pm$0.88)$\times 10^7$ and 2.48 ($\pm$3.28)$\times$ 10^6 to 1.37 ($\pm$0.31) $\times$ 10^7 for placebo and mushroom,

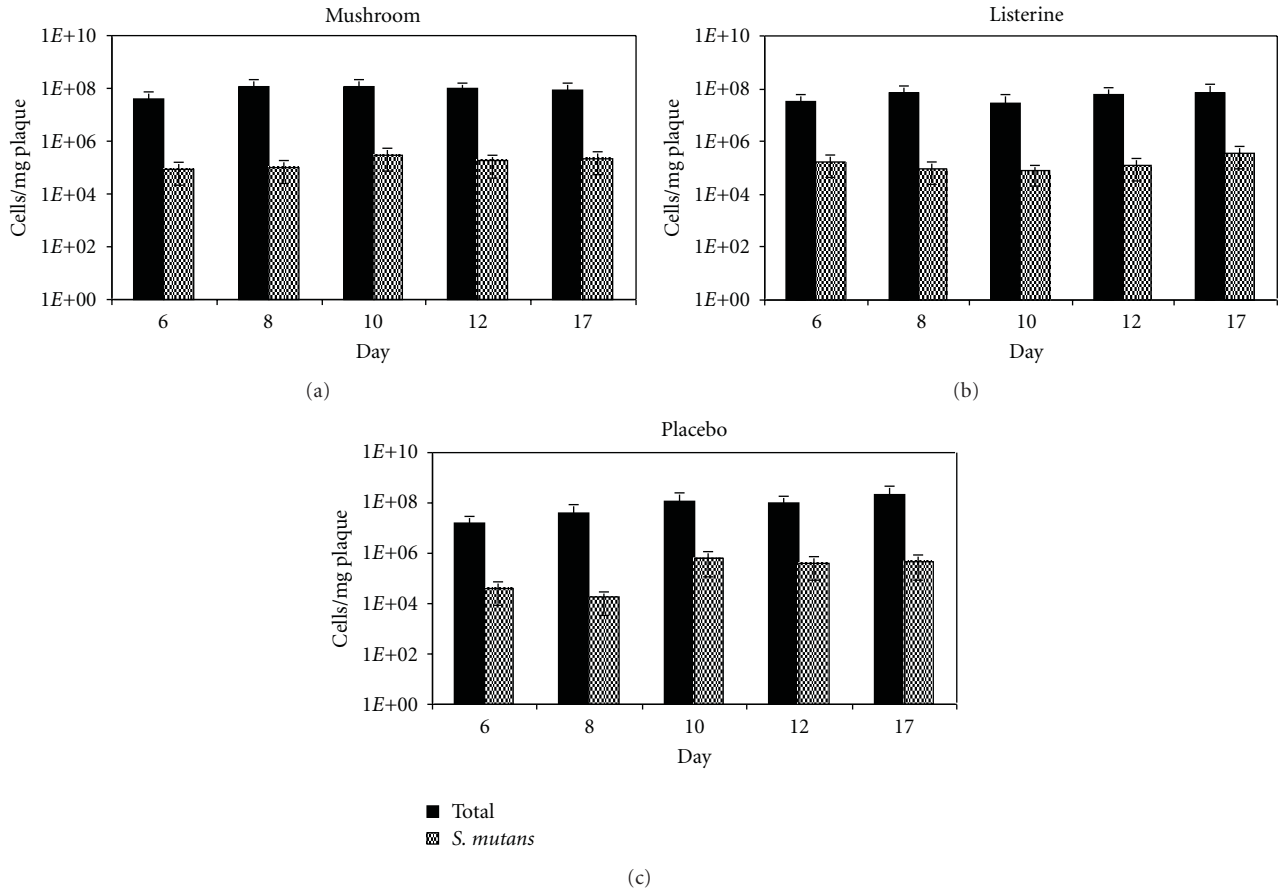

Figure 3: Numbers of *S. mutans* and total counts in dental plaque samples of the three cohorts enrolled in the study. Standard error bars represent the standard deviation ($n = 30$).

respectively. Listerine, on the contrary, prevented any cell number increase of *N. subflava* and *V. dispar* and, thus values at day 17 were quite close to the initial (day 6) count (i.e., 2.95 ($\pm$3.71) $\times$ 10^6 and 3.67 ($\pm$4.41) $\times$ 10^6, resp.). The cell number increase of *S. sanguinis* was only partially affected by Listerine (from 1.87 ($\pm$3.40) $\times$ 10^5 to 1.51 ($\pm$3.20) $\times$ 10^6). Although differences were seen, especially as far as the placebo group versus mushroom or Listerine was concerned, none of them proved statistically significant.

4. Discussion

The aim of this clinical trial was to evaluate the possible beneficial effects of an LMM mushroom extract formulated as a mouthrinse in preventing dental plaque deposition in volunteers. In addition, its activity was compared with the activity of Listerine, a widely used mouthwash and with that of a placebo composed of colored and aromatised water. The rationale of this study was based on the proven *in vitro* activity of this mushroom extract in terms of antimicrobial, antiadhesive, antiplaque and plaque disaggregating activity against several oral microorganisms including those involved either in dental caries or in gingivitis/periodontitis [13].

The results on the whole indicate that a mouthrinse containing an LMM fraction of mushroom extract was capable of significantly reducing dental plaque deposition as compared with placebo, but this activity was not significantly higher than that displayed by Listerine. Worthy of note, however, is the fact that in the present clinical trial Listerine failed to prove significantly more active than placebo, although lower scores were determined. Equally interesting are the results obtained when GI was evaluated. The mushroom mouthwash showed a statistically significant advantage over both placebo and Listerine. This result may be attributed to the ability displayed by the mushroom mouthwash to reduce plaque deposition, but an anti-inflammatory activity or a sort of gingival cell protection by the mushroom extract may be suspected. Indeed, the LMM fraction of mushroom extract, at concentrations that did not affect the viability of KB gingival cells, is capable of modulating the expression of different genes induced by both live and heat-killed *P. intermedia* and *A. naeslundii*, thus supporting the hypothesis that this foodstuff extract can modulate the responses of gingival cells to periodontopathogens [20]. The effects of the mushroom mouthrinse on both PI and GI are reminiscent of those observed in oolong tea [11] and pomegranate [21] extract which significantly inhibit dental plaque deposition in volunteers but differ from those of nondialysable material of cranberry juice [12] for which no effect was detected on either gingival or plaque indices after a 42-day study.

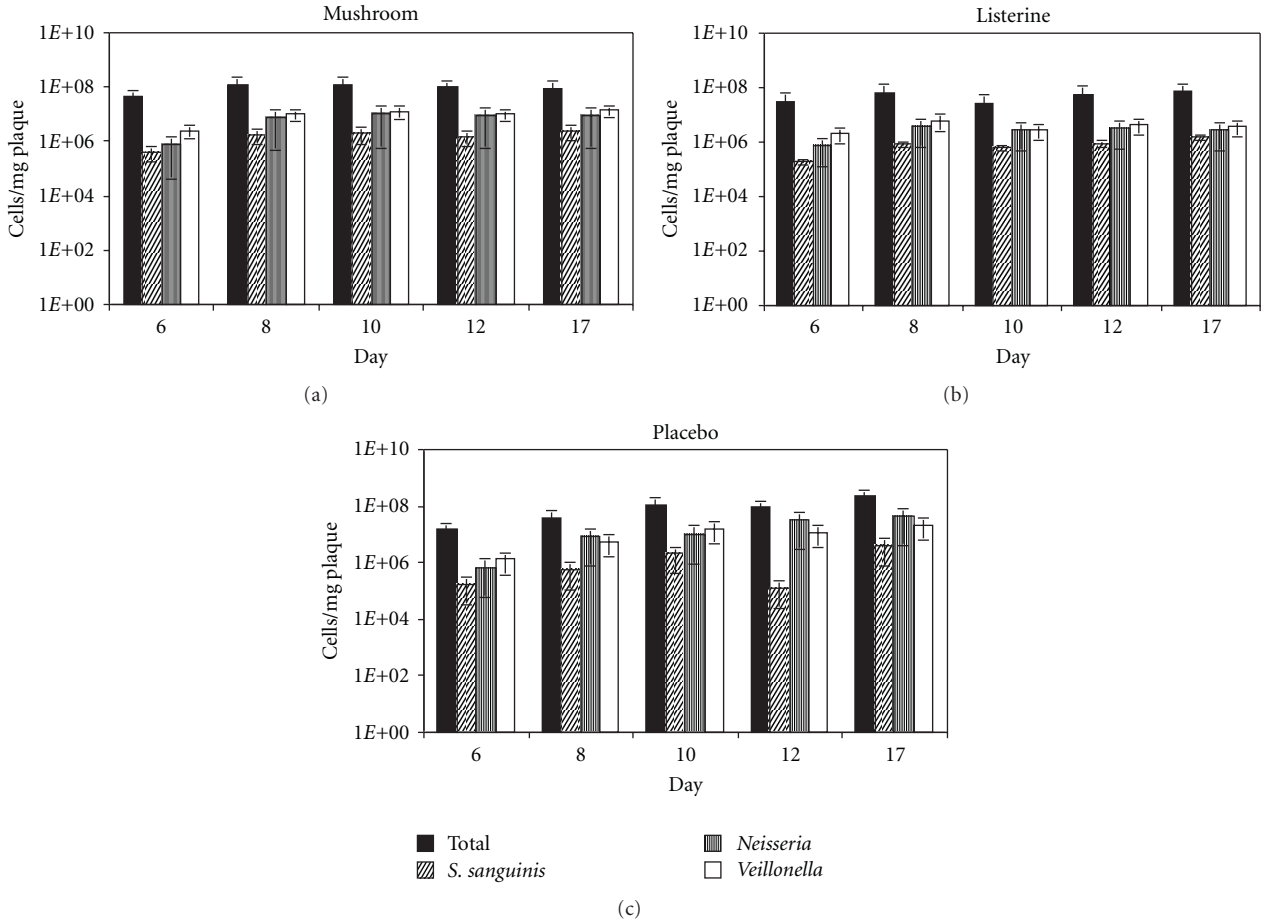

Figure 4: Numbers of each of the investigated taxa associated with oral health and total counts in dental plaque samples of the three cohorts enrolled in the study. Standard error bars represent the standard deviation ($n = 30$).

Chlorhexidine, which is capable of affecting both the clinical parameters PI and GI, is endowed, however, with a potent antibacterial activity [22, 23].

Although the mushroom mouthrinse is capable of significantly affecting clinical parameters, no significant reduction of total cell count, or of the counts of specific microbial components of dental plaque, was demonstrated. Some findings, however, deserve attention: as far as the effect on total plaque bacteria count is concerned, mushroom extract and Listerine may be equivalent in that they are capable of a certain degree of inhibition in comparison with the results of the placebo group, even if this activity failed to prove statistically significant. The partial activity against total plaque bacteria was studied in greater detail by analysing the effects on selected bacteria responsible for different oral conditions, such as the evolution towards gingivitis as indicated by the strict anaerobes *F. nucleatum*, *P. intermedia*, *A. naeslundii* and *L. casei*, caries following *S. mutans,* and the relationship between *N. subflava*, *V. dispar,* and *S. sanguinis* and oral health [19]. Against bacteria involved in gingivitis, mushroom extract and Listerine behaved similarly, inhibiting the increase in cell number in dental plaque of three out of four microorganisms tested (not including *L. casei*) to such an extent that the final number (day 17) is less than the half that of the placebo group. Although inhibition of the cell number increase is calculated as being about half the control value, it is worth noting that this inhibition is mainly attributable to strict oral anaerobes, whose role in the evolution of gingivitis towards periodontitis is fully ascertained [3, 4]. Against cariogenic *S. mutans* both mushroom and Listerine exerted a certain action, with cell counts on day 17 being roughly 50% lower than the control values. Also of interest are the results of the effects of the three mouthrinses on bacteria associated with oral health: a good candidate for formulating a mouthrinse should affect only potential oral pathogens, leaving that portion of oral microbiota associated with oral health unaltered. Mushroom extract seems to comply with this requirement in that it showed no effect mainly on gram-negatives (*N. subflava* and *V. dispar*) and only partial inhibition of *S. sanguinis*. From this point of view, the action of mushroom extract would appear to be superior to that of Listerine, with the latter being an inhibitor also of the microbiota associated with oral health. These results are in agreement with those obtained by Ciric et al. [24] who have tested the activity of the same mushroom fraction in an artificial mouth model (constant depth film fermentor) and shown that mushroom fraction lowered the numbers of

some pathogenic taxa without affecting the taxa associated with oral health.

Although the microbiological results of the effect of the mushroom mouthrinse would appear to be in contrast with *in vitro* results which show antimicrobial, antiadhesive, antiplaque, and plaque disaggregating activities [13, 14], this incongruity may be explained by the fact that these activities were not relevant during the clinical trial or, alternatively, the experimental design was incapable of detecting them if present. Thus, on the basis of the promising effects on clinical parameters obtained with the mushroom mouthrinse, further studies conducted both *in vitro* and in humans are advisable.

The possibility of using mouthrinses containing active components of natural origin for daily oral hygiene may constitute a novel approach in order to alter biofilm formation on tooth and gum surfaces. Their potential for controlling dental caries and/or gingivitis/periodontitis warrants further investigation.

Acknowledgment

The research leading to these results has received funding from the European Union's Sixth Framework Programme (FP6) under contract FOOD-CT-2006-036210 (project NUTRIDENT).

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