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(54) Title: PRODUCTS WITH ORAL HEALTH BENEFITS

(57) Abstract: The invention provides novel compositions for promoting oral health e.g. for ameliorating, controlling or reducing the risk of dental caries or periodontal disease such as gingivitis or periodontitis. The compositions are generally functional foods or oral preparations which comprises one or more extracts, for example particular low molecular weight extracts, of natural products (Shiitake mushroom, chicory and\or raspberry). Also provided are compositions utilising quinic acid; adenosine; inosine; trans-aconitic acid; oxalic acid; adenosine; cis-aconitic acid and succinic acid, or salts thereof, for promoting oral health.

PRODUCTS WITH ORAL HEALTH BENEFITS

Technical field

The present invention relates generally to agents derived from natural products and compounds related thereto which have benefits in promoting oral health, for example in controlling or reducing the risks of dental caries and periodontal diseases when presented in oral preparations or foodstuffs.

Background art

Foods and health

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 regarded not only as being an indispensable source of nutriment, but are also considered to be beneficial in many ways or as the cause of serious chronic diseases with strong socio-economic implications. Foods that have some particular beneficial effects on health, are generally defined as functional foods (1,2). Their activity is determined by a more or less specific and selective interaction of their minor components with one or more physiological functions of the organism.

Food and dental disease

Caries

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 agents of enamel caries are considered to be *Streptococcus mutans* and *Streptococcus sobrinus;* additional aetiological agents are lactobacilli and actinomyces, the former being considered as secondary invaders, while the latter are 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 disease can be considered to involve three main stages: 1) adhesion of bacteria to the tooth; 2) production of a glycocalyx resulting in the formation of a bacterial community embedded in the glycocalyx (i.e. a biofilm) which is known as dental plaque, 3) fermentation of sucrose within the biofilm to, mainly, lactic acid which demineralises the enamel resulting in the production of the caries lesion. The main virulence properties of S. *mutans* and S. *sobrinus* are, therefore, their ability to adhere to the tooth surface together

with their rapid metabolism of sucrose to organic acids and to extra-cellular polysaccharides. In particular, attachment to the tooth surface is a crucial step in cariogenesis and involves a variety of bacterial and host components. Bacterial surface structures (e.g. lipoteicoic acid, antigens I/II) are responsible for the initial interactions of the bacteria with enamel and with the acquired pellicle. Subsequently, adherence depends mainly on insoluble glucan synthesis mediated by cell-free or cell-bound glycosyltransferase (GTF).

Several approaches to caries prevention are possible: (i) elimination of fermentable sugars from the diet, (ii) elimination of the causative organisms, (iii) prevention of bacterial adhesion and/or plaque formation, (iv) interference with bacterial metabolism e.g. by fluorides, (v) enhancing acid resistance of the tooth enamel e.g. 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 anti-adhesive activity and inhibitory activity against matrix formation have been demonstrated.

Recently, the anti-cariogenic properties of food components have been verified in *in vivo* tests using both animals and humans. Extracts obtained from different teas and their polyphenol components have been investigated thoroughly for their anti-cariogenic activity. Polyphenols in tea have been shown to reduce caries development in animals because they decrease the cell surface hydrophobicity of S. *mutans* and its ability to synthesize adherent water-insoluble glucan from sucrose (13-17). 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 and 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 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

Periodontal diseases are a heterogeneous group of inflammatory conditions that involve the supporting tissues of the teeth. They include gingivitis, in which only the gingivae are 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.

Of these diseases, gingivitis is extremely common and most adults demonstrate some loss of bony support and loss of probing attachment (28). Brown e*t al.* (29) has shown that only 15% of the adult population studied had either gingivitis or periodontitis. Gingivitis without periodontitis occurred in 50%, periodontitis (pockets 4 mm or deeper) in 33% and advanced and end-stage periodontal destruction in 8% and 4%, respectively. It is widely accepted that sex, socio-economic and educational status, age and marital quality determine periodontal status (30). These diseases are more prevalent among the less economically and educationally privileged groups in the social hierarchy (31). Finally, tobacco smoking has been shown to be one of the main risk factors for periodontal disease (32-34).

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, for example, oral hygiene products such as toothpastes, mouthwashes etc. Accumulation of dental plaque at gingival margins due to inadequate dental hygiene leads to the inflammation of the gingivae, defined as gingivitis (35). It can be defined as 'a non-specific 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 (36). The bacteria and their extracellular products present within 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 gingival margin plaque, disrupting pre-formed 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 (37). Folate also appears to protect against the disease (38). Green tea polyphenols have *in vitro* inhibitory effects on the adhesion of oral bacteria to epithelial cells (39). Furthermore, it has been shown that the high molecular weight material of cranberry juice is effective in inhibiting coaggregation between different representative bacteria and *Fusobacterium nucleatum* (40). Adhesion of streptococci is inhibited by hop bract polyphenols (41) and by several tea materials (16,42) 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).

Despite the identification of some foods and beverages to date which are reported to have oral health benefits in caries and periodontal disease, it can be seen that the provision of further agents providing such benefits would provide a contribution to the art.

Disclosure of the invention

The present inventors have evaluated a number of natural products (foods and beverages) not previously identified as having anti-caries and/or anti-gingivitis potential using relevant high-throughput bioassays especially designed for the purpose. Promising foods/beverages have been fractionated and re-tested in these assays in an iterative process which has resulted in the identification of the active constituents.

More specifically the present inventors have demonstrated that extracts and fractions from Shiitake mushrooms *{Lentinula edodes),* chicory *(Cichorium intibus.* var. Silvestre - Radicchio di Treviso tardivo IGP) and raspberries *(Rubus idaeus)* have activity in a number of anti-caries and anti-gingivitis assays.

As noted above an extract from *Lentinus edodes* has previously been studied in rats (19) for its caries-inhibiting effect. However there is no suggestion in that publication regarding anti-gingivitis potential.

A more recent publication related to the inhibitory effects of mushroom extracts on sucrose-dependent oral biofilm formation (Yano et al, Appl Microbiol Biotechnol. 2010 Mar;86(2):615-23. Epub 2009 Nov 10). The activity demonstrated to the extracts was generally attributed to enzymes such as alpha-glucanases. JP201 0077028 A201 00408 apparently relates to a water-soluble compound, apparently being a protein .
Shouji et al (Caries Res 2000;34:94-98) report an anti-caries effect of a component from Shiitake mushrooms. Hirasawa et al (International Journal of Antimicrobial Agents 11 (1999) 151-157) report three kinds of antibacterial substances from Shiitake mushrooms. Shiitake mushrooms are also referred to in JP publications JP63253019 A19881 020 and Jp 2005132872 A20050526.

As described below, the identification of these active fractions (and specific compounds found therein) forms the basis for various novel materials (e.g. agents, functional foods, and oral healthcare products) and methods for improving oral health. In other aspects of the invention the identification of active constituents may be used to inform selective breeding of the parent plant to increase the content of the active constituents could be achieved.

Thus in various aspects of the invention there is provided:

- Use of a composition comprising an extract of one or more of Shiitake mushroom, chicory, or raspberry to promote oral health.
- A method of promoting oral health in an individual, which method comprises use of a composition comprising of an extract of one or more of Shiitake mushroom, chicory, or raspberry.
- A composition for promoting oral health which composition comprises an extract of one or more of Shiitake mushroom, chicory, or raspberry.

- Use of an extract of one or more of Shiitake mushroom, chicory, or raspberry in the manufacture of a composition to promote oral health.

In aspects of the invention relating to extracts of Shiitake mushroom, the extract will preferably be used in ameliorating, controlling or reducing the risk of periodontal disease such as gingivitis or periodontitis (e.g. to inhibition of the activities of one or more target organisms associated with gingivitis) and\or will be used as a low molecular mass (LMM) fraction as described below.

The "composition" may be an oral preparation or foodstuff (which may by way of non-limiting example be a dietary supplement) - for example a beverage, chewing gum, toothpaste, mouthwash or so on.

The foodstuff may be a "functional food" by which is meant 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. Foods depleted in, or enriched with, specific components, are also considered to be *functional* foods. These *various* embodiments are discussed in more detail hereinafter

The term "oral composition" as used herein means a composition that is delivered to the oral surfaces. The oral composition is a product, which in the ordinary course of usage, is not intentionally swallowed for purposes of systemic administration of particular therapeutic agents, but is rather retained in the oral cavity for a time sufficient to contact substantially all of the dental surfaces and/or oral tissues for purposes of oral activity.

By "extract" is meant a composition comprising a preparation of the components of a substance (here Shiitake mushroom, chicory, or raspberry) typically in concentrated form, prepared by treatment of the substance either mechanically (e.g., by pressure treatment) or chemically (e.g., by distillation, precipitation, enzymatic action or high salt treatment) or using chromatography or other separation or fractionation techniques. Thus the "extract" may also be a "fraction" where it has been prepared by fractionation of the original substance. The present inventors have identified a number of fractions and sub-fractions with beneficial activities and these are discussed in more detail hereinafter

The composition may consist or consist essentially of the extract. In this embodiment the extract itself may be a "functional food".

'"Promoting oral health" herein may include one or more of ameliorating, controlling or reducing the risk of dental caries (e.g. by controlling or reducing plaque build up); ameliorating, controlling or reducing the risk of periodontal disease (e.g. gingivitis or periodontitis). This may be demonstrated by showing benefit in the oral health of an individual treated with the oral composition compared to a control composition. In certain embodiments promoting oral health may include treating dental caries or periodontal disease. It may also include preventing dental caries or periodontal disease. It may also include ameliorating dental caries or periodontal disease.

Thus also provided is:

- An anti-caries or anti-gingivitis oral preparation comprising, consisting of, or consisting essentially of an extract of one or more of Shiitake mushroom, chicory, or raspberry.

The present inventors have identified particular compounds in these extracts, and shown activity for these isolated compounds in assays relevant to the promotion of oral health. These compounds include: quinic acid, succinic acid, trans-aconitic acid and inosine. Of these quinic acid and succinic acid are preferred. Other compounds identified by the present inventors as having activity in these assays are Shikimic acid; oxalic acid; adenosine; uridine; cis-aconitic acid and epicatechin.

Thus, compositions comprising, consisting, or consisting essentially of one or more of these compounds may also be used in the various aspects of the present invention, in place of the defined extracts obtained from the relevant plants. Put another way, the level of these compounds may be artificially increased in a foodstuff or oral composition (e.g. by adding the pure compound to supplement the foodstuff or orally acceptable preparation) in order to promote the oral health benefits or effects of that composition in accordance with the present invention. The compound will be present in the composition as an active ingredient. In certain embodiments it may be the sole active ingredient in respect of the promotion of oral health.

The manner by which oral health may be promoted may be by inhibition of the activities of one or more of target organisms associated with caries or gingivitis, for example one or more organisms selected from the list consisting of:

(i) *Streptococcus mutans*

(ii) *Actinomyces naeslundii*

(iii) *Lactobacillus casei*

(iv) *Fusobacterium nucleatum*

(v) *Prevotella intermedia*

More specifically the compositions of the invention will generally have one or preferably more than one of the following counteractive effects against one or more of the target organisms. These counteractive effects include the ability to:

(i) prevent adhesion of the target organisms to, and induce detachment from, hydroxyapatite

(ii) prevent biofilm formation by the target organisms

(iii) elicit an antibacterial effect against the target organisms

(iv) prevent co-aggregation by the target organisms

(v) disrupt signal transduction in S. *mutans*

(vi) disrupt pre-existing biofilms of the target organisms

(vii) prevent adhesion to, and invasion of, gingival epithelial cells by those target organisms associated with gingivitis *(A. naeslundii, P. intermedia)*

(viii) inhibit bacteria-induced host cell pro-inflammatory cytokine production by those target organisms associated with gingivitis, and thereby (for example) reduce gingival inflammation

(ix) inhibit acid production by caries-associated organisms, for example plaque acidogenicity (e.g. as measured following sucrose rinse).

Such counteractive effects for the extracts of the invention are described in the Examples below.

Thus another aspect of the invention provides:

- A method of counteracting any of the effects of the target organisms (associated with caries or gingivitis) described above by contacting an oral surface with an oral composition described herein.

By "oral surface" is meant the hard and soft tissues of the oral cavity. As used herein, "hard tissues" refers to tissues such as the teeth and periodontal support in an oral cavity, such as that of a mammal. "Soft tissues" refers to tissues such as the gums, the tongue, the surfaces of the buccal cavity and the like. Preferably the composition contacts the dental enamel and\or gingival surface.

The compositions of the invention may be applied with the purpose of reducing inflammation or disease (gingivitis, periodontitis) in soft tissues or hard tissues (e.g. caries and/or sensitivity) and\or inhibiting or preventing the accumulation of calculus, plaque, and tartar on the hard tissues e.g. dental enamel.

The compositions may be those which are effective in these activities where used on a daily basis.

Compositions and extracts for use in the aspects and embodiments of the present invention may be prepared, by way of non-limiting disclosure, as described in the Examples and other disclosure below.

These and other aspects and embodiments of the present invention will now be discussed in more detail.

Homoaenates and fractions from Shiitake mushroom

Extracts

A preferred extract for use in the present invention may be that obtainable by homogenization (e.g. for 2 minutes) and centrifugation (e.g. for 10 minutes at 8000 rpm) followed by filtering (e.g. using filter paper filter) to remove the "juice" from the solid part.

Fractions

A so-called low molecular mass (LMM) fraction for use in the present invention may be one less than around 1, 2, 3, 4, or 5 kDa. By way of non-limiting example a less than about 5 kDa LMM fraction of mushroom homogenate may be that obtainable by ultrafiltration (e.g. using the Vivaflow 200 complete system with a 5,000 MW cut-off filter and retaining the diafiltrate). Such LMM fractions will typically exclude enzymes, for example.

A so-called high molecular mass (HMM) fraction for use in the present invention may be one greater than around 1, 2, 3, 4, or 5 kDa. By way of non-limiting example a >5 kDa HMM fraction of mushroom homogenate may be that obtainable by ultrafiltration (e.g. using the Vivaflow 200 complete system with a 5,000 MW cut-off filter and retaining the retentate) followed by dialysis with a 5,000 MW cut-off to further eliminate the LMM components.

A preferred fraction is an LMM fraction.

Sub-fractions

Preferred sub-fractions of the LMM fraction for use in the present invention may be those obtainable by gel filtration (see e.g. Example 8). By way of non-limiting example preferred sub-fractions may be one or more of those obtainable by use of a system with the following dimensions: 300 mm x 10 mm, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water (flow rate, 1.0 mL min-1).

Based on such a system the following sub-fractions may be identified with reference to Figure 5:

M1 - peaking between 36 and 41 mins
M2 - peaking at about 59 mins
M3 - peaking at about 81 mins
M4 - peaking at about 106 mins
M5 - peaking at about 154 mins

Preferred sub-fractions are or correspond to M4 and M5 obtainable in this manner.

Sub-sub-fractions

Preferred sub-sub-fractions of the LMM fraction for use in the present invention may be those obtainable by reverse phase HPLC semi-preparative chromatography (see e.g. Example 8). By way of non-limiting example preferred sub-sub-fractions may be one or more of those obtainable from sub-fractions M4 and M5 by use of a semipreparative HPLC column such as the C18 LiChrospher® 250 * 10 mm, 10 μm, using conditions: volume injected, 1 mL; column temperature, 25 °C; UV spectra were recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm.

Based on such a system sub-sub-fractions may be identified with reference to Figures 6 and 7.

Sub-fraction M4 produced 8 sub-sub-fractions (M4.1-8) with dry masses as shown in Example 8, section 8.3.

A preferred sub-sub-fraction is or corresponds to M4.7.

Sub-fraction M5 produced 11 sub-sub-fractions (M5.1-11) with dry masses as shown in Example 8, section 8.3

A preferred sub-sub-fraction is or corresponds to M5.6.

Compounds

Based on the analysis above, the present inventors demonstrated the presence of the following compounds in the preferred sub-sub fractions.

M4.7: aconitic acid, adenosine and oxalic acid;

M5.6: quinic acid, inosine, oxalic acid and succinic acid.

Any one or more of these compounds, for example as an additive to an oral composition, may be utilised in the aspects of the invention described herein. As shown in Example 11 such compounds have been shown by the present inventors to have activities relevant to the improvement of oral health.

Homoaenates and fractions from chicory

A preferred variety is *Cichorium intybus.* Var. *silvestre.*

Extracts

A preferred extract for use in the present invention may be that obtainable by homogenization (e.g. for 1 minute) and centrifugation (e.g. for 10 minutes at 8000 rpm) followed by filtering (e.g. using filter paper filter) to remove the "juice" from the solid part.

Fractions

A so-called low molecular mass (LMM) fraction for use in the present invention may be one less than around 1, 2, 3, 4, or 5 kDa. By way of non-limiting example a less than about 5kDa LMM fraction of chicory homogenate may be that obtainable by ultrafiltration (e.g. using the Vivaflow 200 complete system with a 5,000 MW cut-off filter and retaining the diafiltrate).

A so-called high molecular mass (HMM) fraction for use in the present invention may be one greater than around 1, 2, 3, 4, or 5 kDa. By way of non-limiting example a >5 kDa HMM fraction of chicory homogenate may be that obtainable by ultrafiltration (e.g. using the Vivaflow 200 complete system with a 5,000 MW cut-off filter and retaining the retentate) followed by dialysis with a 5,000 MW cut-off to further eliminate the LMM components.

A preferred fraction is an LMM fraction.

Sub-fractions

Preferred sub-fractions of the LMM fraction for use in the present invention may be those obtainable by gel filtration (see e.g. Example 8). By way of non-limiting example preferred sub-fractions may be one or more of those obtainable by use of a system with the following dimensions: 300 mm x 10 mm, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water (flow rate, 1.0 mL min-1).

Based on such a system the following six sub-fractions may be identified with reference to Figure 8:

C1 - peaking between 37 and 41 mins
C2 - peaking at about 54 mins
C3 - peaking at about 69 mins
C4 - peaking at about 84 mins
C5 - peaking at about 135 mins

A preferred sub-fraction is or corresponds to C1 obtainable in this *manner.*

Sub-sub-fractions

Preferred sub-sub-fractions of the LMM fraction for use in the present invention may be those obtainable by reverse phase HPLC semi-preparative chromatography (see e.g. Example 8). By way of non-limiting example preferred sub-sub-fractions may be one or more of those obtainable from sub-fractions M4 and M5 by use of a semipreparative HPLC column such as the C18 LiChrospher® 250 * 10 mm, 10 pm, using conditions: volume injected, 1 ml_; column temperature, 25 °C; UV spectra were recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm.

Based on such a system sub-sub-fractions may be identified with reference to Figure 9.

Sub-fraction C1 produced 15 sub-sub-fractions respectively (C1.1-15) with dry masses as shown in Example 9, section 9.3

A preferred sub-sub-fraction is or corresponds to C1.7.

Compounds

Based on the analysis above, the present inventors demonstrated the presence of the following compounds in the preferred sub-sub fractions.

C1.7 = oxalic acid and quinic acid.

Any one or more of these compounds, for example as an additive to an oral composition, may be utilised in the aspects of the invention described herein. As shown in Example 11 such compounds have been shown by the present inventors to have activities relevant to the improvement of oral health.

Homopenates and fractions from raspberry

Extracts

A preferred extract for use in the present invention may be that obtainable by homogenization (e.g. for 5 minute) and centrifugation (e.g. for 10 minutes at 8000 rpm) followed by filtering (e.g. using filter paper filter) to remove the "juice" from the solid part.

Optionally a base (e.g. NaOH) may be added to the extract to increase pH (e.g. to around or between pH 4 or 5).

Fractions

A so-called low molecular mass (LMM) fraction for use in the present invention may be one less than around 1, 2, 3, or 3.5 kDa. By way of non-limiting example a less than about 3.5kDa LMM fraction of raspberry homogenate may be that obtainable by dialysis e.g. using dialysis material with a cut off 3,500 Da. In this way, a dialysate containing all the compounds with molecular masses lower than 3,500 Da can be obtained.

A so-called high molecular mass (HMM) fraction for use in the present invention may be one greater than around 1, 2, 3, or 3.5 kDa. By way of non-limiting example a >3.5 kDa HMM fraction of raspberry homogenate may be that obtainable as the retentate from the dialysis described above.

A preferred fraction is an LMM fraction.

Optionally a base (e.g. NaOH) may be added to the fraction to increase pH (e.g. to around or between pH 4 or 5).

Sub-fractions

Preferred sub-fractions of the LMM fraction for use in the present invention may be those obtainable by solid-phase extraction (see e.g. Examples below). By way of non-limiting example preferred sub-fractions may be one or more of those obtainable by:

(i) concentrating the LMM fraction to dryness in a rotary evaporator at <30°C

(ii) dissolving the residue in 20 ml of phosphate buffer (PB, pH 7.0)

(iii) adjusting the pH to 7.0 with NaOH solution

(iv) passing the sample through a 20cc (5 g) tC18 cartridge conditioned with methanol (10 ml), water (2 x 10 ml), and PB (pH 7.0, 10 ml) at a flow rate < 2 ml/min.

Based on such a system the following six sub-fractions may be identified:

(i) R 1 sub-fraction containing the polar substances (among which there are organic acids and sugars) elutable with 25 ml of PB, pH 7.0

(ii) R 2 obtainable by eluting afterwards with 25 ml of ethyl acetate

(iii) R 3 was obtainable by eluting afterwards with 25 ml of a methanol- Millipore grade water mixture (20%-80%)

(iv) R 4 was obtainable by eluting afterwards with 25 ml of a methanol- Millipore grade water mixture (50%-50%)

(v) R 5 was obtainable by eluting afterwards with 25 ml of methanol

A preferred sub-sub-fraction is or corresponds to R1.

Sub-sub-fractions

Preferred sub-sub-fractions of the LMM fraction for use in the present invention may be those obtainable by as follows:

(i) dilute 5 ml aliquot of R 1 was diluted with HCl (0.01 M) and adjusted to pH 3.0 with HCl solution (4M).

(ii) passing the sample through a 20cc (5 g) tC18 cartridge conditioned with methanol (20 ml), water (2 x 20 ml), and HCl =0.01 M at a flow rate < 2 ml/min.

Based on such a system the following three sub-sub-fractions may be identified:

R1a consists of the most polar substances not retained by the Sep-Pak® Vac 20cc (5 g) tC18 cartridge.

R 1b was obtained by eluting afterwards with 25 mL of Millipore grade water,

R1c was obtained by eluting afterwards with 25 ml of methanol.

Processes for producing extracts

The invention this further provides processes for obtaining a composition as described above. Such processes may be those used as described herein, or alternative methods of chromatography or fractionation such as will occur to those skilled in the art in the light of these processes i.e. which provide the same fractions, but by different means.

Thus in one aspect the process comprises:

(i) providing an extract which is a less than 5kDa LMM sub fraction of Shiitake mushroom by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting sub-fractions corresponding to sub-fraction M4 or M5 shown in Figure 5;

(ii) optionally further providing an extract which is an LMM sub-sub-fraction of the sub-fraction corresponding to M4 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to M4.7 shown in Figure 6;

(iii) optionally further providing an extract which is an LMM sub-sub-fraction of the sub-fraction corresponding to M5 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to M5.6 shown in Figure 7.

Alternatively the process comprises:

(i) providing an extract which is a less than 5kDa LMM sub fraction of chicory by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting sub-fractions corresponding to sub-fraction C1 shown in Figure 8;

(ii) optionally further providing an extract which is an LMM sub-sub-fraction of sub-fraction C1 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to C1.7 shown in Figure 9.

Alternatively the process comprises providing an extract which is a less than 3.5 kDa LMM sub fraction of raspberry by:

(i) concentrating the LMM fraction to dryness in a rotary evaporator at <30°C

(ii) dissolving the residue in an aqueous carrier and optionally adjusting the pH towards neutrality;

(iii) using chromatography to sub-fractionate the solution into sub-fractions and selecting the sub-fraction corresponding to the sub-fraction obtainable by passing the solution through a 20cc (5g) tC18 cartridge conditioned with methanol (10 ml), water (2 x 10 ml), and PB (pH 7.0, 10 ml) at a flow rate < 2 ml/min and selecting the polar sub-fraction elutable with 25 ml of PB, pH 7.0.

Activities of homogenates and fractions in gingivitis-relevant assays

As demonstrated by the present disclosure, of the extracts and fractions, LMM fractions of mushroom and chicory were generally the most active in the assays relevant to gingivitis.

Such activities, for example compositions of the present invention, are demonstrated in Example 3, Example 7, Example 9 and Example 10.

Of the mushroom LMM sub-fractions, M4 and M5 were considered to be the most promising in the anti-gingivitis activity battery of tests.

Of the mushroom LMM sub-sub-fractions, when the dry masses of the sub-sub-fractions were taken into account and the specific activities determined, sub-sub-fractions M4.7 and M5.6 were found to be the most active.

The main constituents in these sub-sub-fractions were identified by HPLC and mass spectroscopy as:

M4.7: aconitic acid, adenosine and oxalic acid;
M5.6: quinic acid, inosine, oxalic acid and succinic acid.

Of the chicory LMM sub-fractions, C 1 was considered to be the most promising in the anti-gingivitis activity battery of tests.

Of the chicory LMM sub-sub-fractions, when the dry masses of the sub-sub-fractions were taken into account and the specific activities determined, sub-sub-fraction C1.7 was found to be the most active.

The main constituents in sub-sub-fraction C1.7 were identified by HPLC and mass spectroscopy as: oxalic acid and quinic acid.

All five sub-fractions of raspberry displayed some activity in most of the assays, but R 1 was considered to be the most promising in an anti-gingivitis activity battery of tests. All three sub-sub-fractions (R1A, R1B, R1C) displayed activity in most of the assays. Because raspberry contains high levels of sugars it is less preferred in the present invention. Nevertheless use of raspberry extracts or fractions from which sugar has been removed also forms part of the present invention.

Thus preferred compositions for use in the invention (based on the extracts, fractions and compounds described above) will preferably demonstrate one or more of the following

activities relevant to the promotion of oral health, and more specifically relevant to the ameliorating, controlling or reducing the risk of gingivitis. Such activities will be compared to a negative control (typically a diluent, buffer or solute) which corresponds generally to that used for the composition of the invention, but from which the active (extracts, fraction or compound) is absent.

Inhibition ofbiofilm formation

Inhibition of biofilm formation by one or more target organisms selected from the list consisting of: V. *dispar, F. nucleatum, A. naeslundii, P. intermedia.* Preferred targets are *A. naeslundii* and\or *P. intermedia,* a most preferred target is P. *intermedia.*

Inhibitory activity against microorganisms

Inhibitory activity against one or both of the target organisms: *A. naeslundii, P. intermedia.* A preferred target is *A. naeslundii.*

Inhibition of co-aggregation

Ability to inhibit co-aggregation of one, two, or more preferably three of the following pairs of target organisms.

P. *intermedia* + *S. sanguinis, F. nucleatum* + *S. sanguinis; F. nucleatum* + *N. subflava; S. sanguinis* + *V. dispar, S. sanguinis* + *N. subflava.*

Disruption of pre-existing biofilms

Ability to disrupt the biofilm of, or kill the organism within a biofilm of, one of both of the target organisms: *A. naeslundii, P. intermedia.*

Prevention of adhesion to gingival epithelial cells

Inhibition of adherence of one or more target organisms *(F. nucleatum, A. naeslundii, P. intermedia)* to epithelial cells (Gingival fibroblast KB cell line) when added simultaneously with the target(s) to the monolayers and\or when used to pretreat the monolayer. Preferred targets are *A. naeslundii* and\or *P. intermedia.*

Prevention of invasion of gingival epithelial cells

Inhibition of *A. naeslundii* internalization by KB cells.

Inhibition of bacteria-induced host cell pro-inflammatory cytokine production

Inhibition of IL-6 release induced by *F. nucleatum* supernatant.

Activities of homogenates and fractions in caries-relevant assays

Such activities, for example compositions of the present invention, are demonstrated in Examples 4 and 6 and 8.

As demonstrated by the present disclosure, of the extracts and fractions, LMM fractions of mushroom and raspberry were generally the most active in the assays relevant to caries.

Of the mushroom LMM sub-fractions, M4 and M5 were considered to be the most promising in the anti-caries activity battery of tests.

Of the mushroom LMM sub-sub-fractions, when the dry masses of the sub-sub-fractions were taken into account and the specific activities determined, sub-sub-fractions M4.7 and M5.6 were found to be the most active.

The main constituents in these sub-sub-fractions were identified by HPLC and mass spectroscopy as:

M4.7: aconitic acid, adenosine and oxalic acid;
M5.6: quinic acid, inosine, oxalic acid and succinic acid.

All five sub-fractions of raspberry displayed some activity in most of the assays, but R1 was considered to be the most promising in an anti-caries battery of tests. All three sub-sub-fractions (R1A, R1B, R1C) displayed activity in most of the assays. Because raspberry contains high levels of sugars it is less preferred in the present invention. Nevertheless use of raspberry extracts or fractions from which sugar has been removed also forms part of the present invention.

Preferred compositions for use in the invention (based on the extracts, fractions and compounds described above) will preferably demonstrate one or more of the following

activities relevant to the promotion of oral health, and more specifically relevant to the ameliorating, controlling or reducing the risk of caries.

Prevention of adhesion to, and induce detachment from, hydroxyapatite

Inhibition of adhesion of one or more target bacteria to hydroxyapatite, which bacteria are selected from: S. *mutans, S. sanguinis, L. casei and A. naeslundii* when added simultaneously with the target(s) to the hydroxyapatite, and\or induce detachment from hydroxyapatite. A preferred target organism is S. *mutans.*

Inhibition ofbiofilm formation

Inhibition of biofilm formation by one or more target organisms selected from the list consisting of: S. *mutans, S. sanguinis, L. casei.* A preferred target organism is S. *mutans.*

Inhibitory activity against microorganisms

Inhibitory activity against one or more of the target organisms: *L. casei, S. mutans, S. sanguinis, A. naeslundii.*

Inhibition of co-aggregation

Ability to inhibit co-aggregation of one or more of the following pairs of target organisms:

S. *mutans +S. sanguinis*
S. mutans + *L. casei*
P. intermedia +F. nucleatum
F. nucleatum + *S. mutans*
F. nucleatum +N. subflava
F. nucleatum + S. *sanguinis*

Disruption of pre-existing biofilms

Ability to disrupt biofilms of one of both of the target organisms: S. *mutans, L. casei.* A preferred target organism is S. *mutans.*

Inhibition of signal transduction in S. mutans

Inhibition of S. *mutans comDE* gene expression.

Activities of compounds in caries- and pinoivitis- relevant assays

In tests of pure compounds against S. *mutans* biofilms and other caries-relevant assays (see Figure 10) quinic acid, succinic acid, cis- and trans-aconitic acid, and oxalic acid all had beneficial effects. Oxalic acid at low concentrations resulted in biofilm disruption similar to that of the positive control agent.

In tests of pure compounds against *A. naeslundii* biofilms and other gingivitis-relevant assays (see Figure 11) succinic acid and oxalic acid both had a beneficial effect.

In accordance with Example 11 preferred compounds are quinic acid, succinic acid, trans-aconitic acid and inosine, with quinic acid and succinic acid being most preferred.

Compounds and salts

Where disclosure is made herein of any particular compound, those skilled in the art will appreciate that it may be convenient or desirable to utilise, prepare, purify, and/or handle a corresponding salt of the compound, for example, an orally or pharmaceutically-acceptable salt. Examples of pharmaceutically acceptable salts are discussed in Berge *et* a/., 1977, "Pharmaceutically Acceptable Salts." J. Pharm. Sci., Vol. 66, pp. 1-19.

For example, if the compound is anionic, or has a functional group which may be anionic (e.g., -COOH may be $-COO^-$), then a salt may be formed with a suitable cation. Examples of suitable inorganic cations include, but are not limited to, alkali metal ions such as Na^+ and K^+, alkaline earth cations such as Ca^{2+} and Mg^{2+}, and other cations such as Al^{+3}. Examples of suitable organic cations include, but are not limited to, ammonium ion (i.e., NH_4^+) and substituted ammonium ions (e.g., NH_3R^+, $NH_2R_2^+$, NHR_3^+, NR_4^+). Examples of some suitable substituted ammonium ions are those derived from: ethylamine, diethylamine, dicyclohexylamine, triethylamine, butylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine, benzylamine, phenylbenzylamine, choline, meglumine, and tromethamine, as well as amino acids, such as lysine and arginine. An example of a common quaternary ammonium ion is $N(CH_3)_4^+$.

If the compound is cationic, or has a functional group which may be cationic (e.g., $-NH_2$ may be $-NH_3^+$), then a salt may be formed with a suitable anion. Examples of suitable inorganic anions include, but are not limited to, those derived from the following inorganic

acids: hydrochloric, hydrobromic, hydroiodic, sulfuric, sulfurous, nitric, nitrous, phosphoric, and phosphorous.

Examples of suitable organic anions include, but are not limited to, those derived from the following organic acids: 2-acetyoxybenzoic, acetic, ascorbic, aspartic, benzoic, camphorsulfonic, cinnamic, citric, edetic, ethanedisulfonic, ethanesulfonic, fumaric, glucheptonic, gluconic, glutamic, glycolic, hydroxymaleic, hydroxynaphthalene carboxylic, isethionic, lactic, lactobionic, lauric, maleic, malic, methanesulfonic, mucic, oleic, oxalic, palmitic, pamoic, pantothenic, phenylacetic, phenylsulfonic, propionic, pyruvic, salicylic, stearic, succinic (which itself may be an active in the composition, as described above) sulfanilic, tartaric, toluenesulfonic, and valeric. Examples of suitable polymeric organic anions include, but are not limited to, those derived from the following polymeric acids: tannic acid, carboxymethyl cellulose.

Unless otherwise specified, a reference to a particular compound also includes salt forms thereof.

Selection of plants and fungi

In light of the disclosure herein it can be seen that the compounds described herein may be used as the basis for selecting or breeding plants having enhanced oral health benefits - for example for use in the amelioration of caries\gingivitis. Thus methods of the invention include:

A method for assessing the potential oral health benefits of a plant or fungus, the method comprising assessing the level of a compound in the plant or fungus, wherein the compound is selected from the list consisting of: quinic acid; adenosine; inosine; shikimic acid; trans-aconitic acid; oxalic acid; adenosine; uridine; cis-aconitic acid and succinic acid.

Preferably the compound is selected from the list consisting of: quinic acid; succinic acid, trans-aconitic acid; inosine. Preferably it is quinic acid or succinic acid.

In the method, plants or fungi including enhanced levels of the compound are selected, and may then be consumed or used to prepare compositions according to the present invention.

Assessment of the compounds may be done by conventional methods - for example by HPLC and mass spectroscopy.

Description of certain aspects and embodiments

Thus aspects of the invention include a composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of a natural product selected from the list consisting of: Shiitake mushroom, chicory, or raspberry. Preferably the composition is for one or more of ameliorating, controlling or reducing the risk of dental caries or periodontal disease, which periodontal disease is optionally gingivitis or periodontitis.

The extract may be obtained or obtainable as described above by homogenization of the natural product and filtering to remove solid matter.

A preferred extract is of a low molecular mass (LMM) fraction of the natural product comprising compounds of less than around 1, 2, 3, 3.5, 4, 4.5 or more preferably 5 kDa.

In preferred embodiments relating to Shiitake mushroom, the extract is of a less than 5kDa LMM sub fraction of Shiitake mushroom obtainable by gel filtration in a 300 mm x 10 mm column, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water having a flow rate, 1.0 ml/min, such as to sub-fractionate the LMM fraction into 5 sub-fractions as follows and selecting the sub-fractions M4 and M5 peaking at about 106 mins or about 154 mins.

A preferred extract is of an LMM sub-sub-fraction of Shiitake mushroom obtainable from sub-fraction M4 by use of a semipreparative HPLC column with the following properties: C18, 250 × 10 mm, 10 μm and conditions: volume injected, 1 mL; column temperature, 25°C; UV spectra recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm, such as to sub-fractionate the LMM fraction into 8 sub-fractions in accordance with Figure 6 and selecting the sub-sub-fraction M4.7.

Another preferred extract is of an LMM sub-sub-fraction of Shiitake mushroom obtainable from sub-fraction M5 by use of a semipreparative HPLC column with the following properties: C18, 250 * 10 mm, 10 μm and conditions: volume injected, 1 mL; column

temperature, 25°C; UV spectra recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm, such as to sub-fractionate the LMM fraction into 11 sub-fractions in accordance with Figure 7 and selecting the sub-sub-fraction M5.6.

In preferred embodiments relating to chicory, the extract is of a less than 5kDa LMM sub fraction of chicory obtainable by gel filtration in a 300 mm x 10 mm column, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water having a flow rate, 1.0 ml/min, such as to sub-fractionate the LMM fraction into 5 sub-fractions as follows and selecting the sub-fraction C1 peaking at between about 37 mins and 41 mins.

A preferred extract is of an LMM sub-sub-fraction of chicory obtainable from sub-fraction C1 by use of a semipreparative HPLC column with the following properties: C18, 250 * 10 mm, 10 µm and conditions: volume injected, 1 mL; column temperature, 25°C; UV spectra recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm, such as to sub-fractionate the LMM fraction into 15 sub-fractions in accordance with Figure 9 and selecting the sub-sub-fraction C1.7.

In preferred embodiments relating to raspberry, the extract is of a less than 3.5 kDa LMM sub fraction of raspberry obtainable by:

(i) concentrating the LMM fraction to dryness in a rotary evaporator at <30°C

(ii) dissolving the residue in 20 ml of phosphate buffer (PB, pH 7.0)

(iii) adjusting the pH to 7.0 with NaOH solution

(iv) passing the sample through a 20 cc (5 g) tC18 cartridge conditioned with methanol (10 ml), water (2 x 10 ml), and PB (pH 7.0, 10 ml) at a flow rate < 2 ml/min.

and selecting the polar sub-fraction elutable with 25 ml of PB, pH 7.0.

As described above, other aspects of the invention relate to the use of pure or isolated compounds for use in compositions for promoting oral health. Such a composition may be a functional food or oral preparation which has been supplemented with a compound selected from the list consisting of: quinic acid; adenosine; inosine; shikimic acid; trans-aconitic acid; oxalic acid; adenosine; uridine; cis-aconitic acid and succinic acid. A preferred list consists of: quinic acid; succinic acid, trans-aconitic acid; inosine. Preferred compounds are quinic acid and succinic acid.

As described herein, the compositions will generally demonstrate 1 or more (2, 3, 4, 5, 6, or 7) of the following activities relevant to the promotion of oral health:

(i) inhibition of biofilm formation by one or more target organisms selected from the list consisting of: *V. dispar, F. nucleatum, A. naeslundii, P. intermedia;*

(ii) ihibitory activity against one or both of the target organisms: *A. naeslundii, P. intermedia;*

(iii) ability to inhibit co-aggregation of one, two, or more preferably three of the following pairs of target organisms: *P. intermedia +S. sanguinis; F. nucleatum* + S. *sanguinis; F. nucleatum + N. subflava; S. sanguinis +V. dispar, S. sanguinis +N. subflava.* A preferred pair is *F. nucleatum* + S. *sanguinis;*

(iv) ability to disrupt the biofilm of, or kill the organism within a biofilm of, one of both of the target organisms: *A. naeslundii, P. intermedia.* A preferred target is *A. naeslundii;*

(v) inhibition of adherence of one or more target organisms selected from: *F. nucleatum, A. naeslundii, P. intermedia,* to epithelial cells when added simultaneously with the target(s) to the monolayers and\or when used to pretreat the monolayer;

(vi) inhibition *of A. naeslundii* internalization by epithelial cells;

(vii) Inhibition of cellular IL-6 release induced by *F. nucleatum* supernatant.

Additionally or alternatively, they may demonstrate 1 or more (2, 3, 4, 5, or 6) of the following activities relevant to the promotion of oral health:

(i) inhibition of adhesion of one or more target bacteria to hydroxyapatite, which bacteria are selected from: S. *mutans, S. sanguinis, L casei* and *A. naeslundii* when added simultaneously with the target(s) to the hydroxyapatite, and\or induce detachment from hydroxyapatite;

(ii) inhibition of biofilm formation by one or more target organisms selected from the list consisting of: S. *mutans, S. sanguinis, L casei;*

(iii) inhibitory activity against one or more of the target organisms: *L casei, S. mutans, S. sanguinis, A. naeslundii;*

(iv) ability to inhibit co-aggregation of one or more of the following pairs of target organisms: S. *mutans +S. sanguinis ; S. mutans + L. casei; P. intermedia +F. nucleatum; F. nucleatum* + S. *mutans; F. nucleatum +N. subflava; F. nucleatum* + *S. sanguinis;*

(v) ability to disrupt biofilms of one of both of the target organisms: S. *mutans, L. casei;*

(vi) inhibition of S. *mutans comDE gene* expression.

By way of non-limiting example, as described below, a mouthwash containing a LMM fraction of shiitake mushroom extract has been shown to have a beneficial effect on oral

health in that it can reduce the accumulation of plaque and can reduce gingival inflammation. Indeed statistical analysis of the data revealed that after 6 days of use, the shiitake mushroom-containing mouthwash was more effective than a positive control (Listerine™) at reducing gingival inflammation.

The extracts or other relevant compounds of the present invention may be provided in convenient formats for direct use, or for incorporation into functional foods or other orally acceptable form.

As demonstrated in the Examples below, one convenient format may be a so-called "microbead" in which the relevant compound is mixed with a polymer solution, or polymer-forming solution, which is then cross-linked with the compound microencapsulated *in situ* (e.g. via a process such as ionotropic gelation - see e.g. J Microencapsul. 2001 Mar-Apr; 18(2):237-45.) Such beads can be filtered and dried and incorporated into orally acceptable compositions. As demonstrated in the Examples below, they can bind well to mucosal membranes and hydrozyapatite.

Preferred beads are prepared from chitosan or an alginate. These are available commercially e.g. in the Protanal™ range. Preferred ratios and compositions are described in Example 14 below, especially Tables 11-13.

Preferred beads are between 100 and 10000 μm in diameter e.g. in a population of dried beads having an average size of between 500 and 2000.

As demonstrated in the Examples, extracts and active constituents could be successfully encapsulated into micro-beads which were resistant to mechanical damage and showed good adhesion to mucosa and to hydroxyapatite. These could be incorporated into functional foods (such as chewing gum) and released in the buccal cavity.

As described herein the composition may be a functional food or oral preparation supplemented with the extract or compound in pure form such as to promote the oral health benefits or effects of the oral composition. In the composition the extract or compound may be present as the sole active ingredient in respect of the promotion of oral health, or other compounds (known to those skilled in the art) may be present to promote this effect.

Examples compositions include those selected from: a foodstuff, beverage, chewing gum, toothpaste, mouthwash, mouth rinse, toothpowder or tooth gel. Examples of such compositions (absent the extracts or compounds of the invention) are well known to those skilled in the art.

Formulations or functional foods containing succinic acid would preferably contain equal to or at least 0.5 µg/ml of succinic acid.

Formulations or functional foods containing quinic acid would preferably contain equal to or at least 3.5 mg/ml of quinic acid.

Formulations or functional foods containing trans-aconitic acid would preferably contain equal to or at least 5.0 µg/ml of trans-aconitic acid.

Formulations or functional foods containing inosine acid would preferably contain equal to or at least 1.0 mg/ml of inosine.

Formulations or functional foods containing adenosine would preferably contain equal to or at least 2.0 mg/ml of adenosine.

Formulations or functional foods containing epicatechin would preferably contain equal to or at least 2.5 mg/ml of epicatechin.

Formulations or functional foods containing cis-aconitic acid would preferably contain equal to or at least 50 µg/ml of cis-aconitic acid.

Formulations or functional foods containing uridine would preferably contain equal to or at least 5.0 mg/ml of uridine.

Formulations or functional foods containing oxalic acid would preferably contain equal to or at least 0.6 Mg/ml of oxalic acid.

Formulations or functional foods containing shikimic acid would preferably contain equal to or at least 0.7 µg/ml of shikimic acid.

Where using extracts rather than pure compounds, such extracts may be provided as described above. Typically these may be further concentrated for commercial use - e.g. 2, 3, 4, or 5 times the LMM fraction concentration obtained using the extraction procedure described above.

Mouthwash

Accepted formulations of the kind to be found in textbooks or on the market, are supplemented with the extracts or compounds of the invention. The resulting product is to be used to wash the mouth by gargling.

A preferred mouthwash used in the Examples below includes an extract or compound of the invention (e.g. LMM shiitake mushroom extract) and other ingredients such as sodium fluoride, as well as colouring and flavorings, such as artificial sweetener.

Lozenge Tablet

Accepted formulations of the kind to be found in textbooks or on the market are supplemented with the extracts or compounds of the invention. The resulting product is to be dissolved in the mouth in the usual manner.

Toothpaste

Accepted formulations of the kind to be found in textbooks or on the market are supplemented with the extracts or compounds of the invention. This is to be used in the normal manner on a toothbrush or applied to the teeth and gums as with the fingers.

Examples of these and oral compositions are well known to those skilled in the art and are found, for example, in published PCT international application WO01/82922A1 . To save space, the full text of WO01/82922A1 is incorporated into the present application by reference, as if the disclosure of which is listed herein word by word.

Chewing Gum

Accepted formulations of the kind to be found in textbooks or on the market are supplemented with the extracts or compounds of the invention. Typical ingredients will include a "gum base" with added sweeteners, softeners, and flavoring. Chewing gum base is typically a complex mixture of ingredients: elastomers, resin plasticizers, minerals, waxes, lipids, and emulsifiers. Elastomers provide elasticity and texture. They include natural rubber, natural gums, and styrene-butadiene rubber and polyvinylacetate.

Plasticizers act as softening agents and along with minerals, regulate cohesiveness. A non-limiting example is given in WO0042861 (Wrigley). The resulting product is to be chewed in the usual manner.

As per the Examples below, chewing gums including active ingredients of the invention may be prepared using standard techniques. For example the gum base may be was softened by heating and optionally glycerol was added. Micro-beads or another suitable formal may be uniformly incorporated into the softened gum-glycerol mixture. The resulting chewing gums obtained can then be molded into a desired shape (e.g. rod) and cooled to room temperature.

A chewing gum unit may for example be between 200 and 1000mg e.g. around 500 mg. The ratio of active ingredient to gurmplasticiser may optionally be between 0.1 - 1: 1, depending on the strength of the active and its loading into the relevant format (e.g. micro-beads).
Preferably the chewing gum will release the active ingredient efficiently on chewing e.g. >50% within 5 minutes, and most preferably >90 or 95% within 20 mins. Where micro-beads are used these will preferably be largely resistant to mechanical damage such as to permit them to be released intact during chewing. As shown in the Examles, micro-beads of the invention could be released undamaged within 20 minutes of chewing (a generally accepted measure of time that chewing gum is typically retained in the oral cavity).

Candy

Accepted formulations of the kind to be found in textbooks or on the market are supplemented with the extracts or compounds of the invention. The resulting product is to be dissolved in the mouth in the usual manner.

Treatment period

As is normal in this technical effect, beneficial counteractive effects of the compounds described herein compared to controls in some cases may only be achieved after use of the compositions over a treatment period of days or weeks - for example a period of equal to or exceeding 1, 2, 3, 4, 5, 6, 7 days, or 1, 2, 3, 4 weeks and so on.

Any sub-titles herein are included for convenience only, and are not to be construed as limiting the disclosure in any way.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

The disclosure of all references cited herein, inasmuch as it may be used by those skilled in the art to carry out the invention, is hereby specifically incorporated herein by cross-reference.

Figures

Figure 1 - Determination of most effective bacterial inducer of IL-6 from the Mono-Mac-6 cells

Figure 2 - Effect of chicory homogenate and mushroom homogenate on IL-6 release induced by F. *nucleatum* supernatant.

Figure 3 - Effect of chicory homogenates on IL-6 release induced by *F. nucleatum* supernatant.

Figure 4 - Effect of mushroom homogenates on IL-6 release induced by *F. nucleatum* supernatant.

Figure 5 - Fractionation of LMM mushroom homogenate by gel filtration to fractions M1-M6.

Figure 6 - Sub-fractionation of mushroom homogenate fraction M4 to sub-sub-fractions respectively (M4.1-4) by HPLC.

Figure 7 - Sub-fractionation of mushroom homogenate fraction M5 to sub-sub-fractions respectively (M5.1-5.11) by HPLC.

Figure 8 - Fractionation of LMM chicory homogenate by gel filtration to fractions C1-C6.

Figure 9 - Sub-fractionation of chicory homogenate fraction C1 to sub-sub-fractions respectively (C1.1-1.15) by HPLC.

Figure 10a) - j) - Biological activities against S. *mutans* of isolated compounds identified in mushroom and chicory extracts:

a) shows the effect of Epicatechin
b) shows the effect of Shikimic acid
c) shows the effect of Quinic acid
d) shows the effect of trans-Aconitic acid
e) shows the effect of Oxalic acid
f) shows the effect of Adenosine
g) shows the effect of inosine
h) shows the effect of uridine
i) shows the effect of cis-Aconitic acid
j) shows the effect of succinic acid

Figure 11a) - j) - Biological activities against *A. naeslundii* of isolated compounds identified in mushroom and chicory extracts:

a) shows the effect of Epicatechin
b) shows the effect of Shikimic acid
c) shows the effect of Quinic acid
d) shows the effect of trans-Aconitic acid
e) shows the effect of Oxalic acid
f) shows the effect of Adenosine
g) shows the effect of inosine
h) shows the effect of uridine
i) shows the effect of cis-Aconitic acid

Figure 12 shows a schematic schedule of a randomized, double-blind controlled trial on the effects of a mouthrinse containing shiitake mushroom extract on dental plaque, plaque acidogenicity and the plaque microbiota

Figure 13 shows changes in plaque-pH after a mouthrinse with 10% sucrose following the three test periods Shiitake, Water and Meridol. n = 30 in the trial described in Figure 12.

Figure 14 shows changes in plaque-pH after a mouthrinse with 10% sucrose when calculated as area under the curve below pH 5.7 ($AUC_{5.7}$) after the three test periods (Shiitake, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

Figure 15 shows changes in plaque-pH after a mouthrinse with 10% sucrose after baseline and the three washout periods (after Shiitake, after Water and after Meridol). n = 30 (n=29 for washout Meridol).

Figure 16 shows the mean number of A) lactobacilli, B) mutans streptococci, C) oral streptococci and D) total number of microorganisms in saliva expressed as CFU/ml saliva after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

Figure 17 shows the proportions of total streptococci vs total flora (left) respective mutans streptococci vs total streptococci (right) in saliva expressed as % (mean) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

Figure 18 shows mean protein concentration *(µg)* in resting (pre-sucrose rinse) and fermenting (post-sucrose rinse) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 28.

Figure 19 shows mean amount of A) acetate, B) lactate and C) minor acids in resting (pre-sucrose rinse) and fermenting (post-sucrose rinse) (µmol/mg protein) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 28.

Figure 20 shows plaque score (mean ± SD) after a mouthrinse with 10% sucrose after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

Figure 21 shows the distribution of plaque scores among the various groups during the course of the study described in Example 13. For each of the cluster of bars, the order is

(left to right) scores PS1 , PS2, PS3, with the leftmost on each day being mushroom, then negative control, then positive control.

Figure 22 shows the mean plaque scores of the various groups at different time points in the study of Example 13.

Figure 23 shows the gingival index scores for the various groups during the study (bars ordered as per Figure 21).

Figure 24 shows the mean gingival index scores of the various groups at different time points in the study.

Figure 25 shows the percentages of *F. nucleatum* in plaque samples from the negative control group (pink, squares ■), the positive control group (yellow triangles, A) and the group administered mushroom extract (blue diamonds, 0). The same coding is used in Figs. 26-32 below.

Figure 26 shows the percentages of *L casei* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 27 shows the percentages of *V. dispar* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 28 shows the percentages of *N. subflava* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 29 shows the percentages of *A. naeslundii* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 30 shows the percentages of *P. intermedia* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 31 shows the percentages of S. *sanguinis* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 32 shows the percentages of S. *mutans* in plaque samples from the negative control group (pink), the positive control group (yellow) and the group administered mushroom extract (blue).

Figure 33 shows a scheme for preparation of micro-beads containing active constituents as described in Example 14.

Figure 34 shows the evaluation of in vitro mucoadhesion ability of various quinic acid-containing micro-beads. The results are expressed as the percentage of micro-beads adhering to the model membrane (pork mucosa) after 4 hours. The Batches in the list are shown left to right in the chart.

Figure 35 show the evaluation of the ability of various quinic acid-containing micro-beads to adhere to hydroxyapatite. The results are expressed as the percentage of micro-beads adhering to 23 mm diameter hydroxyapatite discs after 4 hours. The Batches in the list are shown left to right in the chart.

Figure 36 shows the quinic acid release from the various quinic acid-containing micro-beads

Figure 37 shows the adhesion of micro-beads containing LMM shiitake mushroom extract to pork mucosa and hydroxyapatite. The results are expressed as the percentage of micro-beads adhering after 4 hours.

Figure 38 shows a photograph of chewing gum containing micro-beads loaded with LMM shiitake mushroom extract.

Examples

Example 1- General approach and selection of starting materials

The present inventors have evaluated a number of plant-derived foods and beverages not previously identified as having anti-caries and/or anti-gingivitis potential using relevant bioassays. Promising foods/beverages have been fractionated and re-tested in these assays in an iterative process which has resulted in the identification of the active constituents.

A number of foods and beverages were selected based on extensive literature search and the expertise and knowledge of the inventors. Of the foods and beverages originally selected for investigation, a sub-set was taken forward for detailed study. This included raspberries *(Rubus idaeus),* Shiitake mushrooms *{Lentinula edodes),* red chicory *(Cichorium intibus. var. Silvestre - Radicchio di Treviso tardivo IGP)* and beer (Guinness).

All species were for obtained from normal commercial over-counter sources.

Suitable types/strains of the chosen foods/beverages were selected and providers of these identified. Homogenates/extracts of the selected foods/beverages were prepared and chemically analysed to provide material suitable for subsequent investigations by the other partners.

Example 2 - Analysis of starting materials & preparation of extracts.

2.1. Determination of protein, lipid and carbohydrate content of selected food/beverages

These analyses were carried out in order to ensure standardisation of different batches of the food/beverages.

Protein determination: The Kjeldahl method was used. This is the standard method of nitrogen determination (AOAC official methods) consisting of three different steps:

1. digestion of the sample in sulphuric acid with a catalyst, which results in conversion of nitrogen to ammonia
2. distillation of the ammonia into a trapping solution
3. quantification of the ammonia by titration with a standard solution

Lipid determination : The Soxlhet method was used. A dried, ground sample was extracted in a Soxhlet apparatus with diethyl ether which dissolves fats, (and other fat soluble substances). The ether was then evaporated from the fat solution. The resulting residue was weighed and referred to as ether extract or crude fat.

Carbohydrate determination: glucose, fructose, and sucrose were determined using an enzymatic assay (D-glucose concentration was determined before and after the enzymatic hydrolysis of sucrose; D-fructose was determined subsequent to the determination of D-glucose). The other sugars (mannose, ramnose, maltose, xylose, etc) were determined by thin layer chromatography which is only a qualitative analysis. The results obtained from these analyses are shown below:

	Raspberry	Red chicory	Mushroom	Beer
Protein	0.84 g/100g	1.85 g/100g	1.43 g/100g	0.33 g/100ml
Lipid	0.20 g/100g	0.20 g/100g	1.56 g/100g	0.00 g/100ml
Moisture content	84.5 g/100g	94.4 g/100g	89.0 g/100g	92.5 g/100ml
Total carbohydrate	14.90 g/100g	3.95 g/100g	7.99 g/100g	3.00 g/100ml
Sucrose %	0.24	trace	0.02	trace
Glucose %	0.63	0.86	0.12	trace
Fructose %	0.71	0.70	trace	trace

2.2 *Determination of those minor components in the selected materials that are known to affect oral health*

These determinations consisted of the quantification of:

1- Fluoride anion (which has anti-caries activity):

this determination was carried out using a fluoride-specific electrode

2- Zinc, strontium, molybdenum, boron and lithium (all inhibit plaque formation and gingivitis).

All these cations were determined using atomic spectroscopy.

3- Selenium, beryllium, copper and lead cations (all promote caries).

All these cations were determined using atomic spectroscopy.

4- Total polyphenols.

This determination was carried out using the Folin-Ciocalteau reagent and spectrophotometric analysis.

The results obtained from these analyses are shown in the table below

	Raspberry	**Red chicory**	**Mushroom**	**Beer**
Fluoride	<0.002 mg/100g	0.003 mg/100g	0.010 mg/100g	0.016 mg/100ml
Zinc	0.76 ppm	1.04 ppm	1.03 ppm	0.04 ppm
Strontium	0.15 ppm	0.02 ppm	0.15 ppm	0.06 ppm
Molybdenum	<0.002 ppm	<0.003 ppm	<0.003 ppm	<0.01 ppm
Boron	0.025 ppm	0.08 ppm	0.38 ppm	0.03 ppm
Lithium	<0.002 ppm	<0.003 ppm	<0.003 ppm	<0.01 ppm
Selenium	<0.001 ppm	<0.001 ppm	<0.002 ppm	<0.0065ppm
Berillium	<0.002 ppm	<0.003 ppm	<0.003 ppm	<0.005ppm
Copper	0.12 ppm	0.02 ppm	0.17 ppm	<0.005ppm
Lead	<0.002ppm	<0.003ppm	<0.003ppm	<0.005ppm

Total polyphenol	0.48 mg/g	0.43 mg/g	0.38 mg/g	1.53 mg/ml

2.3 Preparation of homogenates/extracts

The following homogenates/extracts were prepared:

Raspberry: juice was obtained by homogenisation of frozen fruits
Mushroom: juice was prepared by homogenisation of frozen fungi
Red chicory: juice was obtained by homogenisation of fresh vegetables
Beer: Guinness beer was de-alcoholated

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.

Raspberry: juice was obtained by homogenisation (for 5 min) and centrifugation (centrifugation speed 8000 rpm, twice for 10 min) from aliquots (400 g) of frozen fruits. The juice, after separation from the solid part, was filtered (paper filter). Ultrafiltration was impossible (due to obstruction of the membrane) but, because of the acidic pH value, the juice was microbiologically uncontaminated. Given the highly acid nature of the juice an appropriate volume of NaOH was added in order to obtain a higher pH value (4.82), freeze-dried and tested for microbiological contamination.

Mushroom: aliquots (400 g) of frozen fungi were homogenized (for 2 minutes) and centrifuged (for 10 minutes at 8000 rpm). The juice, after separation from the solid part, was filtered (paper filter) and then sterilised by ultrafiltration. The sterile juice was tested for microbiological contamination, freeze-dried and re-tested for microbiological contamination.

Red chicory: aliquots (500 g) of fresh vegetable were homogenized (for 1 min) and centrifuged (for 10 minutes at 8000 rpm). The juice, after separation from the solid part, was filtered on paper filter and then sterilised by ultrafiltration. The sterile juice was tested for microbial contamination, freeze-dried and re-tested for microbiological contamination.

Beer: aliquots (325 ml) of Guinness beer were submitted to elimination of CO_2 (AOAC official methods) and de-alcoholated (bath temperature: 50°C, vacuum: 30 bar for 20 min). The resulting beverage was tested for microbiological contamination, freeze-dried and re-tested for microbiological contamination.

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2.4. Analysis of homogenates/extracts

Protein, sucrose, glucose, and fructose content and minor components were determined in the homogenates/extracts as described above. The results obtained from these analyses are shown below

	Raspberry	**Red chicory**	**Mushroom**	**Beer**
Protein	0.53 g/100g	trace	trace	0.33 g/100ml
Sucrose %	0.55	trace	0.03	trace
Glucose %	1.40	1.52	0.18	trace
Fructose %	1.58	1.22	trace	trace
Fluoride	<0.05mg/L	0.05mg/L	0.15mg/L	0.16mg/L
Zinc	1.71 µg/ml	1.84 µg/ml	1.45 µg/ml	0.04 µg/m
Strontium	0.34 µg/ml	0.03 ng/ml	0.21 µg/ml	0.06 µg/ml
Molybdenum	<0.005µg/ml	<0.005 µg/ml	<0.005 µg/ml	<0.005 µg/ml
Boron	0.56 µg/ml	0.13 µg/ml	0.52 µg/ml	0.03 µg/ml
Lithium	<0.005µg/ml	<0.005 µg/ml	<0.005 µg/ml	<0.005 µg/ml
Selenium	<0.003µg/ml	<0.003 µg/ml	<0.003µg/ml	0.0065 µg/ml
Berillium	<0.005µg/ml	<0.005 µg/ml	<0.005µg/ml	<0.005µg/ml

Copper	0.280 µg/ηιI	0.040 µg/ΓηI	0.236 µg/ml	<0.005µg/ml
Lead	<0.005Mg/ml	<0.005ng/ml	<0.005Hg/ml	<0.005µg/ΓπI
Total polyphenol	1.07 mg/ml	0.75 mg/ml	0.53 mg/ml	1.53 mg/ml

Example 3 - Initial assessment of anti-gingivitis activities of the homogenates/extracts

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).* The assays were used to assess the ability of the homogenates/extracts to:

- Prevent biofilm formation by the target organisms
- Elicit an antibacterial effect against the target organisms
- Prevent co-aggregation by the target organisms
- Disrupt pre-existing biofilms of the target organisms
- Prevent adhesion to, and invasion of, gingival epithelial cells by those target organisms associated with gingivitis
- Inhibit bacteria-induced host cell pro-inflammatory cytokine production by those target organisms associated with gingivitis

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 below.

All bacteria were cultured in Brain heart Infusion broth (BHIB) except for *Streptococcus 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 *Lactobacillus* case/) 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 x 10^5 cfu ml^{-1} (S. *mutans, S. sanguinis, L. casei, V. disparand A. naeslundii) or* 5-8 x 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, Streptococcus sanguinis* and *Lactobacillus casei)* or anaerobic conditions (P. *intermedia, A. naeslundii* and *V. dispaO,* 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 at least twice.

Homogenate/ extract	*V. dispar*				*F. nucleatum*				*A. naeslundii*				*P. intermedia*			
	2x	1x	0,5x	0.25x	2x	1x	0,5x	0.25x	2x	1x	0,5x	0.25x	2x	1x	0,5x	0.25x
Beer	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	+++	++
Raspberry	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Chicory	+++	+++	+++	+	+++	+++	+++	++	+++	+++	+++	++	+++	+++	+++	+++
Mushroom	+++	++	++	+	+++	+++	+++	++	+++	+++	++	++	+++	+++	++	++

(81-100% inhibition = +++, 51-80% inhibition = ++, 25-50 % inhibition = +, <25% inhibition = ±, 0 = no effect; 2X to 0.25X denotes the concentration of the homogenate/extract used)

It can be seen that even at the lowest concentration tested, all homogenates/extracts were able to inhibit biofilm formation by the target organisms to some extent.

3.2 Antibacterial activity of homogenates/extracts

All the extracts were assayed for their antibacterial activities in a standard Minimum Inhibitory Concentration (MIC) assay.

Determination of minimal inhibitory concentration (MIC)

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 Brain Heart Infusion (BHI) broth. After overnight growth, the bacterial culture was diluted in broth to contain 10^5 cfu/mL. Two-fold dilutions of test samples and fractions in 0.1 ml of BHI broth were placed into wells of flat-bottomed microtitre plates (Nunc 96-well flat-bottomed microtitre plates; Nunc, Roskilde, Denmark). 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 above, 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,

	Chicory	Mushroom	Beer	Raspberry
P. intermedia	1X	0.25X	>1X	1X
A. naeslundii	>1X	0.125X	0.25X	0.25X

It can be seen that all homogenates/extracts showed some inhibitory activity against one or both of the target organisms. The tables show the concentration with respect to the concentration of the original extract supplied. E.g. 1X = original concentration of extract.

3.3 Prevention of co-aggregation 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. All combinations of the strains used were tested for co-aggregation activity and the following were used in subsequent assays; S. *sanguinis & P. intermedia, S. sanguinis & F. nucleatum, N. subflava & F. nucleatum.*

The homogenates/extracts were assayed for their ability to inhibit co-aggregation as described below:

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 BHI broth . After overnight growth, cells were harvested, washed with coaggregating buffer (1 millimolar tris (hydroxy-methyl) aminomethane; 0.1 mmol/L magnesium chloride; 0.1mmol/L sodium chloride; 0.02 percent sodium azide adusted to pH, 8.0), adjusted to an optical density of 1.5 at 400 nanometers (UV-Vis. Spectrophotometer) and stored at 4°C until use. The ability of test sample or fraction to inhibit co-aggregation of selected pairs of bacteria was tested by adding equal volumes (0.05ml) of bacterial suspension of one pair to equal volume of serial two fold dilution of test sample or fraction in co-aggregating buffer followed by adding equal volume of the bacterial suspension of the other co-aggregating member in 12x75 millimeter test tube. After vigorous vortex of the mixture and further incubation at room temperature for 2 minutes co-aggregation was scored. The last dilution of the sample causing complete inhibition of co-aggregation was recorded and expressed either as final concentration (w/v) or as percent of undiluted sample.

	Chicory	**Mushroom**	**Beer**	**Raspberry**
P. intermedia + *S. sanguinis*	0	+	0	+
F. nucleatum + *S. sanguinis*	0	0	0	+
F. nucleatum + *N. subflava*	+	++	0	0
S. sanguinis + *V. dispar*	0	0	0	+
S. sanguinis + *N. subflava*	+	+	+	0

0 = no inhibition of co-aggregation, ++ = complete inhibition of co-aggregation, + = partial inhibition of co-aggregation

As can be seen, all homogenates/extracts showed some ability to inhibit co-aggregation of at least one pair of target organisms. Mushroom and raspberry exhibited some ability to inhibit co-aggregation of three target pairs.

3.4 Disruption of pre-existing 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. Mature biofilms of each of the test organisms were grown on cellulose nitrate membrane filters and incubated with the test compounds for 1 minute. The number of live and dead cells disrupted from the biofilm was assessed as well as the number of live and dead cells remaining. The protocol used is described below:

Single species biofilms of each of the test strains were cultivated on sterile nitrocellulose membrane filters (0.2μm pore size). Aerobic strains were cultured at 37°C for 24h in 5 % $C0_2$/air on Columbia Blood agar. Anaerobic strains were cultured at 37°C for 48h in an anaerobic chamber. After incubation, the biomass of one whole agar plate was transferred into 1ml BHI broth using a sterile swab. Sterile membrane filters were aseptically located on appropriate agar plates (15-20 maximum per plate) and 10μl inoculum added prior to incubation at 37°C for 24 h at 5% $C0_2$ or 48 h at anaerobic conditions. Membrane filters were carefully transferred into 750μl of the selected test or control agent (in triplicate) in separate microfuge tubes and incubated for 1 min. The positive control consisted of 1.75 mM sodium dodecyl sulphate (SDS) and the negative of reduced transport fluid (RTF). After a contact time of 1 minute was achieved, membrane filters were transferred to 1 ml Neutralising Broth (diluted to a final concentration of 10 % v/v in RTF) and agitated at 200 rpm for 20 s. Membrane filters were transferred to 1ml RTF and vortexed at full speed for 1 min. The original suspension (in Neutralising Broth) was centrifuged at 13000 rpm for 1 min and the pellet resuspended in 1ml RTF. *BacLight* LIVE/DEAD viability stain (Molecular Probes) was used to distinguish between viable and non-viable cells as follows according to manufacturer's instructions. 5μl dye suspension was placed onto a glass slide beneath a 13 mm circular coverslip in preparation for counting. Both live (green) and dead (red) cells were counted at five locations on the slide to determine the mean number of cells. From this data the total number of live and dead cells attached or detached from the nitrocellulose filter was calculated.

The table shows the proportion (%) of cells disrupted from the biofilm after 1 minute incubation with the extracts. Numbers in parentheses indicate the percentage of dead cells.

	Chicory	Mushroom	Beer	Raspberry
A. naeslundii	38.3 (4.4)	52.2 (4.3)	22.1 (11.9)	31.7 (6.1)
P. intermedia	26.9 (13.3)	30.6 (19.1)	67.3 (6.3)	55.0 (19.3)

As can be seen all homogenates/extracts showed some ability to disrupt biofilms of the target organisms. In many cases, a high proportion of the disrupted organisms were killed by the homogenates/extracts.

3.5. Prevention of adhesion to, and invasion of, gingival epithelial cells

3.5.1 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 labelling

All bacteria were cultured in Brian heart Infusion broth (BHIB) except for *Streptococcus mutans* which was grown in BHIB (0.5x) supplemented with sucrose (final concentration, 0.2%). Cultures were incubated at 37"C in 5% $C0_2$/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} were added to the growth medium. Cells were harvested at stationary phase by centrifugation (5,000 x *g* for 10 min at 4°C), and washed twice with 10 mM phosphate buffer (PB), pH 7.0; pellets were re-suspended in either 10 mM PB, pH 7.0, or BHIB or phosphate buffered saline (PBS: 0.1 M Na_2HP0_4, 0.1 M KH_2P0_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% fetal 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% $C0_2$ 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 un-fractionated whole material was tested following three different experimental approaches (a, "simultaneous test"; b, "KB cell pre-treatment test"; c, "bacterial pre-treatment test"). In later experiments when fractions of the test material were analyzed, bacterial adherence was assessed by the "simultaneous test" only.

a) "Simultaneous test". KB monolayers were prepared in 96 well, flat bottom microtiter plates, using Dulbecco's Modified Eagle's Medium (DMEM)- high glucose prepared as described above without antibiotics; before the assay, monolayers were washed twice with PBS (0.1 M Na_2HP0_4, 0.1 M KH_2P0_4, 0.15 M NaCl, pH 7.2 to 7.4). Suspensions of labeled bacteria (P. *intermedia* and *A. naeslundii)* were prepared in PBS containing different concentrations of test materials (pH adjusted to 7) (final bacterial concentration, 4-6 x 10^8 cfu mL^{-1}). Aliquots (100 µl) of the bacterial suspensions were added to KB monolayers, and incubated at 37°Cfor 1 h in 5% $C0_2$ 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., III.). 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.

b) "KB cell pre-treatment test". PBS aliquots (100 µl) containing different concentrations of the un-fractionated whole materials were added to KB monolayers prepared as described in (a). Controls (no fraction added) were included. After 1h incubation at 37°C in 5% $C0_2$ atmosphere with gentle shaking, monolayers were washed with PBS, and radiolabeled bacterial suspensions in PBS (100 µl) were added (final bacterial concentration, 4-6 x 10^7 cfu mL^{-1}) to the wells. Plates were incubated at 37°C in 5% $C0_2$ atmosphere with gentle shaking. After 1h incubation, samples were processed as described in (a).

c) "Bacterial pre-treatment test". Radiolabeled bacteria were prepared culturing strains in the presence of the test un-fractionated materials at concentrations not inhibiting bacterial growth (= 0.5 MIC). Controls (no fraction added) were included in all treatments. Bacteria were harvested at stationary phase by centrifugation (5,000 x *g* for 10 min at 4 °C), and washed twice with PBS; pellets were then re-suspended in PBS (final bacterial concentration, 6-8 x 10^7 cfu mL^{-1}). Aliquots (100 µi) of these suspensions were added to the wells and plates were incubated at 37°Cin 5% $C0_2$ atmosphere with gentle shaking. After 1h incubation, samples were processed as described in (a).

The tested substances did not show cytotoxicity with the exception of mushroom. This was therefore used at 0.5x concentration (sub-cytotoxic).

Homogenate /extract	*P. intermedia*			*A. naeslundii*			*F. nucleatum*		
	Simult. (a)	KB cell pretreat. (b)	Bacteria pretreat. (c)	Simult. (a)	KB cell pretreat. (b)	Bacteria pretreat. (c)	Simult. (a)	KB cell pretreat. (b)	Bacteria pretreat. (c)
	% inhibition of adhesion								
Beer 2x	66	8	7	75	-	-	30	10	10
1x	7			10			4		
Raspberry	67	30	53	72	6	-	70	7	7
2x	6			10			8		
1x									
Chicory 2x	5	8	5	68	-	-	28	10	4
1x	-			30			10		
Mushroom	-	10	-	8	-	-	-	-	-
0.5x									

As can be seen, the highest inhibition values were obtained when homogenates/extracts and bacteria were added simultaneously to the monolayers. Most of the homogenates/extracts (mushroom was an exception) were able to inhibit adherence of the target organisms to epithelial cells.

3.5.2 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, monalayers 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 x 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% $C0_2$ atmosphere with gentle shaking, monolayers were washed with PBS to remove non adherent bacteria. To evaluate total culturable 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 culturable 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 culturable bacteria per monolayer to total culturable 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.

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.

Homogenate/extract	**Inhibition of adhesion of *A. naeslundii* to KB cells (%)**	**Inhibition of internalization of *A. naeslundii* into KB cells (%)**
Beer 2x	53	93
Raspberry 2x	66	69
Chicory 2x	81	98
Mushroom	Toxic to KB cells	Toxic to KB cells

As can be seen most of the homogenates/extracts (mushroom was an exception) were able to inhibit *A. naeslundii* internalization. In the case of Raspberry and Chicory, such activity was due mainly to inhibition of adherence. In contrast, Beer was able to specifically inhibit bacterial internalization into KB cells.

3.6. Inhibition of host cell pro-inflammatory cytokine production induced by the target organisms

The pro-inflammatory cytokines (IL-1 , IL-6 and TNF) released by host cells in response to sub-gingival bacteria are considered to be the primary mediators 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 ability of the test materials to inhibit cytokine production by monoMac 6 cells (a human monocytic cell line) in response to bacteria (S. *sanguinis, A. naeslundii, F. nucleatum* and *P. intermedia)* was evaluated.

Maintenance of Mono-Mac-6 cells

1. 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).
2. Cells were cultured in 75-cm^2 flasks at 37°G in a humidified atmosphere of 5% $C0_2$ in air. Weekly, cells were split at a ratio of 1:5 by centrifugation at 1500rpm for 5 min and resuspended in fresh medium. Cells were then fed with fresh medium once a week.

Inhibition of bacteria-induced host cell pro-inflammatory cytokine production

1. Mono-Mac-6 cells were centrifuged at 1500rpm for 5 min and resuspended in media with 2% (v/v) FCS.
2. Viable cells were dispensed into 24 well tissue culture plates at $2x10^6$/500 µl/well.
3. The selected test or control agent (in triplicate) was then added to cells neat and at dilutions of 1:10 and 1:100.
4. Bacterial strains were inoculated into 10ml of the appropriate broth and grown in appropriate conditions
5. Bacterial cultures were then diluted in fresh broth and grown to exponential growth stage, as determined spectrophotometrically.
6. 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 days incubation under the appropriate conditions (see Table 1) plates were counted to determine the CFU/ml used in the experiment.

7. 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).
8. The number of bacteria added to Mono-Mac-6 cells were judged on the OD of bacterial cultures and previously determined CFU/ml at a particular OD.
9. 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.
10. Bacteria were centrifuged onto the monolayer at 2000rpm for 10 minutes at room temperature and then plates incubated at 37°C in an atmosphere containing 5% $C0_2$ for 5 h.
11. 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-1, IL-6 and TNF/commercially available kits for the detection of IL-1 , IL-6 and TNF.
12. For determination of cytokine mRNA, mRNA was extracted from stimulated cells using the Qiagen RNeasy Protect Cell Mini Kit, as per manufacturer's instructions.

Reverse Transcription

1. 5 µg of RNA in 15 µl of DEPC-treated water from each sample was used.
2. 1 µl of OligodT (Sigma Genosys, UK) were added and samples heated to 70°C for 10 minutes to denature the RNA.
3. The Eppendorfs containing reaction mixture were then placed on ice to chill and 7µl of master mix I, 4 µl first strand buffer, 2 µl dTT (Gibco BRL, UK), 1 µl10 mM dNTPs (Gibco BRL, UK) were added.
4. After addition of master mix I, samples were transferred to 42°C and incubated for 2 minutes. 5 µl of master mix II; 0.5 µl Superscript II reverse transcriptase (Gibco BRL, UK) and 4.5µl DEPC-treated water were then be added and the samples incubated at 42°C for a further hour.
5. Samples were heated to 70°C for 10-15 minutes to inactivate the reverse transcriptase, then diluted 1:4 with DEPC water and stored at -20°C.

PCR

1. Transcripts for all genes will be determined by PCR using 5µl of the cDNA template from samples prepared as above.

2. 45 µl of master mix containing 5 µl PCR buffer (10x), 1.5 µl $MgCl_2$ (50 mM), 1 µl dNTPs (dATP, dCTP, dGTP, dTTP each at a concentration of 25 mM), 0.5 µl Taq polymerase (Gibco BRL, UK), 0.5 µl forward primer (50 µM), 0.5 µl reverse primer (50 µM), 36.5 µl pyrogen free/DECP-treated water will be added to each sample.
3. Tubes will be gently vortexed and placed into an automated DNA thermal cycler with a heated lid (Eppendorf Mastercycler).
4. Programs and primer sequences will be taken from a previous publication (Jung et al., 1995)

Agarose gel electrophoresis

1. Into a flask, 1 g agarose powder (Bioline, UK) and 100ml TBE buffer (1x) will be placed and heated in the microwave for 2 minutes.
2. Once dissolved, the gel will be cooled to hand temperature and 10 µl of ethidium bromide (500 µg/ml (Sigma, UK) added. The gel will carefully be poured into a gel casting unit, and the appropriate sized comb then placed into the unit and the gel allowed to set for 1 hour.
3. To each sample, 12.5 µl of sample buffer (Promega) will be added and then mixed. Once the gels have set, they will be placed into the appropriate gel system with TAE buffer (1x).
4. 20 µl of PCR product will be loaded into the wells and run for 60 minutes at 100 volts (50 mAmps). Gels will be visualised and photographed under UV illumination, using an Alphalmager photo system (Alpha Innotech, Cannock, UK).

Determination of most effective bacterial inducer of IL-6 from the Mono-Mac-6 cells

The cells were exposed to each target organism and the quantity of IL-6 released into the supernatant, was assayed. Results are shown in Figure 1. The supernatant from F. *nucleatum* displayed the greatest IL-6 inducing activity and therefore was used in subsequent experiments.

Results

As shown in Figure 2, The chicory homogenate (at a 1:2 dilution) and mushroom homogenate (at a 1:10 dilution) were able to inhibit IL-6 release induced by the *F. nucleatum* supernatant.

Example 4 - Initial assessment of anti-caries activities of the homogenates/extracts

In order to test the extracts for their potential anti-caries 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 were used to assess the ability of the homogenates/extracts to:

- prevent adhesion of the target organisms to, and induce detachment from, hydroxyapatite
- prevent biofilm formation by the target organisms
- elicit an antibacterial effect against the target organisms
- prevent co-aggregation by the target organisms
- disrupt signal transduction in S. *mutans*
- disrupt pre-existing biofilms of the target organisms
- inhibit acid production by caries-associated organisms

4.1 Prevention of adhesion to, and induction of detachment from, hydroxyapatite (HA)

4.1.1 Prevention of adhesion

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 ½ MIC) were added to the beads.

Bacterial growth and labeling

All bacteria were cultured in Brain heart Infusion broth (BHIB) except for *Streptococcus mutans* which was grown in BHIB (0.5x) supplemented with sucrose (final concentration, 0.2%). Cultures were incubated at 37°C in 5% $C0_2$/air (S. *mutans, Streptococcus sanguinis* and *Lactobacillus casei)* To radiolabel bacteria, 10 µCi [methyl-^{3}H]thymidine (25 Ci mmol^{-1}) mL^{-1} were added to the growth medium. Cells were harvested at stationary phase by centrifugation (5,000 x *g* for 10 min at 4 °C), and washed twice with 10 mM phosphate buffer (PB), pH 7.0; pellets were re-suspended in either 10 mM PB, pH 7.0, or BHIB or phosphate buffered saline (PBS: 0.1 M Na_2HP0_4, 0.1 M KH_2P0_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.

Preparation of hydroxyapatite (HA) beads

Fifty mg aliquots of spheroidal HA beads (Sigma Aldrich, UK, code 21223) were washed with 1 mM PB, pH 7.0, in glass tubes and autoclaved. Beads were collected by centrifugation (100x 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 un-stimulated donors, clarified by centrifugation (15,000 x gfor 30 min at 4°C), and sterilized through 0.22 µνη 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 un-fractionated whole materials was tested using three different experimental approaches (a, "simultaneous test"; b, "HA pre-treatment test"; c, "bacterial pre-treatment test"). When fractions of the starting material were tested (see later) bacterial adherence to HA beads was analyzed by the "simultaneous test" only.

(a) "Simultaneous test". 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 x 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 (100x *g*, 1 min, 4°C), washed twice with 10 mM PB to remove non adherent 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.

(b) "HA pre-treatment test". Aliquots (1 mL) of 10 mM PB, pH 7.0, containing different concentrations of the test un-fractionated materials (pH adjusted to 7) were added to saliva treated beads (50 mg). Controls (no fraction added) were included. After 1h incubation at 37°C, the beads were washed with 10 mM PB, pH 7.0. One mL aliquots of the radiolabeled bacterial suspensions (final bacterial concentration, 6-8 x 107 cfu mL^{-1}) were then added to the beads, and the mixtures were incubated at room temperature on a Rotomix test tube rotator. After 1h incubation, samples were processed as described in (a).

(c) "Bacterial pre-treatment test". Radiolabeled bacteria were prepared culturing strains in the presence of the test un-fractionated materials at concentrations not inhibiting bacterial growth (= 0.5XMIC). Controls (no fraction added) were included in all treatments. Cells were harvested at stationary phase by centrifugation (5,000 x *g* for 10 min at 4°C), and washed twice with 10 mM PB, pH 7.0; pellets were then re-suspended in 10 mM PB, pH 7.0, (final bacterial concentration, 6-8 x 10^7 cfu mL^{-1}). Aliquots (1 mL) of these suspensions were added to saliva coated HA beads (50 mg), and the mixtures were incubated at room temperature on a Rotomix test tube rotator. After 1h incubation, samples were processed as described in (a).

Homogenate/extract	**% inhibition of adherence to hydroxyapatite**								
	S. mutans			*S. sanguinis*			*L. casei*		
	a	b	c	a	b	c	a	b	c
Beer 2x	31	0	0	22	0	0	87	0	0
1x	32			27			24		
Raspberry 2x	79	36	0	96	96	0	94	98	29
1x	81			92			93		
Chicory 2x	81	41	0	61	51	20	96	53	95
1x	75			55			89		
Mushroom 2x	78	27	0	86	36	0	91	65	87
1x	44			70			80		

As can be seen, the highest inhibition values were obtained when homogenates/extracts and bacteria were added simultaneously to the hydroxyapatite. All homogenates/extracts inhibited adhesion of the target bacteria to hydroxyapatite to some extent

4.1.2. 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 x 10^7 cfu mL^{-1}). After 1 h incubation, the beads were collected by centrifugation (100x g, 1 min, 4°C), and washed twice with 10 mM PB, pH 7.0, to remove non adherent bacteria. HA was then re-suspended in the same buffer (1mL) 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 above. Immediately after beads re-suspension (time zero) and after 1 and 2 h incubation, the mixtures were centrifuged (200 x g, 5 min, 4°C), and the supematants were transferred to PICO-FLUOR™ 15 scintillation fluid. Radioactivity was assayed in a liquid scintillation counter and, on the basis of cell labeling efficiency, the number of bacteria present in the supernatant, corresponding to detached cells, was evaluated. The percent of detached *vs.* 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.

Homogenate/extract	% detachment from hydroxyapatite beads					
	S. mutans		*S. sanguinis*		*L. casei*	
		Fold diff.		Fold diff.		Fold diff.
Beer	2		1		3	
2x	9	4x	5	5x	12	4x
1x	6	3x	4	4x	5	2x
Raspberry	2		1		5	
2x	5	3x	6	6x	7	1.4x
1x	10	5x	6	6x	7	1.4x
Chicory	3		1		2	
2x	20	7x	11	11x	17	9x
1x	16	5x	5	5x	22	11x
Mushroom	1		1		5	
2x	38	38x	59	59x	29	6x
1x	21	21x	14	14x	28	6x

As can be seen, all homogenates/extracts induced detachment of the target bacteria from hydroxyapatite to some extent

4.2 Prevention of biofilm formation by the target organisms

The capability of the homogenates/extracts to prevent biofilm formation was evaluated by the microtitre plate assay.

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 x 10^5 cfu mL^{-1} (S. *mutans, S. sanguinis, L. casei* 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 5% CO_2/air (S. *mutans, S. sanguinis* and *L. casei)),* with incubation media changed every 24 h . 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 at least twice.

Homogenate/extract	**S. *mutans***			**S. *mutans* + sucrose**			***L. casei***		
	2x	1x	0.5x	2x	1x	0.5x	2x	1x	0.5x
Beer	**+++**	**++**	**+**	**+++**	**++**	**+**	**++**	**±**	**±**
Raspberry	**+++**	**+++**	**+++**	**+++**	**+++**	**++**	**++**	**++**	**++**
Chicory	0	0	0	0	**+**	**±**	**+++**	**+**	**+**
Mushroom	**++**	**+**	**±**	**++**	**++**	**±**	**+++**	**++**	**+**

(81-100% inhibition = +++, 51-80% inhibition = ++, 25-50 % inhibition = +, <25% inhibition = ±, 0 = no effect; 2X to 0.25X denoted the concentration of the homogenate/extract used)

As can be seen, all homogenates/extracts inhibited 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

4.3 Antibacterial activity of homogenates/extracts

All the extracts were assayed for their antibacterial activities in a standard Minimum Inhibitory Concentration (MIC) assay as described previously.

	Chicory	Mushroom	Beer	Raspberry
L. casei	>1X	0.25X	1X	0.25X
S. mutans	>1X	0.25X	1X	0.25X

As can be seen, mushroom, raspberry, and beer exhibited some inhibitory activity against *L casei* and S. *mutans.*

4.4 Prevention of co-aggregation by the 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. All combinations of the strains used were tested for coaggregation activity and the following were used in the assays: S. *mutans & L casei, S. mutans* & S. *sanguinis*

	Chicory	Mushroom	Beer	Raspberry
S. mutans + *S. sanguinis*	+	++	+	0
S. mutans + *L. casei*	0	0	0	0

As can be seen, chicory, mushroom, beer and cranberry inhibited co-aggregation of at least one pair of organisms to some extent.

4.5 Effect on signal transduction

Some materials may affect bacterial triggering/suppression of signal transduction systems and this may have an effect on colonization of teeth and the induction/progression of disease. The effect of homogenates/extracts on S. *mutans comDE* gene expression was determined as described below:

1. S. *mutans* was grown in Brain Heart Infusion (BHI) broth at 37°C, 5% $C0_2$ to the early log phase, OD_{600} = 0.2
2. inoculum was exposed to the tested homogenates/extracts in BHI for 2 hours.

Extraction of total RNA from S. mutans

1. After being exposed to the tested agents, the above bacterial suspension was centrifuged, washed with PBS and resuspended in Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA (Tarn et al. 2006 J Antimicrob Chemother. 57(5):865-71).
2. 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).
3. The suspensions obtained were centrifuged and the RNA-containing supernatant was *transferred to a new microcentrifuge* tube.
4. 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.
5. The RNA pellet was washed with ethanol, centrifuged and the purified RNA was resuspended in diethyl pyrocarbonate-treated water (Invitrogen, Carlsbad, CA, USA).
6. Due to the sensitivity of the PCR, residual contaminating DNA was removed by RNase-free DNase (Wang et al. 2002. J Microbiol Methods 51:119-21),
7. the RNA concentration was determined spectrophotometrically according to the A_{260}/A_{280} ratio, using a Nanodrop instrument (ND-1000 Spectrophotometer, Nanodrop Technologies, Wilmington, USA).
8. The integrity of the RNA was assessed by 1.5% agarose gel electrophoresis.

Reverse transcription and quantitative real-time PCR.

1. Reverse transcription and quantitative real-time PCR was performed as described previously (Shemesh et al. 2006. Carbohydr Res. 341: 2090-7).
2. A reverse transcription (RT) reaction mix (20 µl) containing 50 ng of random hexamers, 10mM dNTPs mix and 1 µg of total RNA sample was incubated at 65°C for 5 min and then placed on ice.

3. 10xRT 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) was added to the reaction mix in each tube.

4. 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.

5. Real-time quantitative PCR 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).

6. The reaction mix (20 µl) containes 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, 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 for, and at 20 sec for 20 sec, and a slow ramp (20 min) from 60 to 95°C.

7. 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 curves was used for transformation of the C_T values to the relative number of cDNA molecules.

8. 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.

9. 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.

10. 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.

11. 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 (3). They were presented in relative units compared with those of the control, grown in the absence of the tested agents to calculate the percent inhibition of *comC/D/E* mRNA expression by test fraction.

The effect of 0.1% (v/v) of the homogenates/extracts on S. *mutans comDE* gene expression is shown below:

Control	Raspberry	Mushroom	Chicory	Beer
100%	51%	21%	39%	38%

As can be seen, all homogenates/extracts inhibited S. *mutans* comDE gene expression to some extent, compared the control.

4.6 Disruption of pre-existing 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. Using the protocol described previously, mature biofilms of each of the test organisms were grown on cellulose nitrate membrane filters and incubated with the test compounds for 1 minute. The number of live and dead cells disrupted from the biofilm were assessed as well as the number of live and dead cells remaining.

The table shows cells disrupted from the biofilm after 1 minute incubation with the extracts. Numbers in parenthesis indicate the percentage of dead cells.

	Chicory	Mushroom	Beer	Raspberry
L. casei	58.8 (3.1)	31.2 (1.6)	51.4 (7.2)	31.0 (7.9)
S. mutans	37.9 (4.9)	75.4 (58.0)	33.2 (8.7)	43.5 (3.8)

As can be seen, all homogenates/extracts were able to disrupt biofilms of both target organisms to some extent

4.7 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 anti-caries therapeutic agent would inhibit fermentation activity (acid production) of oral microorganisms, especially those that are known to be involved in caries aetiology (e.g. S. *mutans).*

The effects of each homogenate/extract on acid production by the target organisms was carried out using an acidogenicity assay developed at the Academic Centre of Dentistry Amsterdam (ACTA) (Damen et al. *Caries Res* 2002; 36:53-57). Biofilms of S. *sanguinis, S.*

mutans, L case/ 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 re-suspended and sampled for organic acid content determination.

Protocol

1. S. *sanguinis, S. mutans, L. casei* and *A. naeslundii* (table 1) derived from stock cultures were inoculated (streaked for single colonies) onto appropriate solid agar media (table 1) to ensure purity.
2. A single bacterial colony was picked and used to inoculate 5 ml of full-strength BHI broth (table **1).**
3. This was incubated overnight yielding approximately 10^9 CFU/ml.
4. 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 medium as stated in table **1.**
5. Biofilms were grown in 10% H_2, 10% $C0_2$ in N_2 at 37°C.
6. The medium was refreshed after 8 h and biofilms grown for another 16 h (in total for 24 h).
7. After 24 h, the spent medium was carefully removed.
8. 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 minutes. Controls included a negative (RTF; Syed and Loesche *Applied Microbiology* 1972; 24:638-644) and a positive (0.05% chlorhexidine solution).
9. After careful removal of the test or control agent, the biofilms were rinsed three times with peptone buffered water (PBW).
10. 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.
11. After the incubation period, the biofilms were suspended in the incubation fluid and sampled into pre-cooled eppendorf tubes. The tubes were set on ice until further processing within one hour.
12. To release acids, all samples were heated at 80°C for 5 minutes and again cooled on ice (Gerardu et al *Caries Res* 2006;40:245-250).
13. Then the vials with plaque were centrifuged at 16,100 x g for 15 minutes at 4°C (Haereus centrifuge, Dijkstra Bv, Lelystad, the Netherlands).
14. The supernatants were transferred into vials with a microspin filter (Ultrafree-MC 0.22 μm, Millipore, Bedford, Mass., USA) and centrifuged at 13,684 x g for 5 minutes at 4°C.

15. Filtered supernatants were stored at -80°C until further processing.
16. Organic acids in the incubation fluid were determined as their anions by capillary electrophoresis on the Waters Capillary Ion Analyzer (Milford, Mass., USA) (Damen *et* a/., 2002):
    a. Sodium salts of formic, acetic, propionic, butyric, succinic and lactic acid were used to prepare single and mixture standard solutions in MilliQ-water.
    b. Calibration curves were made for each acid separately.
    c. As an internal standard, $NaN0_3$ were included in all samples.
    d. Formic, butyric, succinic, propionic, acetic, and lactic acid were determined in duplicate samples and expressed as nmol acid/sample in 0.2 ml of incubation fluid.

Table 1

Growth conditions for strains used

Strains	Medium	Details
	Solid Media	
Streptococcus sanguinis *Streptococcus rnutans* *Lactobacillus casei*	Blood Agar (LAB 28) LabM	After autoclaving and cooling to approx 50°C add 5% v/v defibrinated horse blood to molten agar at 50°C. Incubate in 5%$C0_2$ at 37°C in a plastic pouch (with $C0_2$GEN Compact Atmosphere Generation System, Code: CD0020, Oxoid Ltd.)
Actinomyces naeslundii	Fastidious Anaerobe Agar (LAB 90) LabM	After autoclaving and cooling to approx 50°C add 5% v/v defibrinated horse blood to molten agar at approx 50°C. Incubate anaerobically at 37°C. In a plastic pouch (with ANAEROGEN Atmosphere Generation System, Code: AN0010 & AN0020, Oxoid Ltd.)
	Liquid Media	
Streptococcus sanguinis	Full and ½ strength	*For biofilm growth in ½-strength BHI:* add 50mM PIPES (P-1851, SIGMA) to increase buffer-

Streptococcus mutans *Lactobacillus casei*	Brain Heart Infusion broth (237500) DIFCO	capacity of the medium (Kara D et al *Eur J Oral Sci* 2006;114:58-63). After autoclaving add filter-sterilized sucrose 2 mg/ml (final concentration) Incubate in 10% H_2, 10% $C0_2$ in N_2 at 37°C in anaerobic cabinet.
Actinomyces naeslundii	Full and ½ strength Brain Heart Infusion broth (237500) DIFCO	*Full-strength BHI for inoculum preparation:* After autoclaving and cooling to approx 50°C supplement with filter sterilized: haemin 5 μg/ml (final concentration). Stock dissolved in 1 M NaOH. Vitamin K (menadione), 1 mg/mL (final concentration). Stock dissolved in 70% ethanol. *½ strength BHI for biofilm growth:* Add 50mM PIPES (P-1851, SIGMA) to increase buffer-capacity of the medium. After autoclaving and cooling to approx 50°C supplement with filter sterilized: sucrose 2 mg/ml (final *concentration)* haemin 5 μg/ml (final concentration). Stock dissolved in 1 M NaOH. Vitamin K (menadione), 1 mg/mL (final concentration). Stock dissolved in 70% ethanol. Incubate in 10% H_2, 10% $C0_2$ in N_2 at 37°C in anaerobic cabinet.

Acid production was either unaffected by the homogenates/extracts or increased.

Example 5 - Initial fractionation of homogenates/extracts

Based on the gingivitis-related data from Example 3, and caries-related data from Example 4, and other criteria, it was decided to take raspberry, chicory and mushroom through for further fractionation and testing as potential beneficial agents.

Fractionation of raspberry, mushroom and chicory extracts was carried out by ultrafiltration or dialysis to provide low and high molecular mass fractions for subsequent bioassay.

5.1 *Fractionation of mushroom homogenate*

The mushroom homogenate was fractionated into low and high molecular weight fractions by ultrafiltration using the Vivaflow 200 complete system (Vivascience AG, Feodor-Lynen-Strasse 21, 30625 Hannover, Germany) comprising a pump (240V), tubing, 500ml sample/diafiltration reservoir, and a membrane 5,000 MWCO PES for ultradiafiltration (Vivasience). In this way, a diafiltrate i.e. a low molecular mass fraction (LMM) containing all the compounds with molecular masses less than 5000 Da, and a retentate, i.e. a high molecular mass fraction (HMM) containing all the compounds with molecular masses greater than 5000 Da.

30% of the components originally present remained in the retentate - this was verified by Gel Filtration Chromatography of the dialysate and retentate. As the retentate contained some components with molecular mass lower than 5,000 Da, the HMM fraction was subjected to dialysis using dialysis membrane tubing with a 5,000 MW cut-off to eliminate the LMM components. The diafiltrate and retentate were sterilized using a 0.20 pm membrane (Vivascience) and then freeze-dried.

5.2 *Fractionation of chicory homogenate*

The chicory homogenate was subjected to ultrafiltration exactly as described above. 50% of the components originally present remained in the retentate - this was verified by Gel Filtration Chromatographic analysis of the dialysate and retentate. As the retentate contained some components with a molecular mass lower than 5,000 Da, the HMM fraction was subjected to dialysis using dialysis membrane tubing with a 5,000 MW cut-off to eliminate the LMM components. The diafiltrate and retentate were sterilized using a 0.20 μm membrane (Vivascience) and then freeze-dried.

5.3 *Fractionation of raspberry homogenate*

Due to its high viscosity and density, this could not be submitted to ultradiafiltration. Consequently, it was fractionated into low and high molecular mass fractions using dialysis. This procedure was carried out using regenerated cellulose "Dialysis sacks" with a cut off 3,500 Da. In this way, a dialysate containing all the compounds with molecular masses lower than 3,500 Da and a retentate containing all the compounds with molecular masses greater than 3,500 Da.

The pH of the retentate and diaiysate were 3.20±0.10 and 2.71± 0.12 respectively. The pH value of both the retentate and diaiysate were brought to pH 4,5-5.0 (using 1.0 M NaOH) so as not to interfere with subsequent assays of their biological activities.
The diafiltrate and retentate were sterilized using a 0.20 μm membrane (Vivascience) and then freeze-dried.

Example 6 - Determination of anti-caries activities of the HMM and LMM fractions of mushroom, chicory and raspberry

The effects of the HMM and LMM fractions of the three test materials 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:

- Prevent adhesion of the target organisms to hydroxyapatite
- Prevent biofilm formation by the target organisms
- Elicit an antibacterial effect against the target organisms
- Prevent co-aggregation by the target organisms
- Disrupt pre-existing biofilms of the target organisms

6.1 Prevention of adhesion of the target organisms to hydroxyapatite

The "simultaneous test" as described above (section 4.1 .1) was used.

Homogenate/extract	**% adherence inhibition**		
	S. *mutans*		***A. naeslundii***
Mushroom			
HMM	54		45
LMM	16		14
Chicory 2x			
HMM	79		46
LMM	4		68
Raspberry 1X			

HMM	22		nt
LMM	62		nt

As can be seen both the LMM and HMM fractions of mushroom, chicory and raspberry were able to inhibit adherence of the target organisms to hydroxyapatite

6.2 Prevention ofbiofilm formation by the target organisms (see 4.2)

Homogenate/extract	% inhibition of biofilm formation	
	S. mutans	***S. sanguinis***
Mushroom 2X		
HMM	0	-
LMM	99	-
Mushroom 1X		
HMM	-	14
LMM	-	87
Chicory 2x		
HMM	0	-
LMM	0	-
Chicory 1x		
HMM	-	17
LMM	-	0
Raspberry 1X		
HMM	8	34
LMM	32	0

As can be seen, the LMM fractions of mushroom and raspberry, but not chicory, were able to inhibit biofilm formation by S. *mutans*

6.3 Elicitation of an antibacterial effect against the target organisms (see 4.3)

Target organism	Homogenate/extract					
	Chicory		Mushroom		raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
S. sanguinis	>2X	1X	>2X	1X	1X	0.125X
L. casei	>2X	1X	>2X	>2X	1X	>1X
S. mutans	>2X	1X	>2X	0.25X	1X	0.125X

As can be seen, the LMM fractions of mushroom, raspberry and chicory generally had a greater inhibitory effect against the target organisms than the HMM fractions

6.4 Prevention of co-aggregation by the target organisms (see 4.4)

Co-aggregating pair	Homogenate/extract					
	Chicory		Mushroom		Raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
P. intermedia + *F. nucleatum*			0	0		
F. nucleatum + *S. mutans*			0	++		
F. nucleatum + *N. subflava*			0	++		
F. nucleatum + *S. sanguinis*	0	++			0	++

0 = no inhibition of co-aggregation, ++ = complete inhibition of co-aggregation

As can be seen, the LMM fractions of mushroom, raspberry and chicory were generally more effective at inhibiting co-aggregation than the HMM fractions

6.5 Disruption of pre-existing biofilms of the target organisms (see 4.6)

The table shows cells disrupted from the biofilm after 1 minute incubation with the extracts. Numbers in parenthesis indicate the percentage of dead cells.

Target organism	Homogenate/extract					
	Chicory		Mushroom		raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
L. casei	33.7 (2.7)	63.2 (2.9)	45.3 (1.7)	60.0 (7.2)	nt	nt
S. mutans	33.5 (18.7)	56.7 (14.9)	31.3 (16.6)	58.1 (29.9)	nt	nt

As can be seen, the LMM fractions of mushroom and chicory were generally more effective at disrupting biofilms of the target organisms than the HMM fractions

Example 7 - Determination of anti-gingivitis 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)* in assays specifically relevant to this disease were carried out.

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

- Prevent biofilm formation by target organism
- Disrupt pre-existing biofilms of the target organisms
- Inhibit adhesion of organisms to epithelial cells
- inhibit bacteria-induced host cell pro-inflammatory cytokine production

7.1 Prevention of biofilm formation by target organism (see 3.1)

Target organism	Homogenate/extract					
	Inhibition of biofilm formation (%)					
	Chicory		Mushroom		raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
	X1	X1	X1	X1	X1	X1
A. naeslundii	7	97	1	99	72	100
P. intermedia	-	-	-	-	74	94

As can be seen the LMM fractions of mushroom, raspberry and chicory were generally more effective at preventing biofilm formation by the target organisms than the HMM fractions

7.2 Disruption of pre-existing biofilms of the target organisms (see 3.4)

Target organism	Homogenate/extract					
	Chicory		Mushroom		raspberry	
	HMM	LMM	HMM	LMM	HMM	LMM
A. naeslundii	4.3 (1.4)	40.4 (7.6)	47.9 (37.5)	51.1 (39.1)	40.3 (30.6	37.5 (30.0)
P. intermedia	8.2 (2.1)	28.7 (6.9)	11.6 (9.2)	63.1 (52.6)	59.0 (20.2	33.9 (23.7)

As can be seen the LMM fractions of mushroom and chicory were more effective at disrupting biofilms of the target organisms than the HMM fractions. In the case of raspberry the HMM fraction was the most effective.

7.3 Inhibition of adhesion of organisms to epithelial cells (see 3.5. 1)

The bacteria and the tested compounds were added simultaneously in the assay.

Target organism	Homogenate/extract											
	Chicory				Mushroom				raspberry			
	HMM		LMM		HMM		LMM		HMM		LMM	
	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1	X2	X1
	(% inhibition of adherence)											
A. naeslundii	60	-	10	-	-	0	-	0	0	0	73	33
P. intermedia	7	-	40	-	-	0	-	0	50	30	35	10

As can be seen, both the LMM and HMM fractions of raspberry and chicory were able to inhibit adhesion of the target organisms to epithelial cells. Neither the LMM or HMM fractions of mushroom displayed inhibitory activity.

7.4 Inhibition of bacteria-induced host cell pro-inflammatory cytokine production (see 3.6)

The results for chicory homogenate and fractions are shown in Figure 3, wherein:

<CH 1:10 = LMM chicory at a dilution of 1:10

>CH 1:2 = HMM chicory at a dilution of 1:2

As can be seen, the crude chicory homogenate was able to inhibit IL-6 production induced by the F.n. supernatant but neither the LMM or HMM fractions were able to inhibit IL-6 production

The results for mushroom homogenate and fractions are shown in Figure **4**, wherein:

<ME 1:10 = LMM mushroom at a dilution of 1:10
>ME 1:2 = HMM mushroom at a dilution of 1:2

As can be seen, the crude mushroom homogenate and the LMM fraction were able to inhibit IL-6 production induced by the *F. nucleatum* supernatant but the HMM fraction was not able to inhibit IL-6 production

The crude raspberry extract and the LMM and HMM fractions were not able to inhibit IL-6 production induced by the *F. nucleatum* supernatant (results not shown).

7.5 *Elicitation of an antibacterial effect against the target organisms (see 3.2)*

Target organism	**Homogenate/extract**					
	Chicory		**Mushroom**		**raspberry**	
	HMM	**LMM**	**HMM**	**LMM**	**HMM**	**LMM**
A. naeslundii	>100	50	>100	>100	50	6.12
P. intermedia	50	50	>100	12.5	>50	12.5

As can be seen the LMM fractions of mushroom, chicory and raspberry displayed a greater antibacterial effect than the HMM fractions.

Example 8 - Further fractionation of LMM mushroom homogenate and identification of constituents with anti-caries activity

8.1 Fractionation of LMM mushroom homogenate

The LMM fraction of mushroom was fractionated further by gel filtration chromatography. An Agilent 1100 liquid chromatography system with an UV wavelength monitor (Agilent, Waldbronn, Germany), was used; chromatograms were recorded at 210 nm. The Agilent Chemstation software was used for control of the system and data processing GFC separation of the LMM fractions (MW <5000 Da) was performed using a Superformance

Universal giasscartridge system (300 mm x 10 mm) (Merck, Darmstadt, Germany). The

Anti-caries Assay		**Mushroom sub-fractions**					
		M1	**M2**	**M3**	**M4**	**M5**	**M6**
Disruption of pre-existing biofilms of *S. mutans* (%)		47	21	33	42	30	23
Killing of *S. mutans* disrupted from pre-existing biofilms (%)		4	5	8	7	8	1
prevent adhesion of *S. mutans* to hydroxyapatite (% inhibition of adhesion)		0	5	0	1	9	8
prevent biofilm formation by the target organisms (% inhibition of biofilm formation)		6	6	2	4	0	6
elicit an inhibitory effect against the target organisms	*L. casei*	>50%	>50%	>50%	>50%	>50%	>50%
	S. sanguinis	>50%	50%	>50%	>50%	50%	50%
	S. mutans	>50%	>50%	>50%	>50%	>50%	>50%

stationary phase was TSK gel Toyopearl HW-40F (exclusion limits 100-10000 Da; particle sizes 45 micron) (Tosoh Biosep GmbH, Stuttgart, Germany). The mobile phase was Millipore grade water (flow rate, 1.0 mL min-1). Blue dextran (MM 2000 kDa) was used as standard to evaluate the dead volume.

The eluant was collected as six fractions as shown in Figure 5.

The resulting six sub-fractions (M1-6) were subjected to the following assays.

- Prevention of adhesion of S. *mutans* to hydroxyapatite
- Prevention of biofilm formation by S. *mutans*
- Elicitation of an antibacterial effect against S. *mutans* and *L casei*
- Disruption of pre-existing biofilms of S. *mutans*

8.2 Anti-caries activities of the sub-fractions

As can be seen, two sub-fractions (M4 and M5) were considered to be the most promising

8.3 Further fractionation of sub-fractions

Sub-fractions M4 and M5 were further fractionated using reverse phase HPLC semi-preparative chromatography. All experiments were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, a thermostatted column compartment, and a DAD. The Agilent Chemstation software was used for control of the

HPLC system. The semipreparative HPLC column was a C18 LiChrospher® 250 * 10 mm, 10 μm (Merck, Darmstadt, Germany)

Chromatographic conditions for gradient elution were as follows:

Time (min)	Flow (ml min^{-1})	0.1% formic acid aqueous solution (%)	MeOH (%)
0	0,4	55	45
30	0,4	55	45
31	0,2	52	48
45	0,2	52	48
50	0,6	52	48
150	0,6	52	48
160	0,4	52	48

Volume injected, 1 mL; column temperature, 25 °C; UV spectra were recorded in the 190-600 nm range, and chromatograms were acquired at 210 nm. The column was equilibrated 20 min before the next injection.

Fractionation of sub-fraction M4 using HPLC:

As shown in Figure 6, sub-fraction M4 produced 8 sub-sub-fractions respectively (M4.1-8). The dry masses of these sub-sub-fractions are shown below:

fraction	dry mass (mg)	%
M4.1	7.8	7.19
M4.2	11.5	10.61
M4.3	28.7	26.47
M4.4	10.2	9.41

M4.5	17.6	16.24
M4.6	3.4	3.14
M4.7	2.4	2.21
M4.8	26.8	24.72
total	108.4	

Fractionation of sub-fraction M5 using HPLC:

As shown in Figure 7, sub-fraction M5 produced 11 sub-sub-fractions (M5.1-11). The dry masses of these sub-sub-fractions are shown below:

fraction	dry mass (mg)	%
M5.1	4.5	1.98
M5.2	10.2	4.49
M5.3	25.7	11.33
M5.4	44.6	19.66
M5.5	7.2	3.18
M5.6	6.6	2.91
M5.7	42.5	18.74
M5.8	6.6	2.91
M5.9	23.2	10.23
M5.10	37.3	16.45
M5.11	18.4	8.1
total	226.8	

8.4 Anti-caries activities of sub-sub-fractions

The various sub-sub-fractions of M4 and M5 were tested for their anti-caries activities using the 4 assays shown below:

Anti-caries Assay	Sub-sub-fractions of Mushroom Sub-fraction M4							
	M4.1	M4.2	M4.3	M4.4	M4.5	M4.6	M4.7	M4.8
Disruption of pre-existing biofilms of	52	11	66	52	47	53	54	60

S. *mutans (%)*								
Killing of S. *mutans* disrupted from pre¬ existing biofilms (%)	35	6	5	1	2	2	1	3
prevent adhesion of S. *mutans* to hydroxyapatite (% inhibition of adhesion)	0	0	0	0	0	16	70	22
prevent biofilm formation by the target organisms (% inhibition of biofilm formation)	0	0	0	0	0	0	0	0

Anti-caries	**Sub-sub-fractions of Mushroom Sub-fraction M5**										
Assay	**M5.1**	**M5.2**	**M5.3**	**MS.4**	**M5.5**	**M5.6**	**M5.7**	**M5.8**	**M5.9**	**M5.10**	**M5.11**
Disruption of pre-existing biofilms of S. *mutans (%)*	24	18	12	10	17	31	24	16	8	8	23
Killing of S. *mutans* disrupted from pre-existing biofilms (%)	9	4	10	4	4	10	10	7	2	6	9
prevent adhesion of S. *mutans* to hydroxyapatite (% inhibition of adhesion)	0	0	0	0	0	18	24	31	15	19	17
prevent biofilm formation by	0	0	33	22	33	32	34	35	34	34	33

the target organisms (% inhibition of biofilm formation)											
elicit an inhibitory effect against the target organisms	0	0	0	0	0	0	0	0	0	0	0

As can be seen above, when the dry masses of the sub-sub-fractions were taken into account and the specific activities determined, sub-sub-fractions M4.7 and M5.6 were found to be the most active.

The main constituents in these sub-sub-fractions were identified by HPLC and mass spectroscopy as:

M4.7: aconitic acid, adenosine and oxalic acid;

M5.6: quinic acid, inosine, oxalic acid and succinic acid.

Example 9 - Further fractionation of LMM mushroom and chicory homogenates and identification of constituents with anti-gingivitis activity

9.1 Fractionation of LMM mushroom and chicory homogenates

The LMM fractions of mushrooms and chicory were fractionated further using gel filtration chromatography as described previously. The chromatogram of the LMM chicory is shown in Figure 8. The eluant was collected as six fractions.

The LMM mushroom and chicory extracts each generated six sub-fractions (M1-6, C1-6) and these were subjected to the following anti-gingivitis assays.

- Elicitation of an antibacterial effect against *A. naes!undii, V. dispar, P. intermedia, F. nucleatum*
- Disruption of pre-existing biofilms of *A. naeslundii* and *P. intermedia*
- Prevention of adhesion to gingival epithelial cells *A. naeslundii* and *P. intermedia*

9.2 Anti-gingivitis activity of mushroom sub-fractions and sub-sub-fractions

Anti-gingivitis Assay		**Mushroom Sub-fractions**					
		M1	M2	M3	M4	M5	M6
Disruption of pre-existing biofilms of *A. naeslundii* (%)		46	29	33	37	39	28
Disruption of pre-existing biofilms of *P. intermedia (%)*		24	37	25	70	53	50
Killing of *A. naeslundii* disrupted from pre-existing biofilms (%)		14	16	5	6	7	4
Killing of *P. intermedia* disrupted from pre-existing biofilms (%)		15	5	0	17	5	8
prevent *A. naeslundii* adhesion to epithelial cells (% inhibition of adhesion)		40	42	40	42	64	25
prevent *P. intermedia* adhesion to epithelial cells (% inhibition of adhesion)		33	27	42	0	10	0
prevent biofilm formation by the target organisms (% inhibition of biofilm formation)		42	40	70	42	60	56
elicit an inhibitory effect against the target organisms	*A. naeslundii*	0	1:4	1:2	1:2	1:2	1:2
	P. intermedia	0	0	0	1:2	1:2	0

As can be seen, sub-fractions M4 and M5 were considered to be the most promising.

Sub-fractions M4 and M5 were further fractionated using reverse-phase HPLC (see Example 8, Section 8.3). Sub-fraction M4 generated 8 sub-sub-fractions (M4.1-8; Figure 6) and sub-fraction M5 generated 11 sub-sub-fractions (M5.1-1 1; Figure 7).

Anti-gingivitis Assay	**Sub-sub-fractions of Mushroom sub-fraction M4**							
	M4.1	**M4.2**	**M4.3**	**M4.4**	**M4.5**	**M4.6**	**M4.7**	**M4.8**

Disruption of pre-existing biofilms of *P. intermedia (%)*	52	11	66	54	47	53	54	60
Killing of *P. intermedia* disrupted from pre-existing biofilms (%)	35	0	5	1	2	2	1	3
prevent *P. intermedia* adhesion to epithelial cells (% inhibition of adhesion)	0	0	0	0	0	0	25	0
prevent biofilm formation by *P. intermedia (%* inhibition of biofilm formation)	0	0	0	0	0	0	23	0

Anti-gingivitis assay	**Sub-sub-fractions of Mushroom sub-fraction M5**										
	M5.1	**M5.2**	M5.3	M5.4	M5.5	M5.6	M5.7	M5.8	M5.9	M5.10	M5.1 1
Disruption of pre-existing biofilms (%)	**24**	**18**	**12**	**10**	**17**	**31**	**24**	**16**	8	8	**23**
Killing of bacteria disrupted from pre-existing biofilms (%)	**9**	**4**	**10**	**4**	**4**	**10**	**10**	**7**	**2**	**6**	**9**
prevent bacterial adhesion to hydroxyapatite (% inhibition of adhesion)	**0**	**0**	**0**	**0**	**0**	18	**24**	**31**	**15**	**19**	**17**
prevent biofilm formation (%	**0**	**0**	**33**	**22**	**33**	**32**	**34**	**35**	**34**	**34**	**33**

inhibition)											
prevent *P. intermedia* adhesion to epithelial cells (% inhibition of adhesion)	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
prevent biofilm formation by *P. intermedia* (% inhibition of biofilm formation)	**0**	**0**	**0**	**0**	**0**	**0**	**12**	**28**	**0**	**0**	**36**
elicit an inhibitory effect against *P. intermedia*	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**	**>1:2**

Once again, sub-sub-fractions M4.7 and M5.6 were found to be the most active taking into account the dry masses of the various fractions and, consequently, their specific activities.

The main constituents in these sub-sub-fractions were identified by HPLC and mass spectroscopy as:

M4.7: aconitic acid, adenosine and oxalic acid;

M5.6: quinic acid, inosine, oxalic acid and succinic acid;

9.3 Anti-gingivitis activity of chicory sub-fractions and sub-sub-fractions

Anti-gingivitis Assay	**Chicory Sub-fractions**					
	C1	C2	C3	C4	C5	C6
Disruption of pre-existing biofilms of *A. naeslundii (%)*	8	29	0	32	36	33
Disruption of pre-existing biofilms of *P. intermedia (%)*	16	57	5	8	10	27

Killing of *A. naeslundii* disrupted from pre-existing biofilms (%)		*0*	0	0	14	0	0
Killing of P. *intermedia* disrupted from pre-existing biofilms (%)		16	24	4	2	8	7
prevent *A. naeslundii* adhesion to epithelial cells (% inhibition of adhesion)		0	0	0	0	0	0
prevent P. *intermedia* adhesion to epithelial cells (% inhibition of adhesion)		45	18	0	0	0	0
prevent biofilm formation by *P. intermedia* (% inhibition of biofilm formation)		0	0	0	37	0	0
elicit an inhibitory effect against the target organisms	*F. nucleatum*	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2
	A. naeslundii	>1:2	>1:2	>1:2	1:2	1:2	1:2
	P. intermedia	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2
	V. dispar	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2

Based on this data, sub-fraction C1 was considered to be the most promising

As shown in Figure 9, sub-fraction C1 was further fractionated using reverse-phase HPLC and this generated 15 sub-sub-fractions (C1.1-15).

The dry masses of these sub-sub-fractions are shown below:

fraction	dry mass (mg)	%
C1.1	6.8	1.9
C1.2	57.8	15.9
C1.3	37.9	10.4
C1.4	62.2	17.1
C1.5	53.5	14.7

C 1.6	17.5	4.8
C 1.7	1.8	0.49
C 1.8	11.6	3.19
C 1.9	5.3	1.45
C 1.10	14	3.85
C 1.11	14.2	3.91
C 1.12	8.9	2.45
C 1.13	50.7	13.94
C 1.14	13.1	3.6
C 1.15	8.3	2.28
total	363.6	

The sub-sub-fractions were tested for their anti-gingivitis activities using the following assays:

- ability to elicit an antibacterial effect against *P. intermedia*
- ability to disrupt pre-existing biofilms

Anti-gingivitis assay	**Sub-sub-fractions of Chicory Sub-fraction C1**														
	C1.1	C1.2	C1.3	C1.4	C1.5	C1.6	C1.7	C1.8	C1.9	C1.10	C1.11	C1.12	C1.13	C1.14	C1.15
Disruption of pre-existing biofilms (%)	15.4	17.6	21.8	23.7	9.0	23.0	23.6	14.5	26.2	11.7	25.9	18.0	26.2	25.1	22.1
Killing of bacteria disrupted from pre-existing biofilms (%)	12.5	12.1	18.2	21.3	9.0	20.4	18.3	14.5	23.6	8.6	25.9	16.1	24.8	25.5	21.1
prevent P. *intermedia* adhesion to epithelial cells (% inhibition of adhesion)	35	0	10	0	0	0	0	0	0	0	0	0	0	0	0

prevent biofilm formation by P. *intermedia* (% inhibition of biofilm formation)	0	0	0	15	0	16	0	21	0	22	27	21	35	0	0
elicit an inhibitory effect against *P. intermedia*	1:2	1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2	>1:2

Based on this data, sub-sub-fraction C1.7 was found to be the most active taking into account the dry masses of the various fractions and, consequently, its specific activity.

The main constituents in sub-sub-fraction C1.7 were identified by HPLC and mass spectroscopy as oxalic acid and quinic acid.

Example 10 - Further fractionation of LMM fraction of raspberry homogenate and assessment of its anti-caries and anti-gingivitis activities

10.1 Fractionation of LMM fraction of raspberry

Further fractionation of the LMM fraction of the raspberry homogenate was undertaken using a solid-phase extraction approach as described below.

A 5 ml aliquot of the LMM (MW<3500Da) raspberry homogenate was concentrated to dryness in a rotary evaporator at <30°C. The residue was dissolved in 20 ml of phosphate buffer (PB, pH 7.0) and adjusted to pH 7.0 with NaOH solution (4M) (sample).

A Sep-Pak® Vac 20cc (5g) tC18 cartridge (Waters, Milford, MA) was conditioned with methanol (10 ml), Millipore grade water (2 x 10 ml), and PB (pH 7.0, 10 ml).

The sample was passed through the conditioned cartridge at a flow rate < 2 ml/min and the following sub-fractions were obtained:

(i) R 1 sub-fraction containing the polar substances (among which there are organic acids and sugars) were eluted with 25 mL of PB, pH 7.0

(ii) R 2 was obtained by eluting afterwards with 25 mL of ethyl acetate,

(iii) R 3 was obtained by eluting afterwards with 25 ml of a methanol- Millipore grade water mixture (20%-80%),

(iv) R 4 was obtained by eluting afterwards with 25 ml of a methanol- Millipore grade water mixture (50%-50%),

(v) R 5 was obtained by eluting afterwards with 25 ml of methanol.

The five sub-fractions were freeze-dried and the residues were dissolved in 5 ml of Millipore grade water.

10.2 Biological activities of fractions

10.2. 1 Anti-gingivitis activities of raspberry sub-fractions

Anti-gingivitis assay		**Raspberry sub-fractions**				
		R 1	R2	R3	R4	R5
Disruption of pre-existing biofilms of *A. naeslundii (%)*		27.7	19.8	19.8	5.5	13.5
Disruption of pre-existing biofilms of *P. intermedia (%)*		38.5	50.0	23.3	18.1	1.8
Killing of *A. naeslundii* disrupted from pre-existing biofilms (%)		3.9	1.6	7.2	1.6	2.7
Killing of *P. intermedia* disrupted from pre-existing biofilms (%)		30.8	50.0	10.0	8.7	1.8
prevent *A. naeslundii* adhesion to epithelial cells (% inhibition of adhesion)	X2	0	0	0	0	0
	X 1	0	0	0	0	0
prevent *P. intermedia* adhesion to epithelial cells (% inhibition of adhesion)	X2	30	0	26	27	16
	X 1	0	0	0	14	0
prevent biofilm formation by the target organisms (% inhibition of biofilm formation)	*A. naeslundii*	75	29	0	0	0
	P. intermedia	14	0	0	19	24
elicit an inhibitory effect against the target organisms						
	A. naeslundii	**>50**	**>50**	**>50**	**50**	**>50**

	P. intermedia	50	>50	>50	50	>50

It can be seen that all five sub-fractions of raspberry displayed some activity in most of the assays but R1 was considered to be the most promising

10.2.2 Anti-caries activities of raspberry sub-fractions

Anti-caries Assay		Raspberry sub-fractions				
		R1	R2	R3	R4	R5
prevent adhesion of *S. mutans* to hydroxyapatite (% inhibition of adhesion)		53	0	0	0	0
prevent biofilm formation by the target organisms (% inhibition of biofilm formation)	*S. mutans*	72	0	0	0	0
	L. casei	0	67	0	0	0
Prevent co-aggregation between *S. sanguinis* and *F. nucleatum*		0	0	0	0	0
elicit an inhibitory effect against the target organisms	*S. mutans*	>50	>50	>50	>50	>50
	L. casei	>50	>50	>50	>50	>50

It can be seen that sub-fraction R1 was considered to be the most promising.

10.3 Further fractionation of raspberry sub-fraction R1

A 5 ml aliquot of R1 was diluted to 20 ml with HCl (0.01 M) and adjusted to pH 3.0 with HCl solution (4M). A Sep-Pak® Vac 20cc (5g) tC1 8 cartridge (Waters, Milford, MA) was conditioned with methanol (20 ml), distilled water (2 x 20 ml), and HCl =0.01 M

The R1 sub-fraction was then passed through the conditioned cartridge at a flow rate < 2 ml/min.

R1A consists of the most polar substances not retained by the Sep-Pak® Vac 20cc (5g) tC1 8 cartridge. The further sub-sub-fractions were obtained as follows:

R1B was obtained by eluting afterwards with 25 ml of Millipore grade water,

R1C was obtained by eluting afterwards with 25 ml of methanol.

The three sub-sub-sub-fractions were freeze dried and the residues were dissolved in 5 ml of Millipore grade water.

10.4 Biological activities of raspberry sub-sub-fractions from R1

10.4. 1 Anti-caries activities of raspberry sub-sub-fractions from R1

Anti-caries assay	**Sub-sub-fraction of raspberry sub-fraction R1**		
	R1A	**R1B**	**R1C**
Inhibition of adhesion of *S. mutans* to HA (%)	31	77	48
Inhibition of *S. mutans* biofilm formation (%)	39	0	0
Disruption of pre-existing biofilms of *S. mutans (%)*	17.1	27.2	19.0
Killing of *S. mutans* disrupted from pre-existing biofilms (%)	17.1	27.2	17.6

It can be seen that all three sub-sub-fractions displayed activity in most of the assays

10.4.2 Anti-caries activities of raspberry sub-sub-fractions from R1

Anti-gingivitis assay	**Sub-sub-fraction of raspberry sub-fraction R1**		
	R1A	**R1B**	**R1C**
Inhibition of adhesion of *P. intermedia* to KB cells (%)	58	0	0
Inhibition of *P. intermedia* biofilm formation (%)	41	34	21
Disruption of pre-existing biofilms of *P. intermedia (%)*	28.3	17.9	3.4
Killing of *P. intermedia* disrupted from pre-existing biofilms (%)	8.7	17.9	3.4

It can be seen that all three sub-sub-fractions displayed activity in most of the assays

Example 11 - Biological activities of isolated compounds

The active constituents identified in the various sub-sub-fractions of the LMM homogenates of mushroom and chicory are shown below:

Fraction	compound
Mushroom fraction M4.7	aconitic acid, adenosine, oxalic acid
Mushroom fraction M5.6	quinic acid, inosine, oxalic acid, succinic acid
Chicory fraction 1.7	oxalic acid, quinic acid

These compounds were then assayed for their activities in a selection of the assays described above as follows:

(i) ability to disrupt biofiims of the target organisms
(ii) ability to kill bacteria in the disrupted biofiims
(iii) ability to inhibit adhesion to hydroxyapatite
(iv) ability to inhibit biofilm formation
(v) ability to inhibit adhesion to epithelial cells

11.1 Ability to disrupt biofiims of the target organisms and to kill the detached bacteria

In all cases, RTF (reduced transport fluid) was used as a negative control and 1.75 mM SDS was used as a positive control.

11. 1. 1 S. mutans as the target organism

The results are shown in Figure 10a) -j), wherein

a) shows the effect of Epicatechin
b) shows the effect of Shikimic acid
c) shows the effect of Quinic acid
d) shows the effect of frans-Aconitic acid
e) shows the effect of Oxalic acid
f) shows the effect of Adenosine
g) shows the effect of inosine
h) shows the effect of uridine
i) shows the effect of c/s-Aconitic acid
j) shows the effect of succinic acid

As can be seen in Figure 10, *Streptococcus mutans* biofiims were disrupted by all of the agents tested, though most of the agents did not cause a significant kill of the bacteria. However, quinic acid did achieve significant kills of S. *mutans.*

Succinic acid resulted in the disruption of approximately 80 % of cells.

Both cis- and trans-aconitic acid resulted in the disruption of over 80 % at all concentrations.

Oxalic acid resulted in disruption similar to that of the positive control agent at the two lower concentrations, and in 95 % disruption at the highest concentration.

11.1.2 Actinomyces naeslundii as the target organism

The results are shown in Figure 11a) - j), wherein
a) shows the effect of Epicatechin
b) shows the effect of Shikimic acid
c) shows the effect of Quinic acid
d) shows the effect of frans-Aconitic acid
e) shows the effect of Oxalic acid
f) shows the effect of Adenosine
g) shows the effect of inosine
h) shows the effect of uridine
i) shows the effect of c/s-Aconitic acid
j) shows the effect of succinic acid

Neither cis- nor trans-aconitic acid resulted in disruption of Actinomyces naeslundii biofilms. However, succinic acid caused a disruption to biofilms similar to that seen with the positive control agent (1.75 mM SDS), approximately 70 %. Oxalic acid yielded the best results with disruption of over 75 % at all concentrations. None of the agents resulted in a significant kill of *A. naeslundii* cells.

11.2 Ability to inhibit adhesion to hydroxyapatite and to inhibit biofilm formation

11.2. 1 Streptococcus mutans as the target organism

Purified component and	**% inhibition of adherence to HA**	**% inhibition of biofilm formation**

concentration	beads	
Inosine 1mg/ml	0	0
0.1mg/ml	0	0
0.01mg/ml	0	0
Uridine 5 mg/ml	0	0
0.5 mg/ml	0	0
0.05 mg/ml	0	0
Shikimic acid 70 ug/ml	0	0
7ug/ml	0	6
0.7ug/ml	0	0
Adenosine 2mg/ml	17	25
0.4mg/ml	0	0
0.04mg/ml	0	0
Quinic acid 35mg/ml	0	77
3.5mg/ml	10	45
0.35mg/ml	10	0
Epicatechin 2.5mg/ml	0	89
0.5mg/ml	0	24
0.05mg/ml	0	8
Succinic acid 50µg/ml	0	14
5µg/ml	0	20
0.5µg/ml	0	19
Oxalic acid 60µg/ml	0	0
6µg/ml	9	0
0.6µg/ml	9	0

Cis-aconitic acid 500µg/ml	5	6
50µg/ml	5	8
5µg/ml	9	9
Trans-aconitic acid 500µg/ml	8	6
50µg/ml	9	8
5µg/ml	9	8

Both adenosine and quinic acid exhibited some ability to inhibit the adhesion of S. *mutans* to hydroxyapatite at mg/ml concentrations. Low concentrations (Mg/ml) of oxalic acid, cis- and trans-aconitic acids also inhibited adhesion but to a limited degree.

Adenosine, quinic acid and epicatechin were able to inhibit biofilm formation by the organism at mg/ml concentrations whereas succinic acid also inhibited at much lower concentrations (Mg/ml).

11.2.2 Actinomyces naeslundii as the target organism

Purified component and concentration	**% inhibition of adherence to HA beads**	**% inhibition of biofilm formation**
Inosine 1 mg/ml	0	0
0.1 mg/ml	0	14
0.01 mg/ml	0	8
Uridine 5 mg/ml	0	10
0.5 mg/ml	0	10
0.05 mg/ml	0	12
Shikimic acid 70 µg/ml	0	6

7 µg/ml	0	0
0.7 µg/ml	0	0
Adenosine 2 mg/ml	0	11
0.4 mg/ml	0	17
0.04 mg/ml	0	9
Quinic acid 35 mg/ml	0	87
3.5 mg/ml	0	2
0.35 mg/ml	0	8
Epicatechin 2.5 mg/ml	3	88
0.5 mg/ml	7	84
0.05 mg/ml	0	57

None of the test compounds achieved substantial inhibition of adhesion of *A. naeslundii* to hydroxyapatite. Quinic acid and epicatechin achieved substantial inhibition of biofilm formation by *A. naeslundii.* Inosine, uridine, shikimic acid and adenosine inhibited biofilm formation to a limited degree.

11.2.3 *Prevotella intermedia* as the target organism

Purified component and concentration	**% inhibition of adherence to KB cells**	**% inhibition of biofilm formation**
Inosine 1 mg/ml	23	32
0.1 mg/ml	0	25
0.01 mg/ml	0	17
Uridine 5 mg/ml	0	11

0.5 mg/ml	0	0
0.05 mg/ml	0	0
Shikimic acid 70 ug/ml	0	0
7 µg/ml	0	0
0.7 µg/ml	0	0
Adenosine 2 mg/ml	0	0
0.4 mg/ml	0	0
0.04 mg/ml	0	0
Quinic acid 35 mg/ml	37	70
3.5 mg/ml	31	19
0.35 mg/ml	0	0
Epicatechin 2.5 mg/ml	0	10
0.5 mg/ml	0	0
0.05 mg/ml	0	0
Succinic acid 50 µg/ml	37	25
5 µg/ml	42	32
0.5 µg/ml	26	34
Oxalic acid 60 µg/ml	7	0
6 µg/ml	0	9
0.6 µg/ml	6	9
Cis-aconitic acid 500 µg/ml	8	23
50 µg/ml	9	20
5 µg/ml	9	25
Trans-aconitic acid 500 µg/ml	31	25
50 µg/ml	45	24
5 µg/ml	47	24

Inosine and quinic acid, at mg/ml concentrations, were able to inhibit adhesion of *P. intermedia* to KB cells. However, succinic and trans-aconitic acids were effective at much lower concentrations (Mg/ml). Inosine and quinic acid were able to inhibit biofilm formation by *P. intermedia* at mg/ml concentrations. However, succinic acid, cis- and trans-aconitic acids were effective at much lower concentrations (ug/ml).

Example 12 - A randomized, double-blind controlled trial on the effects of a mouthrinse containing shiitake mushroom extract on dental plaque, plaque acidogenicity and the plaque microbiota

The objective was to determine the effect of a low molecular weight fraction of the edible mushroom Shiitake *{Lentinua edodes)* extract in preventing caries in a Swedish adult population. More specifically, its effect on dental plaque acidogenicity, plaque formation and the plaque microbiota was studied.

12. 1 Material and methods

Study design

This study was carried out as a double-blind randomized, placebo-controlled, three-leg cross-over clinical trial. It was carried out at the Department of Cariology, Institute of Odontology, Sahlgrenska Academy at University of Gothenburg, Sweden.

The subjects came to the research laboratory at 8 occasions; for a first clinical examination and information procedure and for a total of 7 test visits (Fig. 1). A total of three products were evaluated where each test period lasted for 14 days. The three test periods were separated by a two-week washout period which also preceeded the first test period and was performed after the last test period. Thus, the total study lasted for 14 weeks.

Fig. 12 shows a schematic schedule of the study.

Professional oral hygiene was performed at the start of each test period as well as directly after each test period (prior to washout). At each of the seven visits the subjects undergone the following data collection in the order mentioned: 1) collection of resting

plaque for protein/acid analyses, 2) plaque-pH measurements, 3) collection of fermented plaque for protein/acid analyses, 4) collection of plaque for microbiological analyses, 5) registration of plaque score, and 6) collection of stimulated saliva.

Test subjects

A total of 65 subjects were screened. Volunteers were identified among students and personnel at the Institute of Odontology as well as individuals in the nearby vicinity. As inclusion criteria they should have a minimum of 20 teeth, ability to lower plaque-pH with at least on pH-unit after a mouthrinse with 10% sucrose solution for 1 min, no metal fillings in the area of the pH measurements, a stimulated salivary secretion rate >0.7 ml/min and not undergone antibiotic treatment during the last 14 days prior to study. Thirty subjects who fulfilled the inclusion criteria were selected for the study. They all got verbal and written information about the study and signed informed consent form.

Apart from the specific instructions given for each test period, the volunteers should prior to each visit refrain from any oral hygiene procedures during the last 3 days prior to each visit as well as eating/drinking during the last 2 hours prior to test. Toothpaste containing 1450 ppm F as NaF (Pepsodent Super Fluor, Unilever Sverige AB, Stockholm) was distributed to all volunteers to be used twice daily througout the whole study.

Test solutions

A mouthwash formulation containing LMM shiitake extract was prepared in two stages as shown below.

Mixture A

	%
Ethanol 96%, Double Rectified	12.000
PEG 40 Hydrogenated Castor Oil (Cremophor RH40)	0.200
Flavour	0.200

Mixture B

	%
Glycerine	6.000
Saccharin 25% solution	0.200
Sodium Fluoride	0.025

Blue No.1 0.5% solution	0.030
Yellow No.5 1% solution	0.020
LMM shiitake mushroom extract	to 100.000

Procedure

Mixtures A & B were prepared separately.
Mixture B was added slowly to A with stirring

The following three products were tested: 1) Shiitake (a mouthwash containing the low molecular weight fraction of Shiitake mushroom extract), 2) water (placebo), and 3) Meridol® (125 ppm AmF + 125 ppm SnF_2, positive control). The active product was distributed in 20 ml aliquots. Also the placebo formulation (Water) was distributed in identical vials containing 20 ml aliquots. The two active and placebo solutions contained identical flavoring and preservative agents. A positive control containing Meridol® (GABA International AB, Munchenstein, Switzerland) was used in vials containing 20 mis. 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 respective solution twice daily. At each rinsing occasion they rinsed twice with 10 mL of the solution for 30 sec after which they spit out the solution. The two rinsing procedures were performed directly after each other. Thus, the total daily exposure was 40 mL for 120 sec. To standardize the sampling procedure after 2 weeks of use of the mouthwash, all volunteers were asked to rinse exactly 3 hours before the visit day 14.

Plaque-pH registration

Changes of plaque acidogenicity was performed before and after a mouthrinse with 10% sucrose by the microtouch method. A microelectrode (Beetrode®, WPI Instruments, Sarasota, FL, USA) was inserted into the plaque in the interproximal area in the left and right upper premolar/moral region. After baseline registration (0 min), the subjects rinsed with the sucrose solution for 1 min after which pH was measured at different time points up to 45 min.

Protein and organic acid analyses

Collection of resting plaque was carried out at the buccal surface of the right upper second molar by use of a sterile carver. Fermented plaque, collected 10 min after start of sucrose rinse, was collected from the contralateral buccal surface. The plaque was transferred to an Eppendorf tube after which the tubes were heated and cooled on ice. The vials with plaque were then centrifuged after which they were stored at -80°C. They were then sent on dry ice by special delivery to ACTA for analyses. To summarize, amount of protein and organic acids (acetate, lactate, formate, propionate, butyrate and succinate) was analyzed by Capillary Ion Electrophoresis.

Microbiological analyses

A stimulated saliva sample was collected by chewing on a piece of parafilm for 5 min. The saliva sample was within 1 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 incubated in its respective atmosphere, the number of colony-forming units (CFU) were counted. The number of mutans streptococci were identified by their characteristic colony morphology on the MSB agar.

Plaque was also collected from the buccal surface of the left and right upper first molars and stored at -80°C.

Five mL of saliva was prior to further handling of saliva put aside into an Eppendorf tube and frozen at -80°C for eventual further analyses regarding contect of active ingredients.

Registration of plaque formation

The amount of plaque was calculated by the TQPIH index. The amount of plaque was for each tooth scored on six surfaces (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual and mesio-lingual) from score 0-5.

Statistical analyses

The mean ± SD of all measurements and individuals were 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 plaque score, the mean score for each tooth was first calculated after which the mean score for the whole dentition was calculated. All microbiological data

were transformed to logarithmic values. The distribution of mutans streptococci and total streptococci in comparison to the total streptococcal flora respectively total oral flora (%) was also calculated. For the answers on the VAS-scale, the distance (in mm) from the left side was measured for each question. Two-way analysis of variance, ANOVA, was used to test the significance of differences among 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 with the Fisher's PLSD. $p<0.05$ was considered statistically significant.

12.2 Results

All 30 subjected completed the study apart from the final washout period for one subject. The mean age of the volunteers were 31 ± 13 years (mean ± SD) including 19 females and 11 males.

Plaque acidogenicity

When comparing the three test solutions, the most pronounced pH-fall was found after rinsing with Water (placebo) and the least attenuated pH-fall for Meridol (Fig. 13, Table 2). The active compound (Shiitake) resulted in an intermediate position. A statistically significant difference when comparing the pH-values at the different time points was found at 2 min between Shiitake and water ($p<0.05$). Also for minimum-pH, there was a numerical difference among the three products with a difference of 0.2 pH-units between Shiitake and both Water respective Meridol (ns). Only minor differences in plaque acidogenicity was found when evaluating maximum pH-decrease as well as AUC_{5-7} and AUC_{6-2} (Fig. 14, Table 3).

Only minor differences in plaque acidogenicity was found when comparing the results from the four washout periods (baseline, after Shiitake, after Water and after Meridol) (Fig. 15, Table 2). For some of the variables, a significant difference was found when comparing data from the baseline measurements and the washout-period after use of Meridol ($p<0.05$).

Table 2. Changes in plaque-pH (mean ± SD) at the different time points up to 45 min after a mouthrinse with 10% sucrose after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

Time (min)	Baseline	Shiitake	Washout Shiitake	Water	Washout Water	Meridol	Washout Meridol
o	6.44 ±1-36	6.49 * 0.51	6.49 ± 1.32	6.14 ± 1.26	6.48 ± 1.30	6.54 0.41	6.S1 ± 1-32
2	5.70 ± 1.14	5.70 ± 0.47	5.71 ± 1.18	5.54 ± 1.20	5.64 ± 1.15	5.97 ± 0.60	5.77 ± 1.16
5	5.50 ± 1.11	5-S5 ± 00.49	5.65 ± 1.13	5.38 ± 1.18	5.52 ± 1.14	S.73 ± 0.56	5.56 ± 1.10
10	5.50 ± 1.10	S.56 ± 0.56	5.64 ± 1.15	5.35 ± 1.15	5·53 ± 1-12	5.6 ± 0.61	5.62 ± 1.15
15	5.57 ± 1.14	*5-66* ± 0.57	5.71 ± 1.10	5-49 ± 1.11	5.69 ± 1.20	5.90 ± 0.61	5.71 ± 1.19
20	5.69 ± 1.17	5-79 ± 0.60	5·86 ± 1.20	5.62 ± 1.23	5-79 ± 1.21	5·99 ± 0.52	5.86 ± 1.21
30	5.89 ± 1.23	5.90 ± 0.61	6.07 ± 1.2.8	5.81 ± 1.30	5-97 ± 1.29	6.11 ± 0.59	6.05 + 1.26
45	0.15 ± 1.29	6.10 ± 0.62	6.15 ± 1.27	6.03 ± 1.29	6.11 ± 1.27	6.20 ± 0.53	6.25 ± 1.30

Table 3. Minimum-pH, maximum pH-decrease, **AUC_{5-7}** and $AUC_{6.2}$ (mean ± SD) after a mouthrinse with 10% sucrose after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

	Minimum-pH	Maximum pH-decrease	AUC_{5-7}	$AUC_{6.2}$
Baseline	5.26 * 1.03	1.18 ± 0.53	9.6 ± 11.3	22.9 ± 18.6
Shiitake	5.36 ± 0.53	1.13 ± 0.53	9.1 ± 11.0	22.2 ± 17.9
Washout Shiitake	5.39 ± 1.08	1.10 ± 0.50	6.0 ± 10.6	17.6 ± 16.9
Water	5.19 ± 1.13	0.95 0.49	8.9 ± 12.7	20.4 ± 19-4
Washout Water	5.31 ± 1.07	1.17 ± 0.47	8.9 ± 11.1	20.7 ± 17.9
Meridol	5·59 ± 0.53	0.95 ± 0.41	5.7 ± 10.4	15.2 ± 16.3
Washout Meridol	5.39 ± 1.09	1.12 ± 0.46	7.1 ± 8.4	18.7 ± 16.1

Oral microorganisms

No statistically significant differences were found for lactobacilli or mutans streptococci in saliva among the three test periods (ns; Table 4, Fig. 16). There was significantly less

number of oral streptococci after Meridol compared to Shiitake ($p<0.05$). Also the total number of microorganisms differed significantly between Shiitake and Meridol ($p<0.001$) as well as between Water and Meridol ($p<0.01$). The lowest proportion of mutans streptococci in comparison to the total number of streptococci was found for Shiitake, while the lowest proportion of streptococci in comparison to the total number of microorganisms was found for Meridol (ns; Table 5, Fig. 16).

Table 4. Microorganisms in saliva expressed as CFU/ml saliva (mean ± SD) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

	Lactobacilli	Mutans streptococci	Total streptococci	Total oral flora
Baseline	3.56 ± 1.42	4.82 ± 1.17	7.40 ± 0.53	7.96 ± 0.31
Shiitake	3.79 ± 1.30	5.05 ± 1.03	7.62 ± 0.45	8.06 ± 0.33
Washout Shiitake	3.79 ± 1.20	4.81 ± 1.04	7.47 ± 0.45	8.0 ó ± 0.33
Water	3.75 ± 1.28	4.88 ± 1.09	7.50 ± 0.36	8.03 ± 0.32
Washout Water	3.70 ± 1.43	4.92 ± 1.15	7.57 ± 0.33	8.io ± 0.33
Meridol	3.70 ± 1.38	4.54 ± 1.16	7.17 ± 0.75	7.85 ± 0.57
Washout Meridol	3.52 ± 1.31	4.75 ± 1.00	7.48 ± 0.42	7-97 ± 0.31

Table 5. Proportions of total streptococci vs total flora respective mutans streptococci vs total streptococci in saliva expressed as % (mean ± SD) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

	Total streptococci / total flora (%)	Mutans ¾¡¾ptococci / total strept@occi (%)
Baseline	35.2 ± 19.4	1-09 ± 1.68
Shiitake	41.0 ± 19.8	0.71 ± 0.93
Washout Shiitake	30·1 * 16.0	1.10 ± 2.46
Water	32.5 ± 1a.6	0.8a ± 1.14
Washout Water	35.0 * 19-0	1.08 ± 1.64
Meridol	31.9 ± 30.2	0.84 ± 1.5a
Washout Meridol	40.4 ± 23.7	0-73 ± 1.44

As shown in Fig. 17, no statistically significant differences were found for any of the groups of oral microorganisms nor for comparison of proportions of bacteria when comparing the four washout periods (ns).

Protein and organic acids in plaque

The numerically least amount of protein was for resting plaque found for the positive control (Meridol) and the largest amount after Baseline (Fig. 18). The amount of protein of fermented plaque was generally somewhat higher compared to the resting plaque with the largest increase found for Meridol and Baseline. For Shiitake and Water only smaller differences were found when comparing the protein content of resting and fermented plaque. Statistical comparisons of these data are in progress.

For organic acids, there was generally a larger numerical variation among both the three test periods and the washout periods compared to protein content (Fig. 19, Table 6). Among the three test periods, the least amount of acetate was for both for resting and fermented plaque found for Meridol. The largest increase after the sucrose rinse was found for placebo (Water). The same was found for lactate with the largest increase found for Water after the sucrose rinse when comparing the two plaque conditions. For the minor acids, a reduction was found between resting and fermented plaque for Shiitake, while an increase was found for Meridol and Water. Minor differences were found for most of the comparison among Baseline and the three Washout periods.

Table 6. Mean amount of A) acetate, B) lactate and C) minor acids in resting (pre-sucrose rinse) and fermenting (post-sucrose rinse) (µmol/mg protein) after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 28.

		Protein (µg)	Acetate (µmol/ mg protein)	Lactate (µmol/ mg protein)	Minor acids (µmol/ mg protein)
Baseline	R	26.3 ± 17.4	0.28 ± 1.74	0.11 ± 0.14	0.14 ± 0.11
	F	40.3 ±31-4	0.48 ± 0.62	0.96 ± 1.81	0.13 ± 0.15
Shiitake	R	17-5 ± 12.5	0.70 ± 0.92	0.10 ± 0.13	0.26 ± 0.39
	F	23 7 ± 19-2	0.83 ± 0.73	1.20 ± 0.99	0.18 ± 0.11
Washout Shiitake	R	20.6 ± 15.4	0.48 ± 0.38	0.12 ± 0.18	0.12 ± 0.0.8
	F	22.4 ± 21.4	0.77 ± 0.64	1.8 ± 1.20	0.15 ± 0.07
Water	R	19.7 ± 11.0	0.48 ± 0.28	0.19 ± 0.41	0.14 ± 0.08
	F	23.6 ± 10.2	0.89 ± 1.15	1.50 ± 2.31	0.19 ± 0.23
Washout Water	R	25.5 ± 17.0	0.61 ± 0.40	0.07 ± 0.08	0.17 ± 0.14
	F	26.3 ± 20.5	0.38 ± 0.24	0.91 ± 1.05	0.12 ± 0.08
Meridol	R	14.7 ± 0.1	0.45 ± 0.49	0.15 ± 0.35	0.17 ± 0.10
	F	27.2 ± 27.7	0.39 ± 0.27	0.42 ± 0.27	0.23 ± 0.42
Washout Meridol	R	17.5 ± 12.1	0.78 ± 1.03	0.18 ± 0.31	0.11 ± 0.11
	F	20.2 ± 12.0	0.87 ± 1.04	1.19 ± 1.46	0.08 ± 0.10

Plaque formation

No significant differences were found between the three test periods (Fig. 10, Table 7). However, the positive control (Meridol) resulted in numerically less plaque compared to the other two test periods (ns).

Table 7. Plaque score (mean ± SD) after a mouthrinse with 10% sucrose after the three test periods (Shiitaki, Water, Meridol) and the baseline and three washout periods (after Shiitake, after Water and after Meridol). n = 30.

	Plaque score
Baseline	2.03 ± 0.87
Shiitake	1.72 ± 0.85
Washout Shiitake	1.76 ± 0.83
Water-	1.72 ± 0.71
Washout Water	1.92 ± 0.79
Meridol	1.57 * 0.55
Washout Meridol	1.84 * 0.83

For the washout periods, significantly less plaque was found after the Shiitake washout period compared to the Water washout ($p<0.05$). No other differences were found.

12.3 Summary of Example 12

The reduced plaque acidogenicity following administration of the shiitake mushroom extract, found by following changes of plaque-pH after a sucrose rinse, indicates that a change in biofilm ecology has occurred. For most variables, the positive control - Meridol - was found to have the strongest effect. The Example also demonstrates that an exposure time longer than two weeks may be advantageous in order to obtain suitable clinical effect of the active compound.

Example 13 - A single centre, randomized, double-blind clinical trial of the effects of a mouthrinse containing shiitake mushroom extract on dental plaque accumulation and gingival index in an Italian population

13.1 Methodology

A total of 90 participants were enrolled among students attending the various degree courses of the Faculty of Medicine of the University of Verona, thus they aged 20-26 years. Thirty were enrolled into the group administered the shiitake mushroom-containing mouthwash, 30 for the positive control (Listerine) group and 30 as a negative control (Coloured water/flavouring) group. All received and signed the informed consensus. The

clinical trial was double blind. The duration of each test was 18 days. The protocol applied was that shown below and in Table 8.

Day 0: instructions to participant and professional oral hygiene

Days 1-6: accurate domiciliary oral hygiene (brushing and flossing)

Day 6: Collection of both plaque and saliva samples

Days 7-17: no oral hygiene, only mouth washing with the appropriate solution, two times a day with 10 ml in double for 30 sec. each

Days 8, 10, 12 and 17: visit including plaque and saliva collection

Day 18: end of the trial and final professional oral hygiene

Table 8. Clinical procedures

Day	**TREATMENT**	***Plaque sample and collection of saliva***
0	**PROFESSIONAL ORAL HYGIENE**	
1	Oral hygiene at home (brushing + dental floss)	
2	Oral hygiene at home (brushing + dental floss)	
3	Oral hygiene at home (brushing + dental floss)	
4	Oral hygiene at home (brushing + dental floss)	
5	Oral hygiene at home (brushing + dental floss)	
6	Oral hygiene at home (brushing + dental floss)	**X**
7	No oral hygiene - twice rinse a day	
8	No oral hygiene - twice rinse a day	**X**
9	No oral hygiene - twice rinse a day	
10	No oral hygiene - twice rinse a day	**X**
11	No oral hygiene - twice rinse a day	
12	No oral hygiene - twice rinse a day	**X**
13	No oral hygiene - twice rinse a day	
14	No oral hygiene - twice rinse a day	
15	No oral hygiene - twice rinse a day	
16	No oral hygiene - twice rinse a day	
17	No oral hygiene - twice rinse a day	**X**
18	**PROFESSIONAL ORAL HYGIENE**	

Clinical parameters:

Plaque Score (PS) and Gingival Index (GI) were determined according to Loe 1963 and 1967.

As far as PS is concerned, total site number to be examined was determined in advance for each subject participating in the clinical trial and, subsequently, the site number corresponding to index 1, index 2 or index 3 was recorded during each visit over time. PS 1 means no plaque deposit, PS 2 means presence of visible plaque, and PS 3 means plaque accumulation.

As far as GI is concerned, the total site number was determined in advance as above. During the following visits over time, the site number corresponding to index 1, index 2 or index 3 was recorded. GI 1 means red gums, GI 2 means bleeding at probing and GI 3 means spontaneous bleeding.

Clinical data were recorded at day 6, 8, 10, 12 and 17 for each participant. The following weight was attributed to the three categories of PS and GI: The site number determined in PS 1 and GI 1 was multiplied for 1, the site number determined in PS 2 and in GI 2 was multiplied for 3, and the site number determined in PS 3 and GI 3 was multiplied for 5.

Microbiology data: Plaque samples were collected at each visit of all the participants in order to evaluate the bacterial counts by qPCR. At the same time, saliva samples were also collected and frozen.

The following were determined:

Total bacterial load (universal primers)

F. nucleatum

L. casei

V. dispar

N. subflava

A. naeslundii

P. intermedia

S. sanguinis

S. mutans

The mean value ± SD was calculated on each collection day for all the participants of a given category (i.e. positive and negative controls and mushroom). Data were then plotted.

13.2 Results

The plaque scores for the various groups during the study are shown in Figures 21 and 22 and in Table 9.

Table 9. Mean plaque scores of the various groups at different time points

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

As can be clearly seen in Figure 22, the mean PI for the mushroom group was lower than that of the negative control group on days 8, 10, 12 and 17 and was lower than that of the positive control (Listerine) group on days 8, 10 and 12. Comparison of the three groups by use of ANOVA/Fischer 's PLSD showed that the mean PI of the mushroom group was statistically significantly lower than that of the negative control group on day 12 ($p<0.05$).

The gingival index scores for the various groups during the study are shown in Figures 23 and 24 and in Table 10.

Table 10. Mean gingival index scores of the various groups at different time points

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 ± 030	0.70 ± 0.34	0.69 ± 0.32
Day 17	0.56 ± 0.31	0.65 ± 0.33	0.59 ± 0.31

As can be clearly seen in Figure 24, the mean GI for the mushroom group was lower than that of the negative control group on days 8, 10, 12 and 17 and was lower than that of the positive control (Listerine) group on days 6, 8, 10, 12 and 17. Comparison of the three groups by use of ANOVA/Fischer's PLSD showed that the mean GI of the mushroom group was statistically significantly lower than that of both the negative control group and the positive control (Listerine) group on day 12 ($p<0.05$).

Microbiological composition of dental plaque samples taken during the trial

The plaque samples taken during the study were analysed using a quantitative PCR method. The proportions of the various species present in the samples are shown in Figures 25 to 32. Figures 29 and 30 show the proportions in plaque of two of the main gingivitis-associated organisms, *A. naeslundii* and *P. intermedia* respectively.

13.3 Summary and conclusions from Example 13

The results of this clinical study have shown that the use of a mouthwash containing a LMM fraction of shiitake mushroom extract has a beneficial effect on oral health in that it can reduce the accumulation of plaque and can reduce gingival inflammation. Statistical analysis of the data revealed that after 6 days of use, the shiitake mushroom-containing mouthwash was more effective than Listerine at reducing gingival inflammation.

Example 14 - Formulation of extracts and active components

14.1 Development of a microparticulate delivery system for quinic acid and LMM shiitake mushroom extract

The LMM shiitake mushroom extract or quinic acid were each incorporated into a variety of polymeric micro-beads using the scheme shown in Figure 33. Several types of micro-beads were used and these were made from chitosan, a range of alginates and a mixture of alginate and chitosan. Scanning electron micrographs were taken of chitosan micro-beads loaded with quinic acid: which showed micro-beads average size 700 - 900 µm (not shown)

The quinic acid-encapsulation efficiency of the various micro-beads was determined (Table 11) as well as their ability to adhere to hydroxyapatite and pork mucosa (Figures 34 and 35).

Table 11 Composition and encapsulation efficiency of micro-beads

Batch n.	Chitosan conc. w/v	Tripolyphosphate conc. w/v	Chitosan :TPP Ratio	Chitosan: quinic acid w:w ratio	Curing time (mins)	Quinic acid encapsulation efficiency
1	2,00 %	6,00 %	1:10	4:1	15'	90.56%

Batch n.	Alginate	Total Alginate conc. w/v	$CaCl_2$ conc	Alginate: $CaCl_2$ v:v ratio	Quinic acid encapsulation efficiency %
2	Protanal LF120	2%	0.05 M	1:10	-
3	Protanal LF200S	2%	0.1 M	1:5	76.02
4	Protanal LF200S: Protanal LF120 1:1 w:w	2%	0.1 M	1:5	51.56
5	Protanal LF200S: Protanal LF120 1:2 w:w	2%	0.1 M	1:5	13.04
6	Protanal LF200S: Protanal LF120 1:1.5 w:w	2%	0.1 M	1:5	58.25
7	Protanal LF200S:Protanal LF120 1:1.5 w:w	2%	0.2 M	1:5	92.18

Batch n.	Total alginate conc w/v *	$CaCl_2$ conc.	Chitosan conc. w/v	Alginate: $CaCl_2$ v:v ratio	Time curing (mins)	Preparation method
8	2%	0.2 M	0.5% in H_2O bid.	1:5	15'	One step
9	2%	0.1 M	0.5% in H_2O bid.	1:5	15'	One step
10	2%	0.1 M	0.5% in CH_3COOH 1% v/v	1:5	15'	Two steps
11	2%	0.2 M	0.5% in CH_3COOH 1% v/v	1:5	15'	Two steps
12	2%	0.5 M	0.1% in CH_3COOH 1% v/v	1:3	30'	One step

The swelling characteristics of the various quinic acid-containing micro-beads was then evaluated in artificial saliva. All batches (with the exception of batch 2 made of 100% alginate LF120) showed similar swelling behaviour, with swelling % between 100 and 105% (data not shown). All batches (with the exception of batch 2) were stable for 4 hours in artificial saliva.

Quinic acid release from the micro-beads was evaluated by soaking weighed amounts of micro-beads in 5 ml of artificial saliva at 35°C and determining the amounts of quinic acid released at various times by HPLC. The results are shown in Table 12.

Table 12 Quinic acid release from the various quinic acid-containing micro-beads

Batch n.	Polymer	Encapsulation efficiency %	Loading %	Quinic acid rel. % (20 mins)
1	Chitosan	90.56	22.14	-
2	Alginate (Protanal LF120)	-	-	-
3	Alginate (Protanal LF200S)	76.02	71,42	12.77
4	Alginate (Protanal LF200S: Protanal LF120 1:1 w:w)	51.56	58.12	15.13
5	Alginate (Protanal LF200S: Protanal LF120 1:2 w:w)	13.04	15.73	37.10
6	Alginate (Protanal LF200S: Protanal LF120 1:1.5 w:w), $CaCl_2$ 0.1 M	58.25	55.16	14.10
7	Alginate (Protanal LF200S: Protanal LF120 1:1,5 w:w), $CaCl_2$ 0.2 M	92.18	61.09	12.74
8	Alginate (Protanal LF200S: Protanal LF120 1:1.5 w:w): CS	87.47	66.01	36.37

Table 12 and Figure 36 show that it is possible to produce micro-beads exhibiting a wide range of quinic acid release characteristics - maximal release was obtained using batches 5 and 8.

14.2 Micro-beads containing LMM shiitake mushroom extract

Various microbead preparations were prepared containing the LMM extract of shiitake mushrooms.

Scanning electron micrographs were used to evaluate micro-beads made of Alginate (Protanal LF200S):CS were (data not shown). By way of example, batch 15, loaded with LMM shiitake mushroom extract showed an average size is between 800 µm - 900 pm

The content and release of the LMM extract from the micro-beads was evaluated by monitoring (using GFC) fraction M4 of the extract (Table 13).

Table 13. Characteristics of micro-beads containing LMM shiitake mushroom extract.

Batch	Polymer	M_4 encapsulation efficiency %	M_4 Loading %	M_4 rel. % (20 mins)
13[a]	Alginate (Protanal LF200S) $CaCl_2$ 0.2 M	48.79	52.23	20.27
14[c]	Alginate (Protanal LF200S: Protanal LF120 1:1.5 w:w), $CaCl_2$ 0.2 M	10	-	-
15[b]	Alginate (Protanal LF200S):CS	50.83	57.00	36.33
16[d]	Alginate (Protanal LF200S: Protanal LF120 1:1.5 w:w): CS	9.34	12.45	-

[a] **Polymer composition as batch 3;**

[b] **Polymer composition as batch 3 , but chitosan coated;**

[c] **Polymer composition as batch 7;** [d] **p_0 lymer composition as batch 8;**

Again a swelling % of around 110% was seen in batches 13 and 15 (data not shown).

The extent of adhesion of the micro-beads containing LMM extract to pork mucosa and hydroxyapatite is shown in Figure 37.

Example 15 - Preparation of chewing gum containing LMM shiitake mushroom extract.

Chewing gums were prepared using a standard technique. The gum base (PG new nutraTA, Gum base Company SpA) was softened at 60°C and 20% glycerol was added.

Micro-beads containing the LMM shiitake mushroom extract were then uniformly incorporated into the softened gum-glycerol mixture. The chewing gums obtained were molded into rods of uniform shape and cooled to room temperature (Figure 38). The total chewing gum mass was about 500 mg and consisted of 280 mg gum base, 70 mg glycerol (plasticizer) and 150 mg micro-beads. The ratio of chewing gum to micro-beads was 2.33: 1 w:w.

The integrity of the micro-beads upon release from chewing gums was evaluated. The chewing gums were placed in a chewing apparatus for 20 mins, and the undamaged micro-beads released in this time were counted (Table 14).

Table 14. Percentage of intact micro-beads released from chewing gum at various times following treatment in a chewing apparatus.

	Percentages of microbeads released undamaged in the times tested		
Sample	5 min	10 min	20 min
A	77.48 %	92.05 %	98.01 %
B	58.06 %	92.26 %	98.71 %
C	66.02 %	93.07 %	96.79 %

The micro-beads were largely resistant to mechanical damage which enabled them to be loaded into chewing gum and released intact during chewing. Most of the micro-beads were released undamaged within 20 minutes of chewing - this is widely accepted as being the average time period that chewing gum is retained in the oral cavity.

Summary of Example 15

LMM extracts of shiitake mushroom and chicory, as well as one of their active constituents (quinic acid), were successfully encapsulated into micro-beads. The microbeads were largely resistant to mechanical damage and showed good adhesion to mucosa and to hydroxyapatite. Microbeads containing LMM shiitake mushroom extract were incorporated into a functional food (chewing gum) and were released undamaged within 20 minutes of chewing.

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41) Tagashira M, Uchiyama K, Yoshimura T, Shirota M and Uemitsu N. 1997. Inhibition by hop bract polyphenols of cellular adherence and water-insoluble glucan synthesis of mutans streptococci. Biosci. Biotechnol. Biochem. 61: 332-35.

42) Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S and Ooshima T. 1999. Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res. 33: 441-45.

Claims

1 A composition for promoting oral health,

which composition is a functional food or oral preparation which comprises one or more extracts of a natural product selected from the list consisting of: Shiitake mushroom, chicory or raspberry.

2 A composition as claimed in claim 1 wherein the composition is for one or more of ameliorating, controlling or reducing the risk of dental caries or periodontal disease, which periodontal disease is optionally gingivitis or periodontitis.

3 A composition as claimed in claim 1 or claim 2 wherein the extract is obtained or obtainable by homogenization of the natural product and filtering to remove solid matter.

4 A composition as claimed in any one of claims 1 to 3 wherein the extract is or is derived from a low molecular mass (LMM) fraction of the natural product comprising compounds of less than around 1, 2, 3, 3.5, 4, 4.5, 5 kDa.

5 A composition as claimed in any one of claims 1 to 4 wherein the extract is derived from a less than 5kDa LMM sub fraction of Shiitake mushroom obtainable by gel filtration in a 300 mm x 10 mm column, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water having a flow rate, 1.0 ml/min, such as to sub-fractionate the LMM fraction into 5 sub-fractions and selecting the sub-fractions M4 and M5 peaking at about 106 mins or about 154 mins.

6 A composition as claimed in claim 5 wherein the extract is of an LMM sub-sub-fraction of Shiitake mushroom obtainable from sub-fraction M4 by use of a semipreparative HPLC column with the following properties: C18, 250 * 10 mm, 10 μm and conditions: volume injected, 1 mL; column temperature, 25 °C; UV spectra recorded in the 190-600 nm range, and chromatograms acquired at 210 nm, such as to sub-fractionate the LMM fraction into 8 sub-fractions in accordance with Figure 6 and selecting the sub-sub-fraction M4.7.

7 A composition as claimed in claim 5 wherein the extract is of an LMM sub-sub-fraction of Shiitake mushroom obtainable from sub-fraction M5 by use of a semipreparative HPLC column with the following properties: C18, 250 * 10 mm, 10 μιτι

and conditions: volume injected, 1 mL; column temperature, 25 °C; UV spectra recorded in the 190-600 nm range, and chromatograms acquired at 210 nm, such as to sub-fractionate the LMM fraction into 11 sub-fractions in accordance with Figure 7 and selecting the sub-sub-fraction M5.6.

8 A composition as claimed in any one of claims 1 to 4 wherein the extract is of a less than 5kDa LMM sub fraction of chicory obtainable by gel filtration in a 300 mm x 10 mm column, with a stationary phase having exclusion limits 100-10000 Da and particle size 45 micron and a mobile phase of water having a flow rate, 1.0 ml/min, such as to sub-fractionate the LMM fraction into 5 sub-fractions as follows and selecting the sub-fraction C1 peaking at between about 37 mins and 41 mins.

9 A composition as claimed in claim 8 wherein the extract is of an LMM sub-sub-fraction of chicory obtainable from sub-fraction C1 by use of a semi preparative HPLC column with the following properties: C18, 250 * 10 mm, 10 μηι and conditions: volume injected, 1 mL; column temperature, 25 °C; UV spectra recorded in the 190-600 nm range, and chromatograms acquired at 210 nm, such as to sub-fractionate the LMM fraction into 15 sub-fractions in accordance with Figure 9 and selecting the sub-sub-fraction C1.7.

10 A composition as claimed in any one of claims 1 to 4 wherein the extract is of a less than 3.5 kDa LMM sub fraction of raspberry obtainable by:

(i) concentrating the LMM fraction to dryness in a rotary evaporator at <30°C

(ii) dissolving the residue in 20 ml of phosphate buffer pH 7.0)

(iii) adjusting the pH to 7.0 with NaOH solution

(iv) passing the sample through a 20cc (5g) tC1 8 cartridge conditioned with methanol (10 ml), water (2 x 10 ml), and PB (pH 7.0, 10 ml) at a flow rate < 2 ml/min.

and selecting the polar sub-fraction elutable with 25 ml of PB, pH 7.0.

11 A composition for promoting oral health, which composition is a functional food or oral preparation which has been supplemented with a compound selected from the list consisting of: quinic acid; adenosine; inosine; trans-aconitic acid; oxalic acid; adenosine; cis-aconitic acid and succinic acid, or a salt thereof.

12 A composition for promoting oral health, which composition is a functional food or oral preparation which has been supplemented with a compound selected from the list consisting of: shikimic acid; uridine; epicatechin, or a salt thereof.

13 A composition as claimed in claim 11 wherein the compound is selected from the list consisting of: quinic acid; succinic acid; or a salt thereof..

14 A composition as claimed in any of claims 11 to 13 wherein the compound, if present, is present at equal to or at least the following concentration:
(i) 0.5 µg/ml of succinic acid or a salt thereof,
(ii) 3.5 mg/ml of quinic acid or a salt thereof,
(iii) 5.0 µg/ml of trans-aconitic acid or a salt thereof,
(iv) 1.0 mg/ml of inosine or a salt thereof,
(v) 2.0 mg/ml of adenosine or a salt thereof,
(vi) 2.5 mg/ml of epicatechin or a salt thereof,
(vii) 50 µg/ml of cis-aconitic acid or a salt thereof,
(viii) 5.0 mg/ml of uridine or a salt thereof,
(ix) 0.6 µg/ml of oxalic acid or a salt thereof,
(x) 0.7 µg/ml of shikimic acid or a salt thereof.

15 A composition as claimed in any one of claims 1 to 14 which demonstrates one or more of the following activities relevant to the promotion of oral health:

(i) inhibition of biofilm formation by one or more target organisms selected from the list consisting of: *V. dispar, F. nucleatum, A. naeslundi, P. intermedia;*

(ii) inhibitory activity against one or both of the target organisms: *A. naeslundi, P. intermedia;*

(iii) ability to inhibit co-aggregation of one, two, or more preferably three of the following pairs of target organisms: *P. intermedia +S. sanguinis; F. nucleatum + S. sanguinis; F. nucleatum + N. subflava; S. sanguinis +V. dispar; S. sanguinis +N. subflava.*

(iv) ability to disrupt the biofilm of, or kill the organism within a biofilm of, one of both of the target organisms: *A. naeslundi, P. intermedia.*

(v) inhibition of adherence of one or more target organisms selected from: *F. nucleatum, A. naeslundi, P. intermedia,* to epithelial cells when added simultaneously with the target(s) to the monolayers and\or when used to pretreat the monolayer;

(vi) inhibition of *A. naeslundii* internalization by epithelial cells;

(vii) Inhibition of cellular IL-6 release induced by *F. nucleatum* supernatant.

16 A composition as claimed in any one of claims 1 to 15 which demonstrates one or more of the following activities relevant to the promotion of oral health:

(i) inhibition of adhesion of one or more target bacteria to hydroxyapatite, which bacteria are selected from: S. *mutans, S. sanguinis, L casei and A. naeslundii when* added simultaneously with the target(s) to the hydroxyapatite, and\or induce detachment from hydroxyapatite;

(ii) inhibition of biofilm formation by one or more target organisms selected from the list consisting of: S. *mutans, S. sanguinis, L. casei*;

(iii) inhibitory activity against one or more of the target organisms: *L casei, S. mutans, S. sanguinis, A. naeslundii;*

(iv) ability to inhibit co-aggregation of one or more of the following pairs of target organisms: S. *mutans +S. sanguinis ; S. mutans + L. casei; P. intermedia +F. nucleatum; F. nucleatum +* S. *mutans; F. nucleatum +N. subflava; F. nucleatum +* S. *sanguinis;*

(v) ability to disrupt biofilms of one of both of the target organisms: S. *mutans L. casei;*

(vi) inhibition of S. *mutans* comDE gene expression.

17 A composition as claimed in any one of claims 1 to 15 which demonstrates one or more of the following activities relevant to the promotion of oral health:

(i) ability to reduce gingival inflammation;

(ii) ability to reduce plaque accumulation or acidogenicity following sucrose rinse.

18 A composition as claimed in any one of claims 1 to 17 wherein the extract or compound is provided in the form of a polymeric microbead, which is optionally provided by ionotropic gelation.

19 A composition as claimed in any one of claims 1 to 18 which is a functional food or oral preparation supplemented with the extract or compound in pure form such as to promote the oral health benefits or effects of the composition.

20 A composition as claimed in any one of claims 1 to 19 where the extract or compound is present as the sole active ingredient in respect of the promotion of oral health.

21 A composition as claimed in any one of claims 1 to 20 wherein composition is selected from a foodstuff, beverage, chewing gum, toothpaste, mouthwash, mouth rinse, toothpowder or tooth gel.

22 A composition as claimed in claim 21 which is a foodstuff, which is optionally a dietary supplement.

23 A method of promoting oral health in an individual, which method comprises use of a composition comprising of an extract of one or more of chicory, Shiitake mushroom, or raspberry.

24 Use of an extract of one or more of chicory, Shiitake mushroom, or raspberry in the manufacture of a composition to promote oral health.

25 An anti-caries or anti-gingivitis oral preparation comprising, consisting of, or consisting essentially of an extract of one or more of chicory, Shiitake mushroom, or raspberry.

26 A method, use or preparation as claimed in any one of claims 21 to 23 wherein the composition is as claimed in any one of claims 1 to 22.

27 A method of counteracting any of the effects of the target organism associated with caries or gingivitis by contacting an oral surface with an oral composition as claimed in any one of claims 1 to 22.

28 A method for assessing the potential oral health benefits of a plant or fungus, the method comprising assessing the level of a compound in the plant or fungus, wherein the compound is selected from the list consisting of: quinic acid; adenosine; inosine; trans-aconitic acid; oxalic acid; adenosine; cis-aconitic acid; succinic acid; shikimic acid; uridine; epicatechin.

29 A process for obtaining a composition as claimed in any one of claims 1 to 4, which process comprises:

(i) providing an extract which is derived from a less than 5kDa LMM sub fraction of Shiitake mushroom by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting sub-fractions corresponding to sub-fraction M4 or M5 shown in Figure 5;

(ii) optionally further providing an extract which is an LMM sub-sub-fraction of the sub-fraction corresponding to M4 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to M4.7 shown in Figure 6;

(iii) optionally further providing an extract which is an LMM sub-sub-fraction of the sub-fraction corresponding to M5 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to M5.6 shown in Figure 7.

30 A process for obtaining a composition as claimed in any one of claims 1 to 4, which process comprises:

(i) providing an extract which is derived from a less than 5kDa LMM sub fraction of chicory by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting sub-fractions corresponding to sub-fraction C1 shown in Figure 8;

(ii) optionally further providing an extract which is an LMM sub-sub-fraction of sub-fraction C1 by use of chromatography to sub-fractionate the LMM fraction into sub-fractions and selecting the sub-sub-fraction corresponding to C1.7 shown in Figure 9.

31 A process for obtaining a composition as claimed in any one of claims 1 to 4, which process comprises providing an extract derived from a less than 3.5 kDa LMM sub fraction of raspberry by:

(i) concentrating the LMM fraction to dryness in a rotary evaporator at <30°C

(ii) dissolving the residue in an aqueous carrier and optionally adjusting the pH towards neutrality;

(iii) using chromatography to sub-fractionate the solution into sub-fractions and selecting the sub-fraction corresponding to the sub-fraction obtainable by passing the solution through a 20cc (5g) tC18 cartridge conditioned with methanol (10 ml), water (2 x 10 ml), and PB (pH 7.0, 10 ml) at a flow rate < 2 ml/min and selecting the polar sub-fraction elutable with 25 ml of PB, pH 7.0.

Figure 1

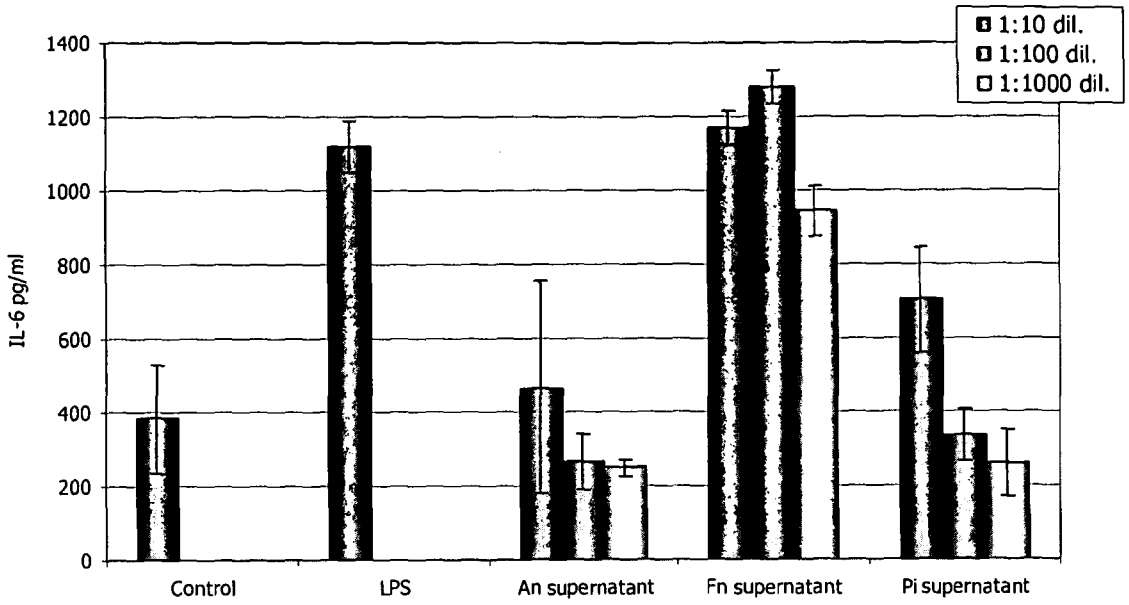
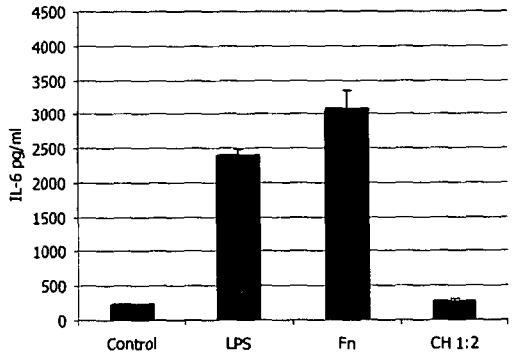


Figure 2



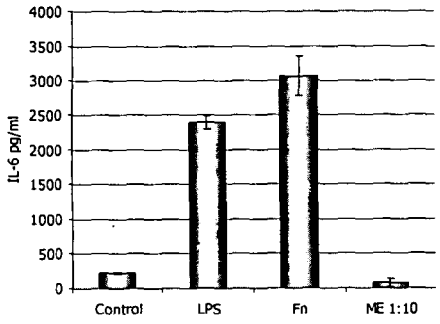


Figure 3

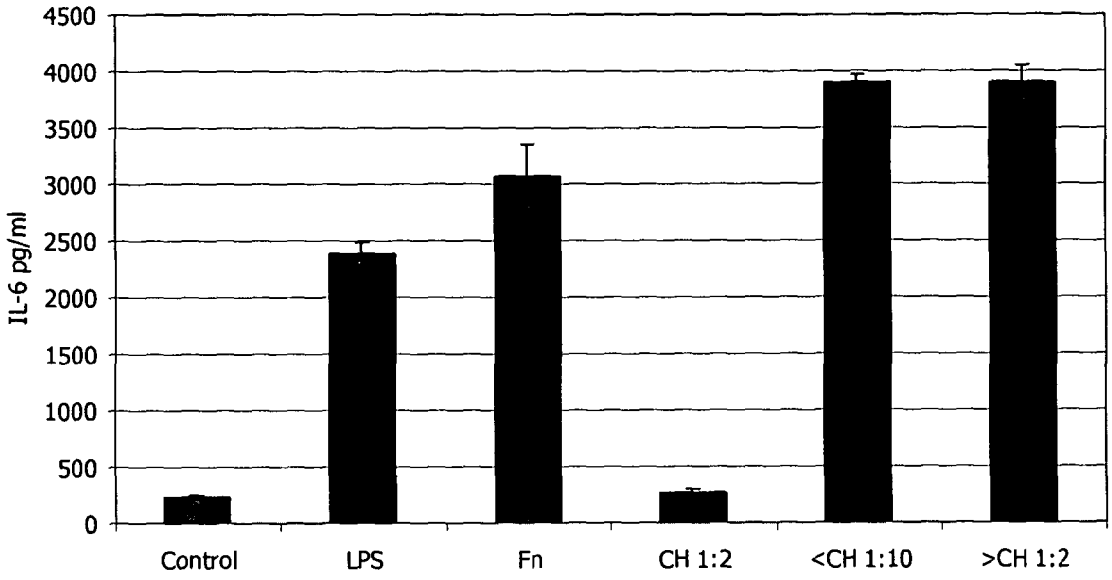


Figure 4

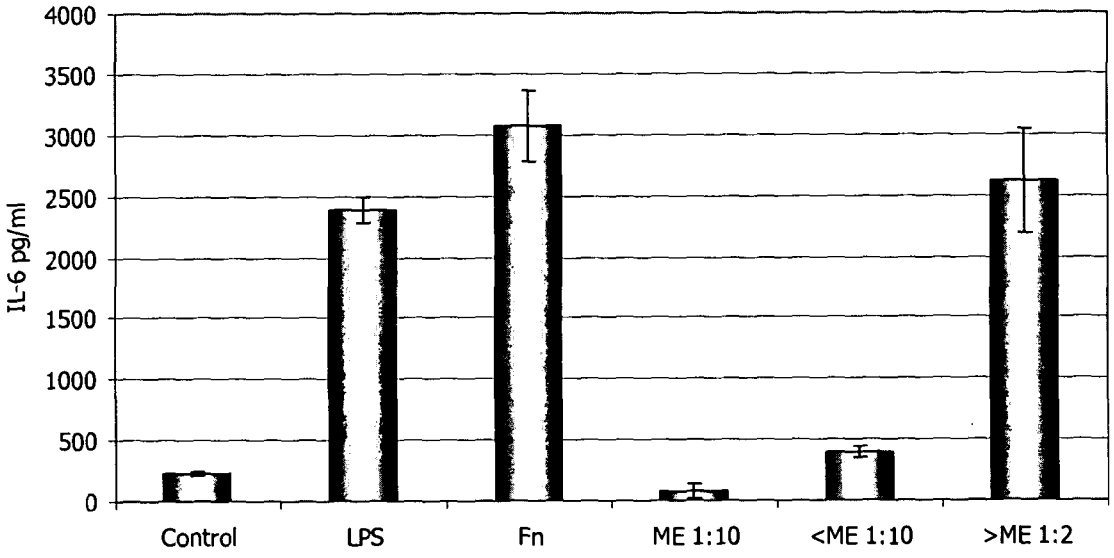
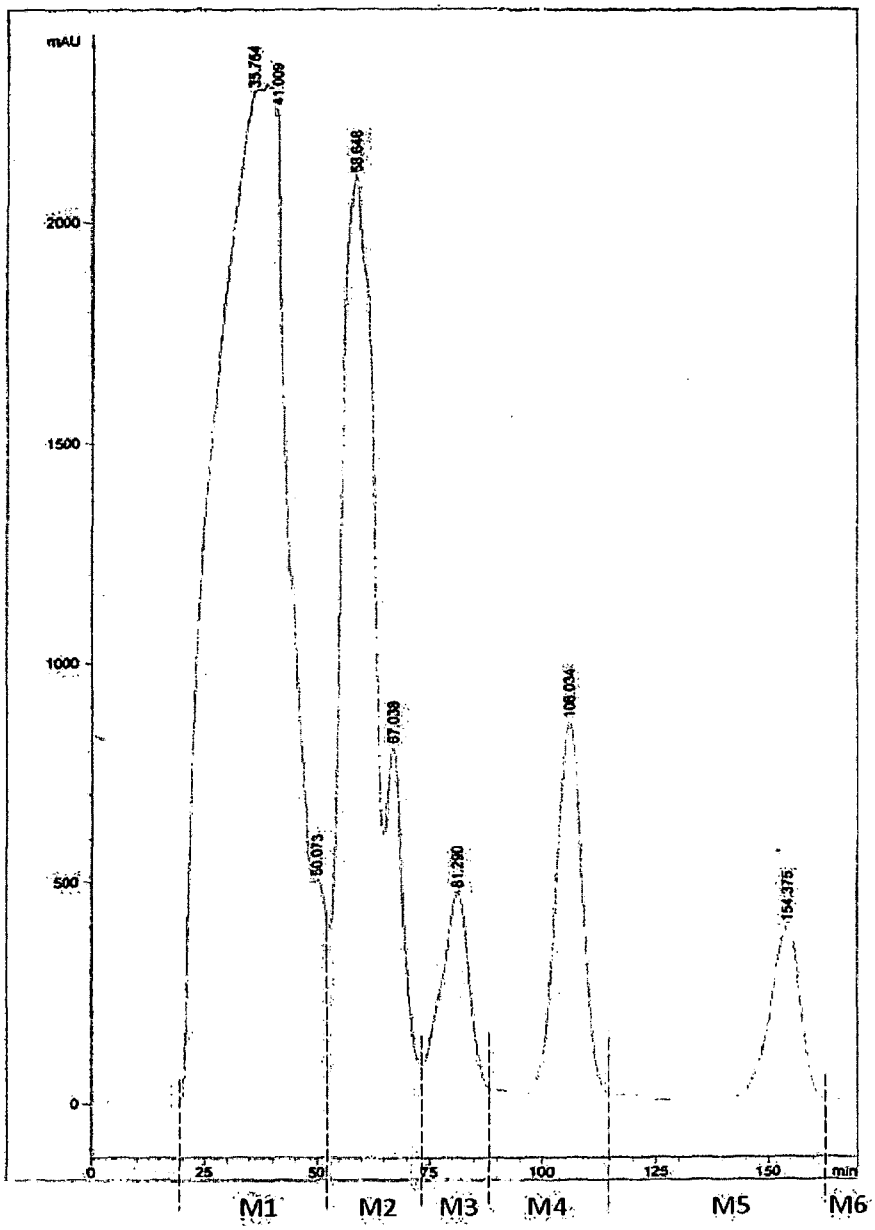


Figure 5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1500
1000
500
0
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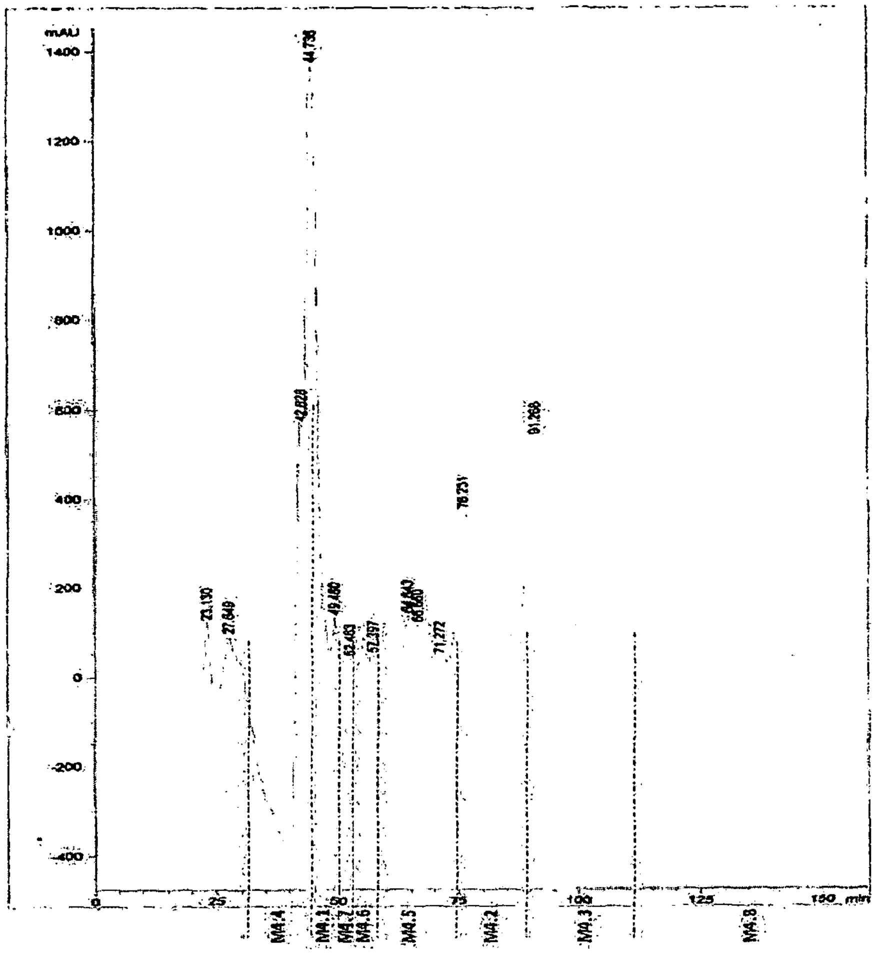
41.009
50.073
58.648
67.038
81.290
108.034
154.375
0
25
50
75
100
125
150
min
M1
M2
M3
M4
M5
M6

Figure 6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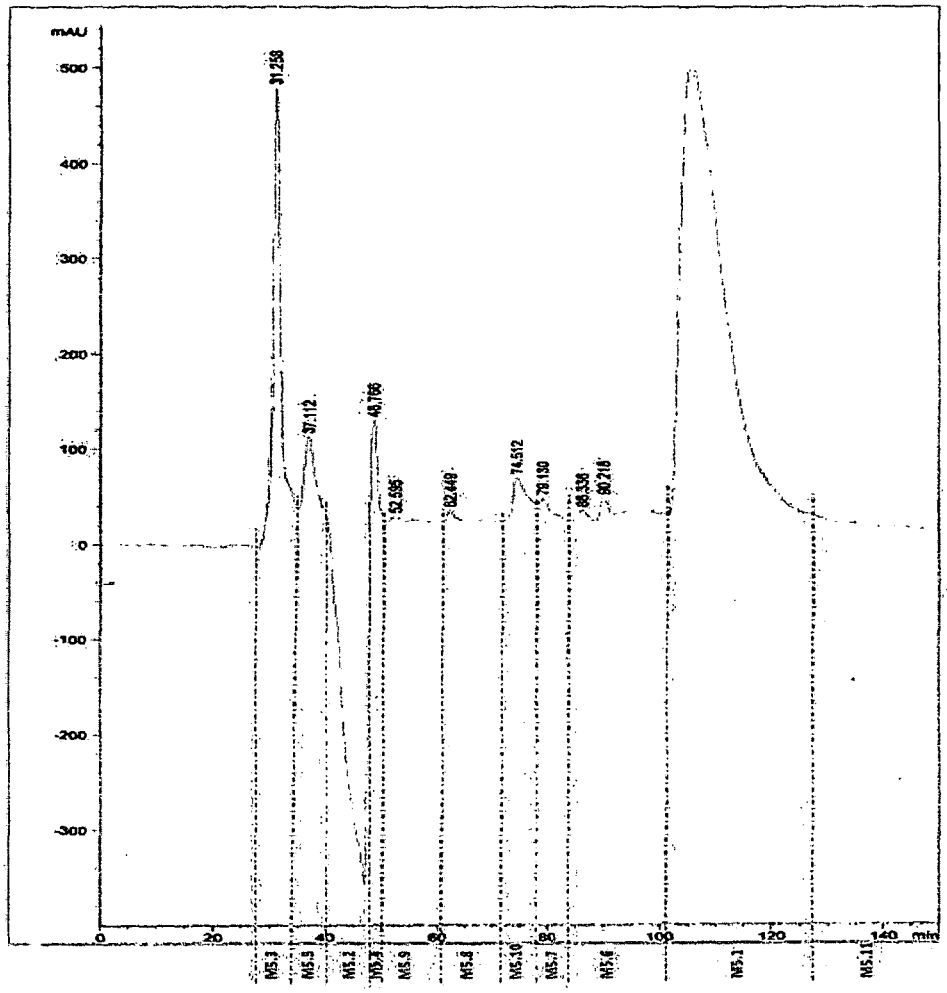
23.130
27.849
49.480
53.403
57.397
65.650
71.272
0
25
50
75
100
125
150
min
M4.4
M4.1
M4.7
M4.6
M4.5
M4.2
M4.3
M4.8

Figure 7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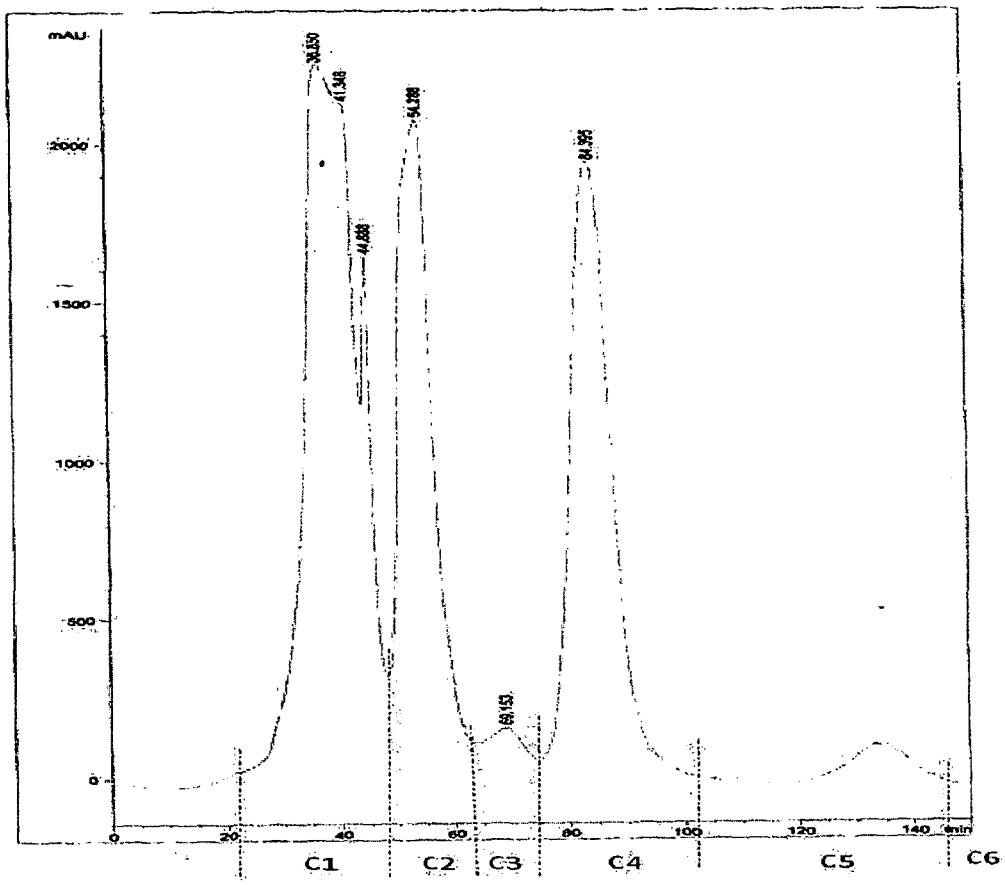
100
120
140
min

Figure 8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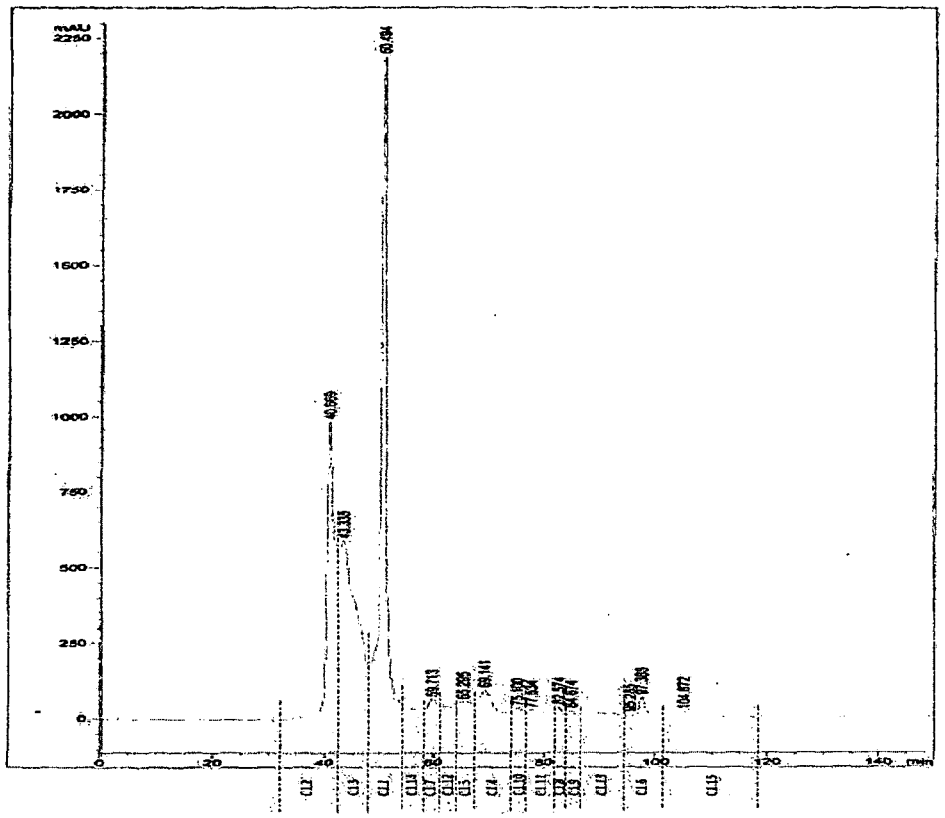
140
min
C1
C2
C3
C4
C5
C6

Figure 9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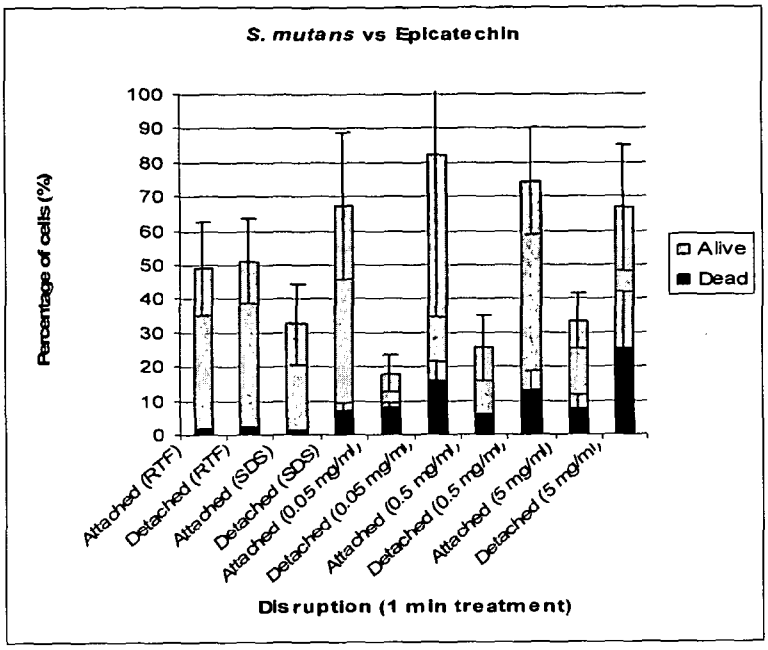
80
100
120
140
min

Figure 10

a)


S. mutans vs Epicatechin
Percentage of cells (%)
100
90
80
70
60
50
40
30
20
10
0
Alive
Dead
Attached (RTF)
Detached (RTF)
Attached (SDS)
Detached (SDS)
Attached (0.05 mg/ml)
Detached (0.05 mg/ml)
Attached (0.5 mg/ml)
Detached (0.5 mg/ml)
Attached (5 mg/ml)
Detached (5 mg/ml)
Disruption (1 min treatment)

b)

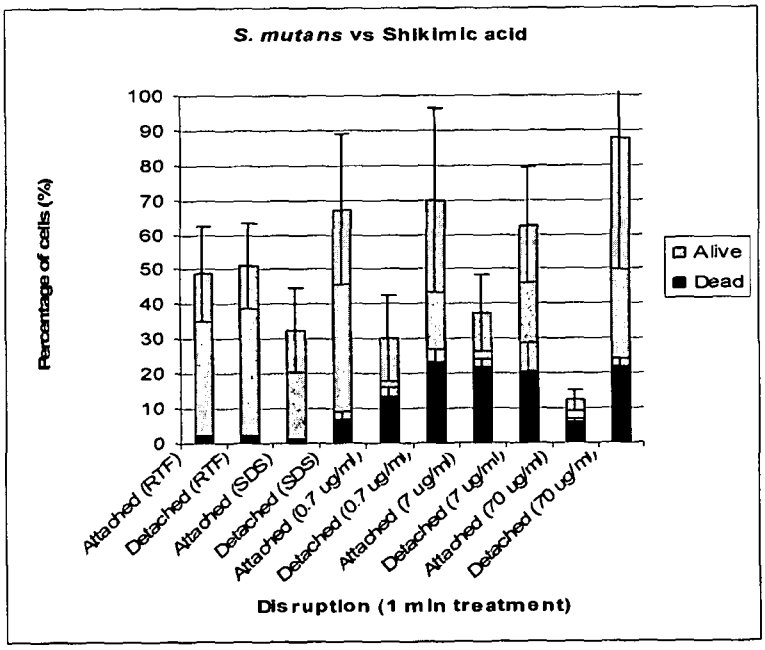
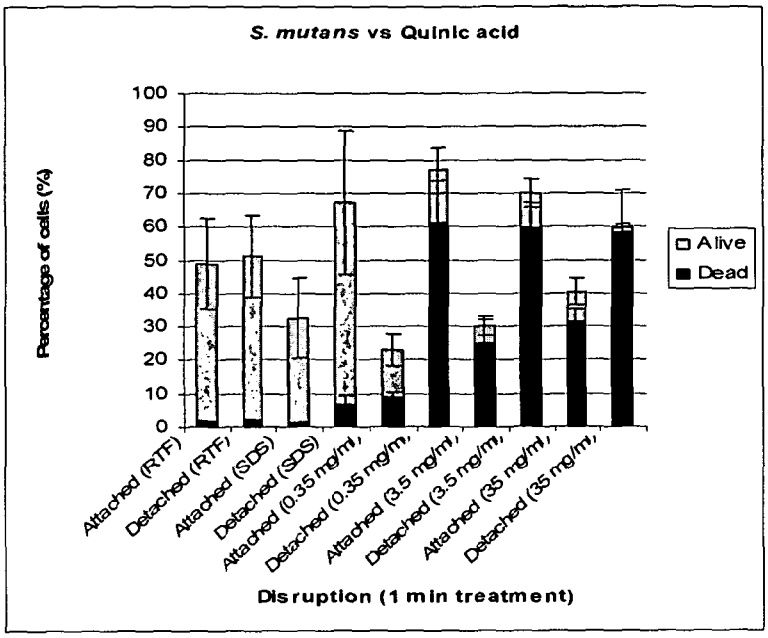

S. mutans vs Shikimic acid
Percentage of cells (%)
100
90
80
70
60
50
40
30
20
10
0
Alive
Dead
Attached (RTF)
Detached (RTF)
Attached (SDS)
Detached (SDS)
Attached (0.7 ug/ml)
Detached (0.7 ug/ml)
Attached (7 ug/ml)
Detached (7 ug/ml)
Attached (70 ug/ml)
Detached (70 ug/ml)
Disruption (1 min treatment)

Figure 10 continued

c)



d)

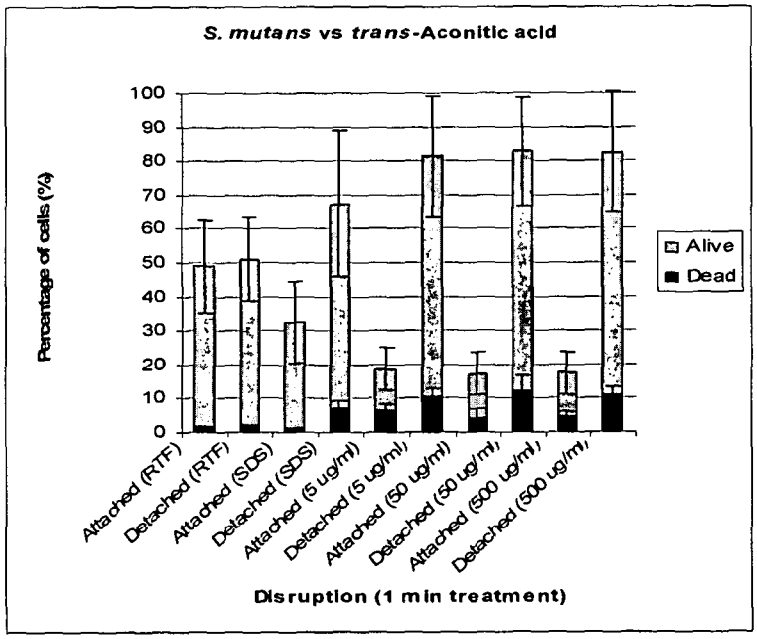
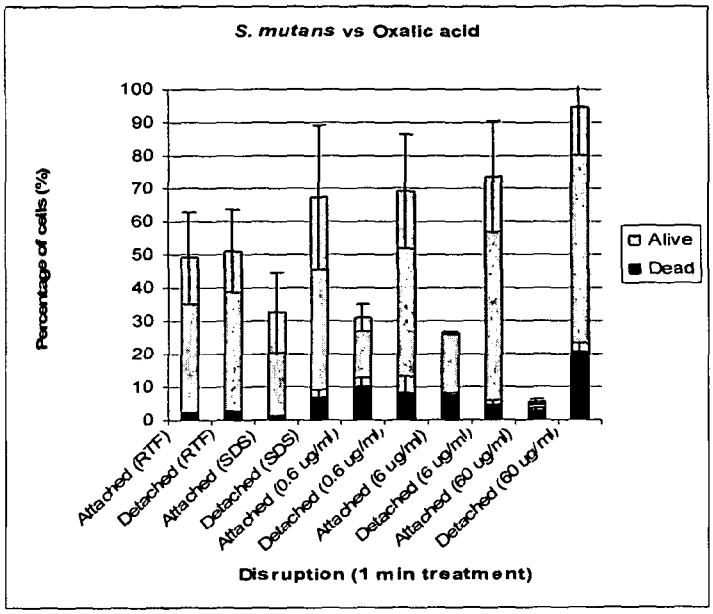


Figure 10 continued

e)



f)

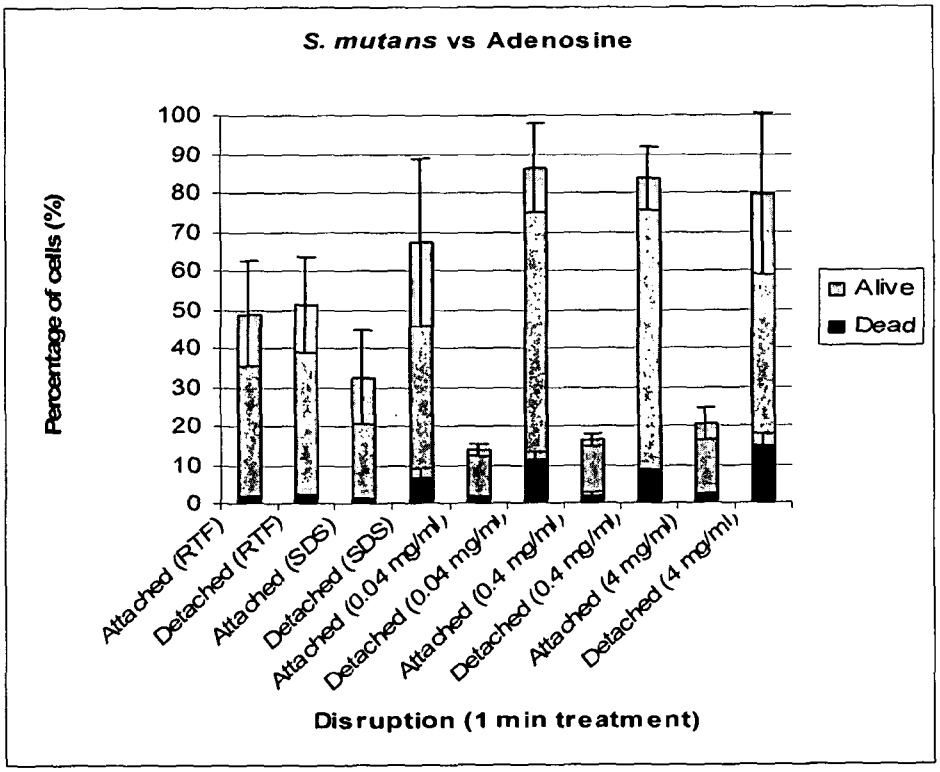
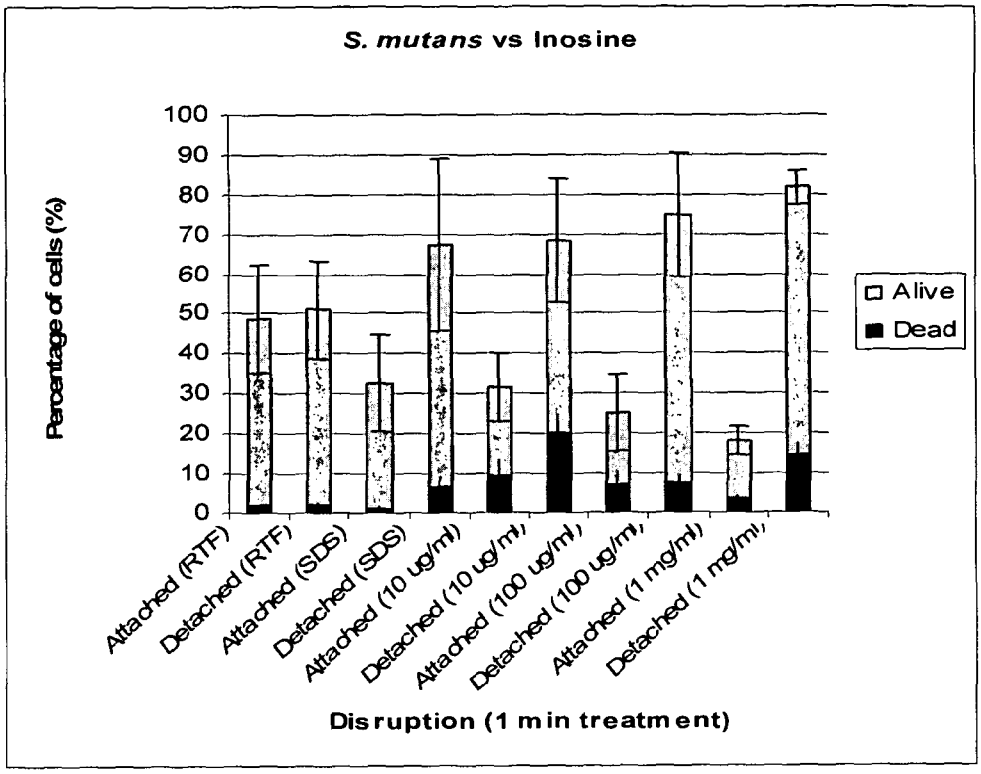


Figure 10 continued

g)



h)

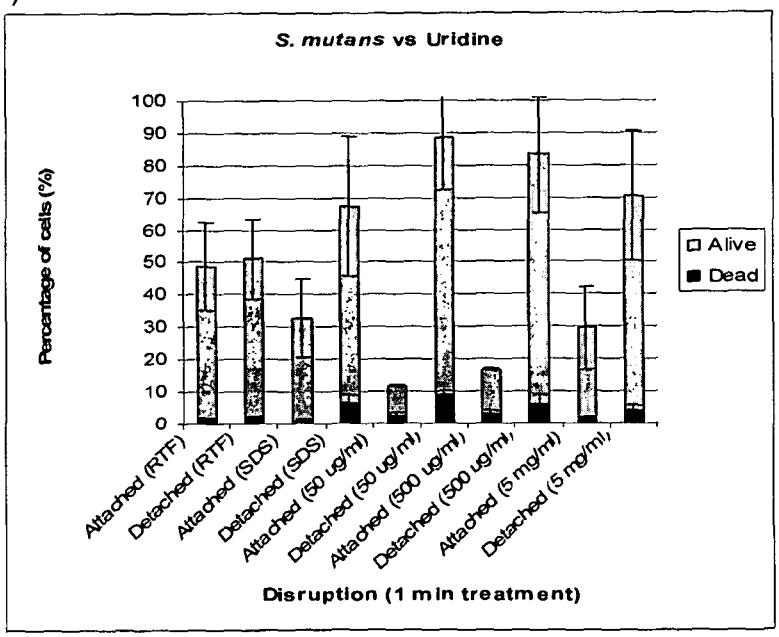
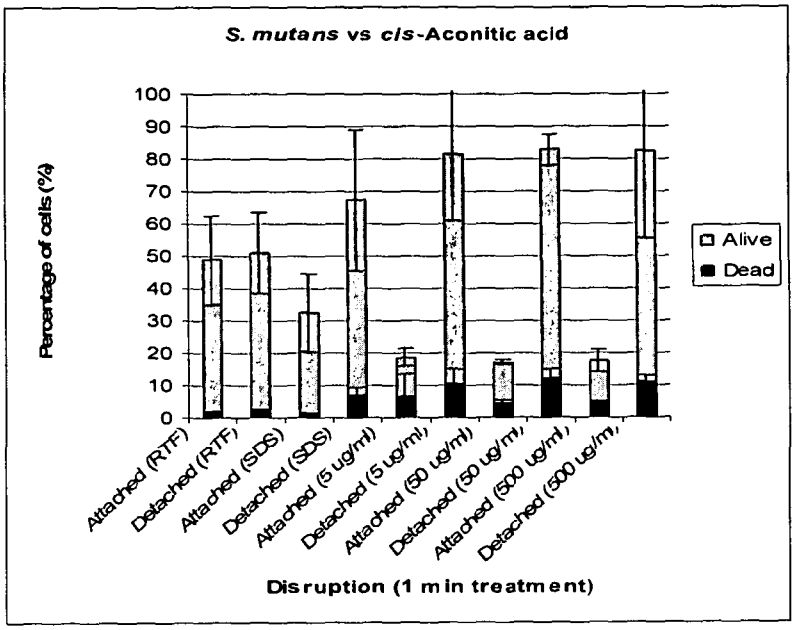


Figure 10 continued

i)



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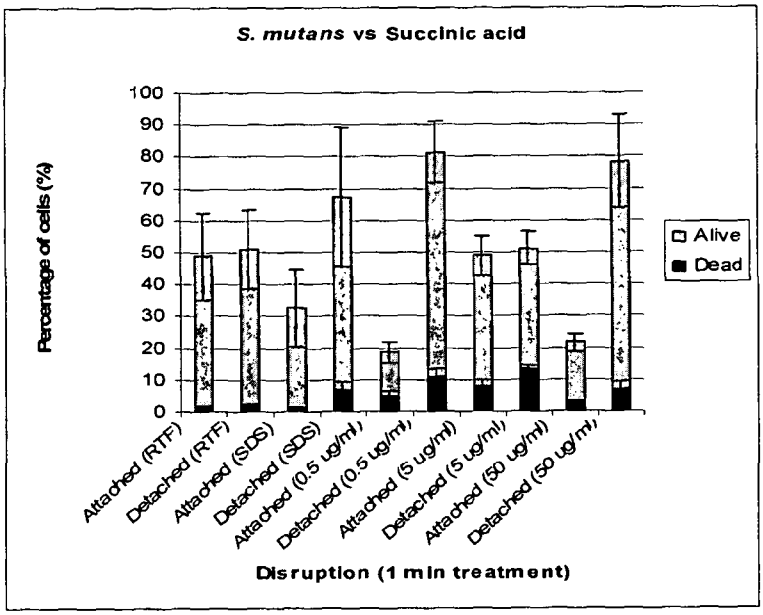
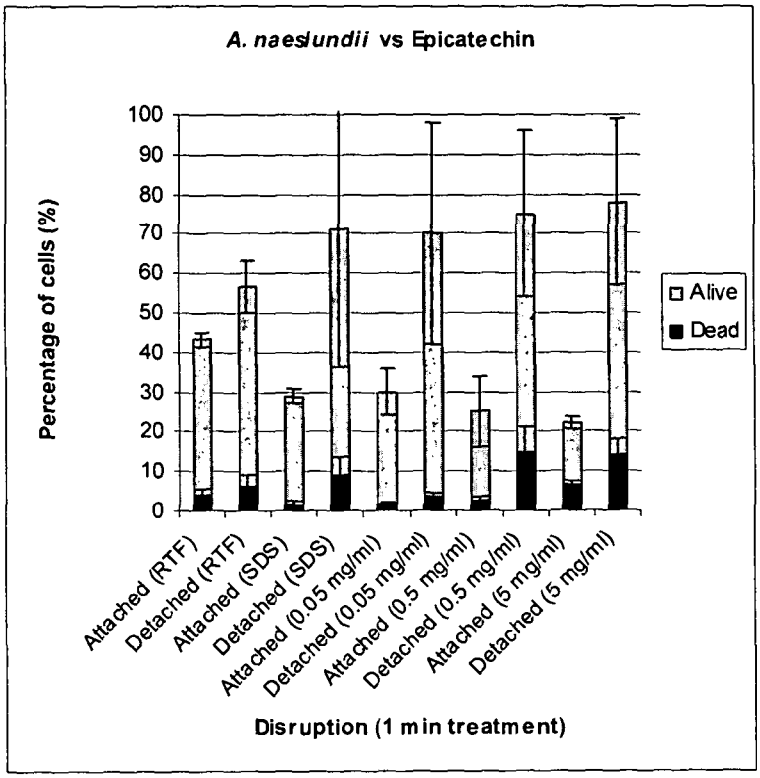
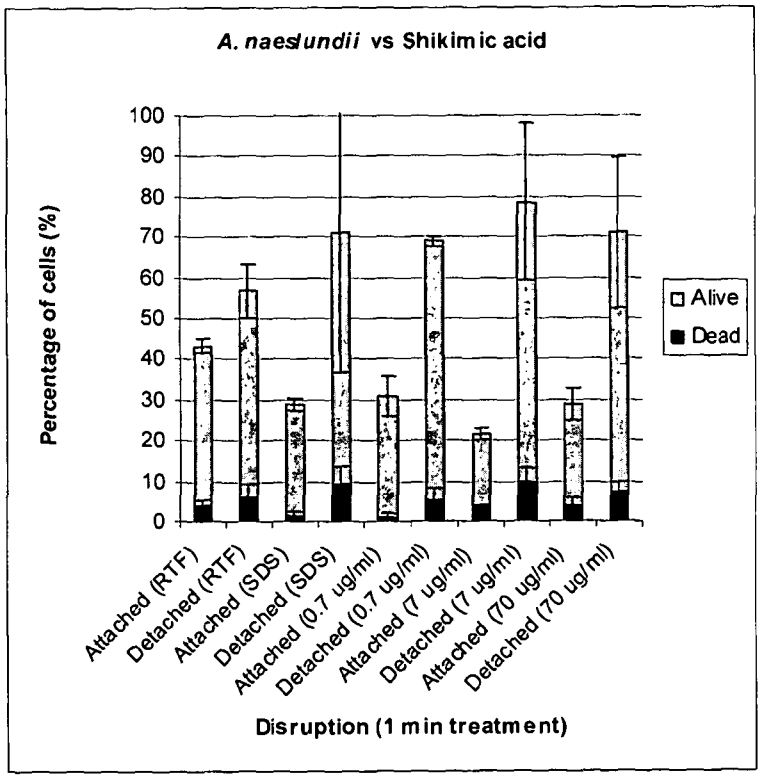


Figure 11

a)


A. naeslundii vs Epicatechin
Percentage of cells (%)
100
90
80
70
60
50
40
30
20
10
0
Alive
Dead
Attached (RTF)
Detached (RTF)
Attached (SDS)
Detached (SDS)
Attached (0.05 mg/ml)
Detached (0.05 mg/ml)
Attached (0.5 mg/ml)
Detached (0.5 mg/ml)
Attached (5 mg/ml)
Detached (5 mg/ml)
Disruption (1 min treatment)

b)


A. naeslundii vs Shikimic acid
Percentage of cells (%)
100
90
80
70
60
50
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30
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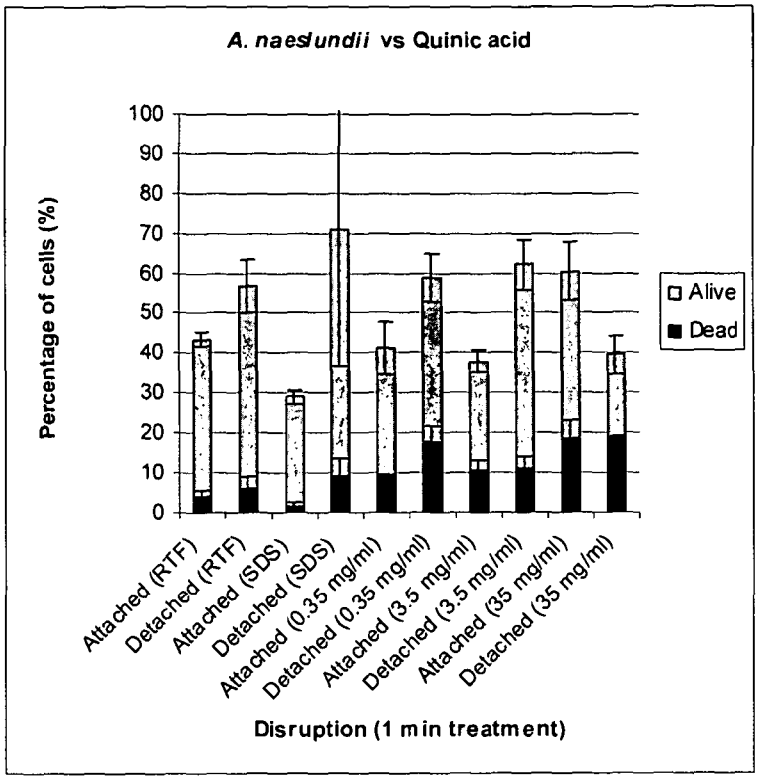
10
0
Alive
Dead
Attached (RTF)
Detached (RTF)
Attached (SDS)
Detached (SDS)
Attached (0.7 ug/ml)
Detached (0.7 ug/ml)
Attached (7 ug/ml)
Detached (7 ug/ml)
Attached (70 ug/ml)
Detached (70 ug/ml)
Disruption (1 min treatment)

Figure 11 continued

c)



d)

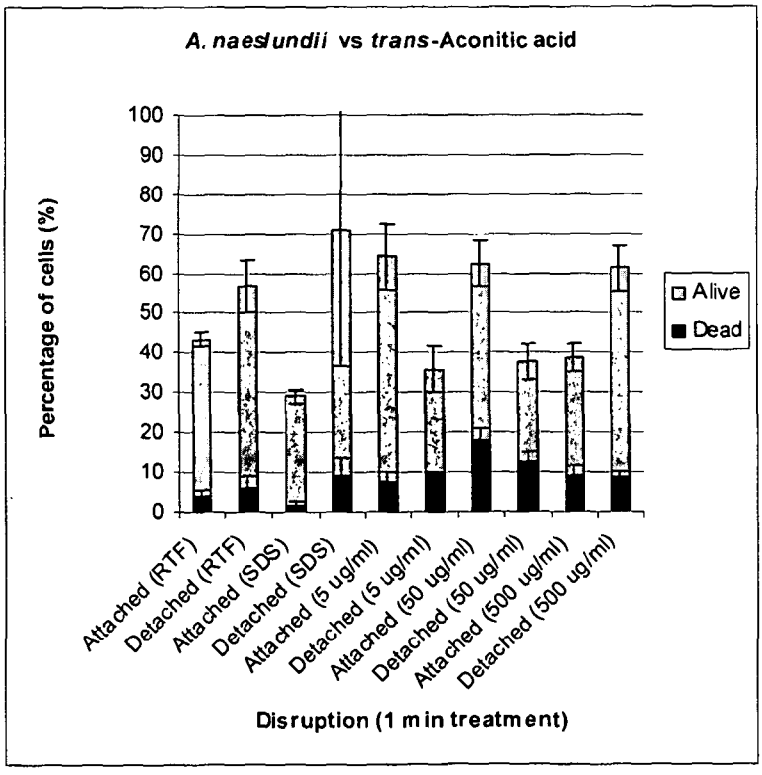
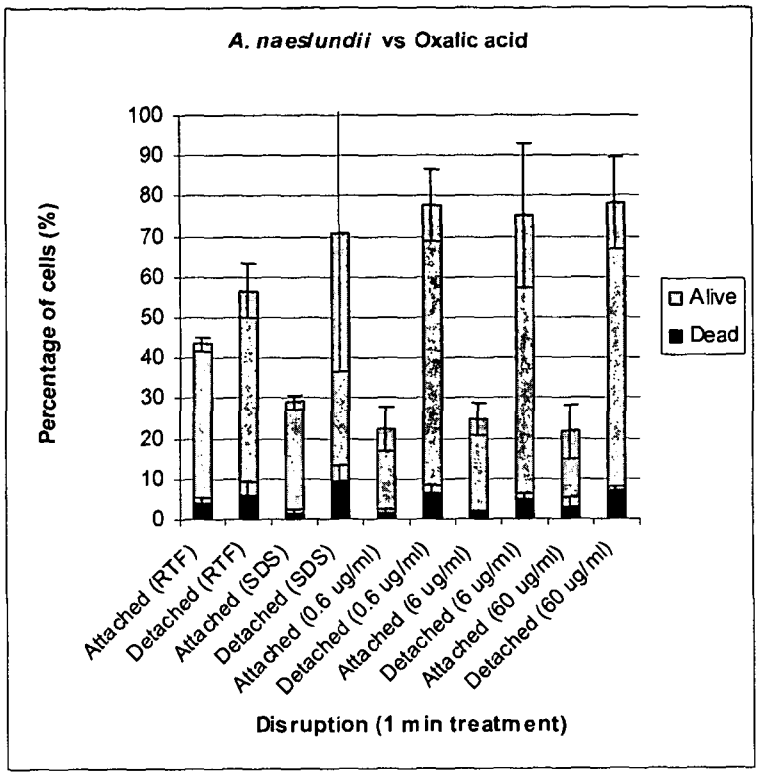


Figure 11 continued

e)



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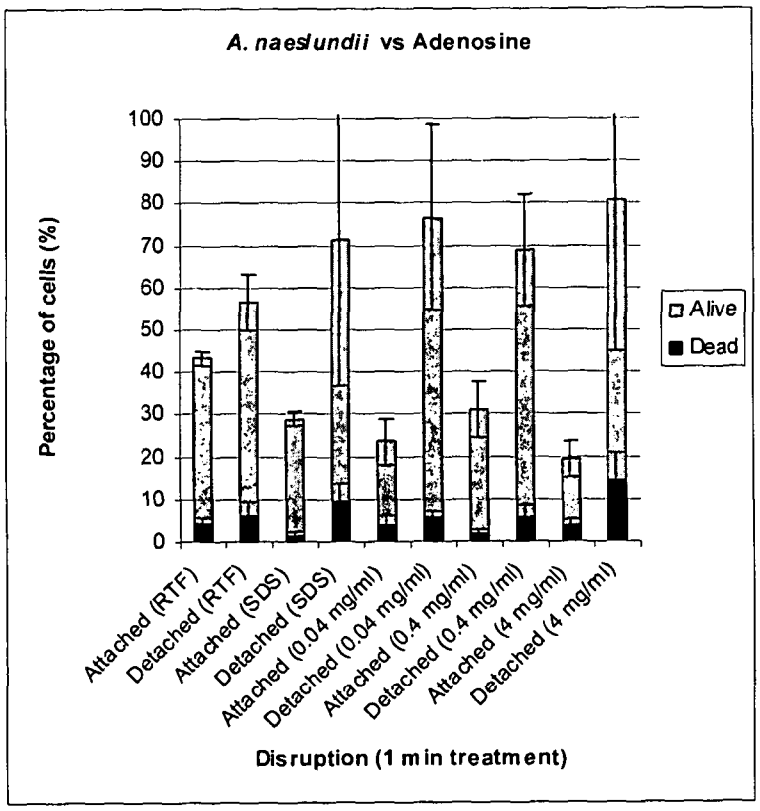
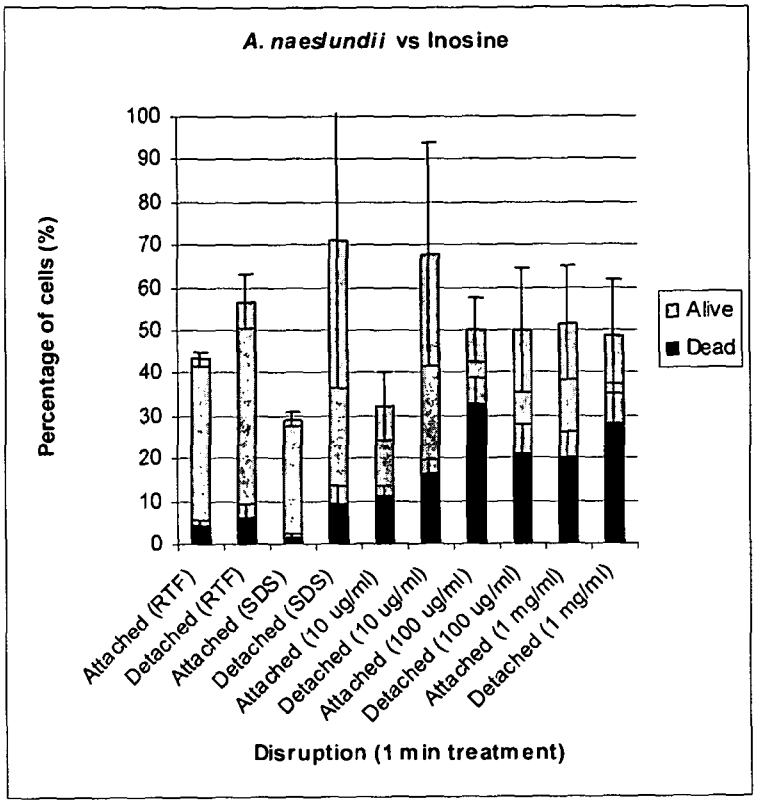


Figure 11 continued
g)



h)

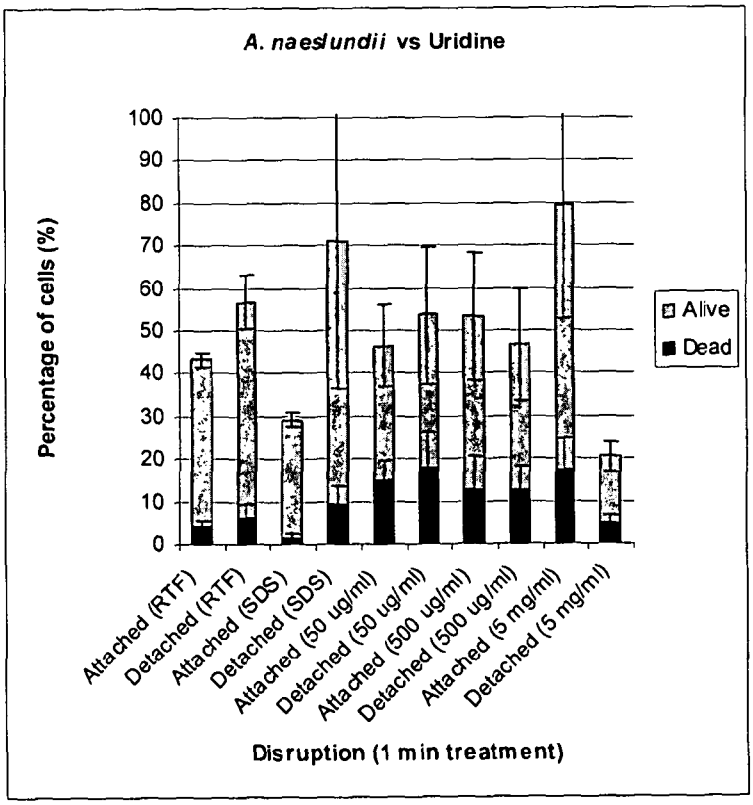
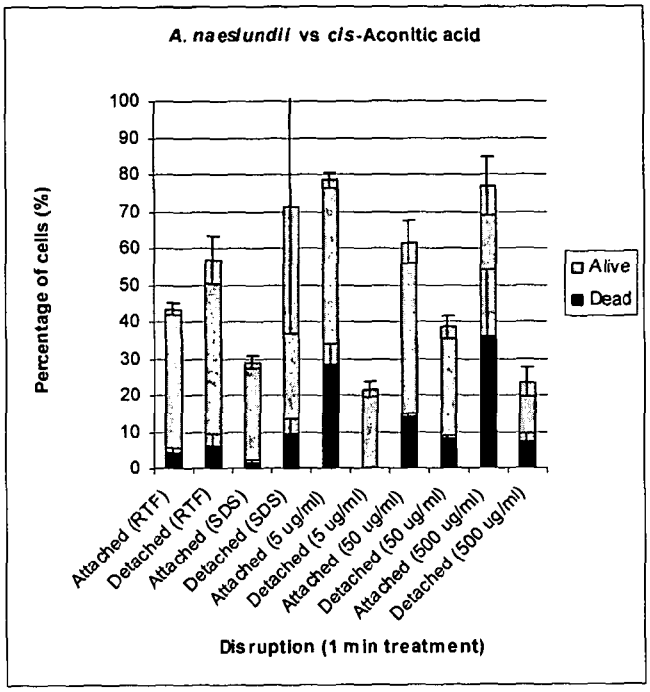
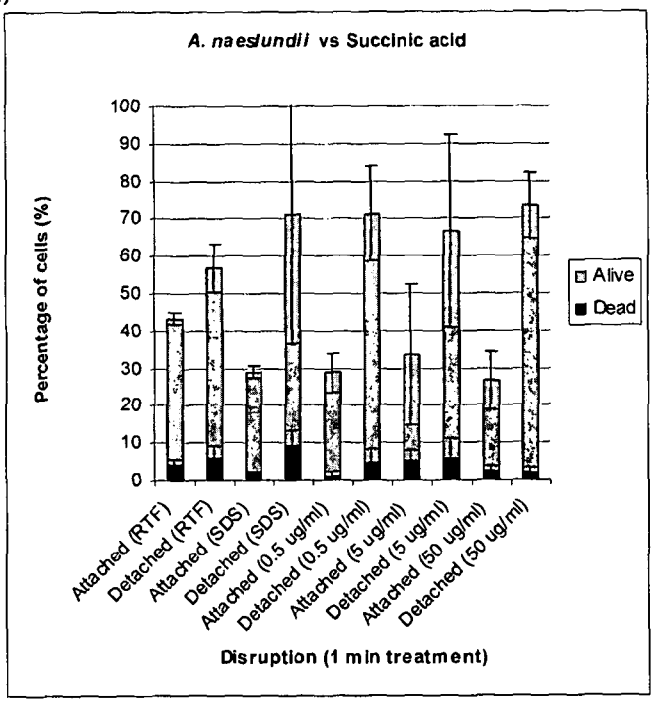


Figure 11 continued

i)



j)



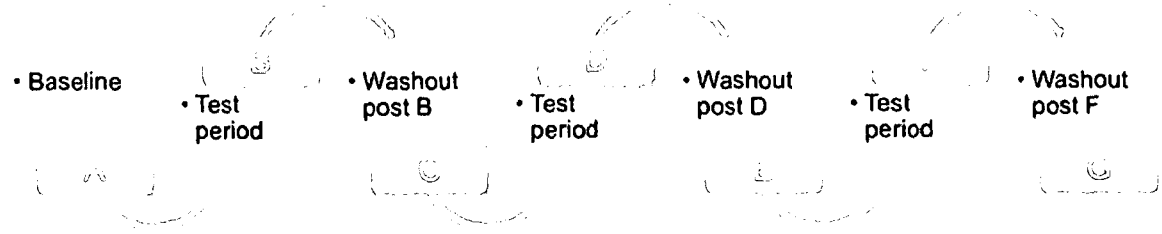


Fig. 12

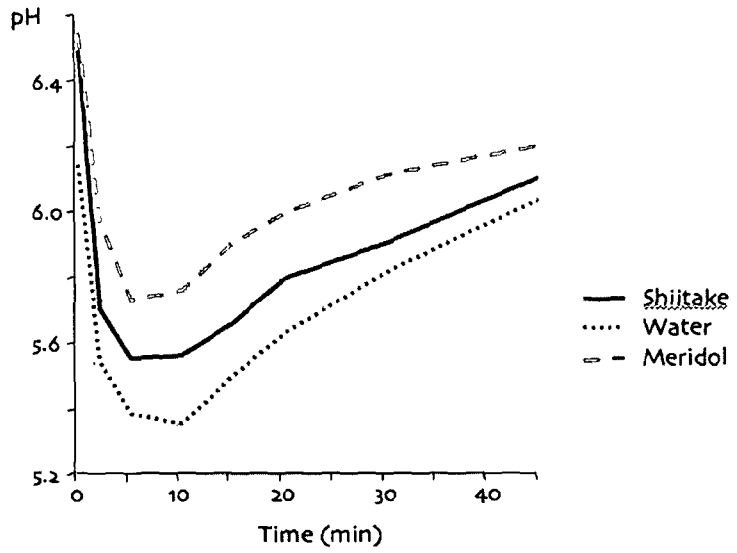


Fig. 13

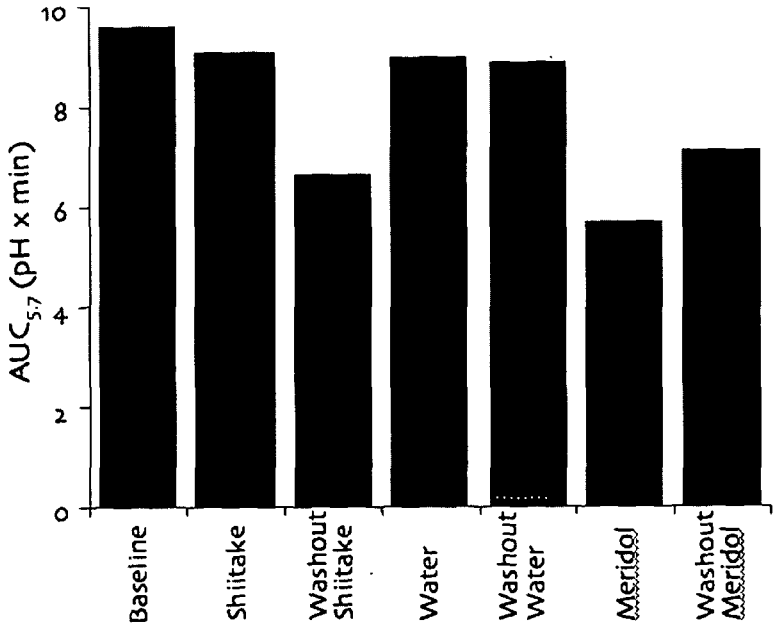


Fig. 14

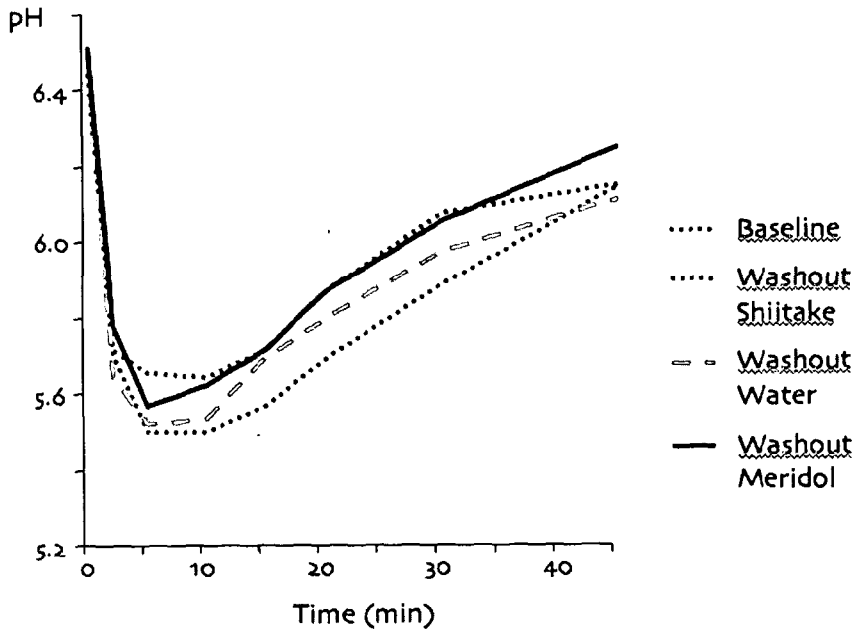


Fig. 15

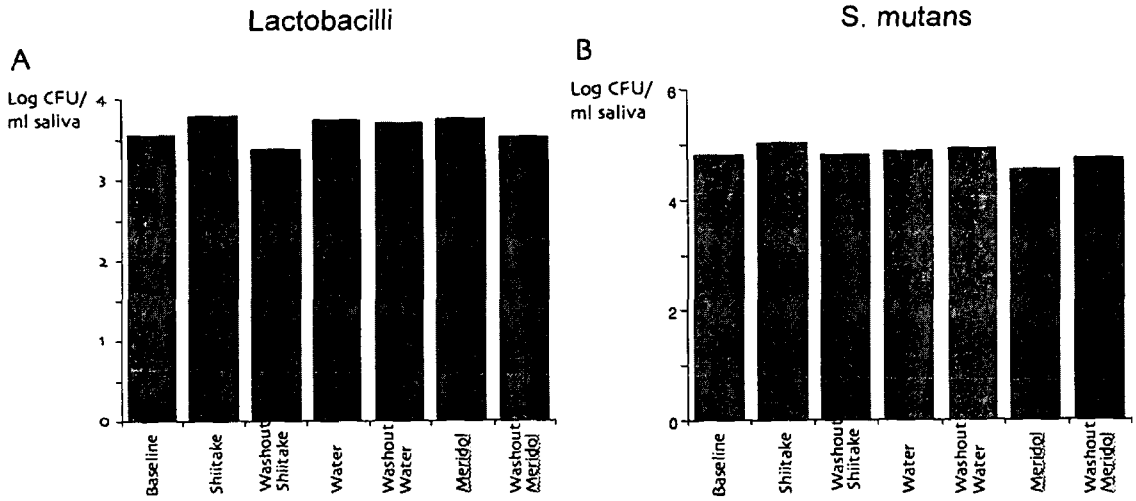


Figure 16A-B

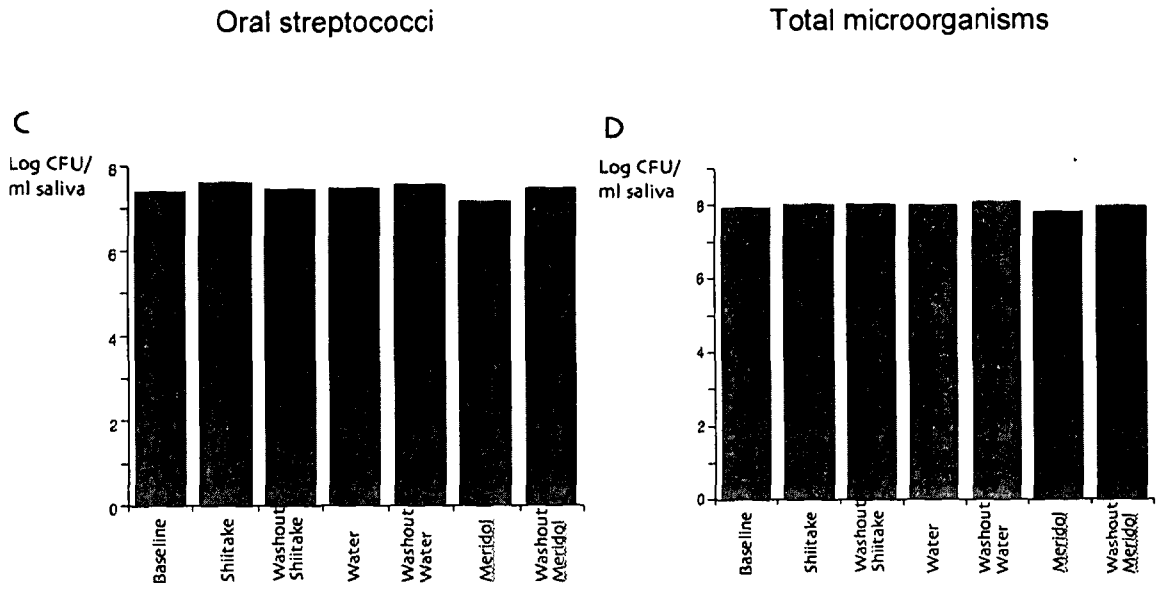


Fig. 16 continued - C-D

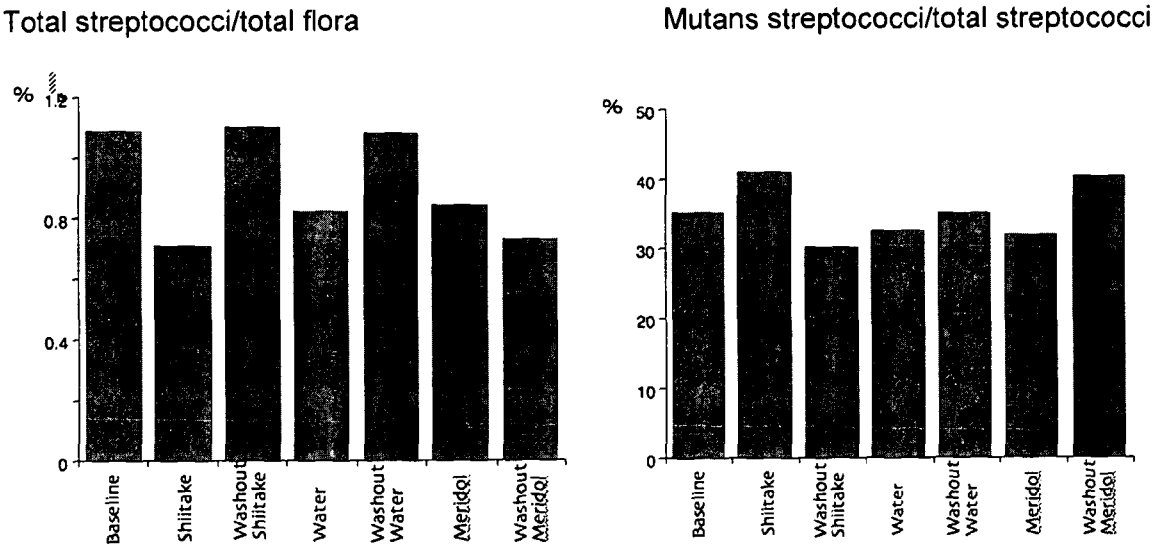


Fig. 17

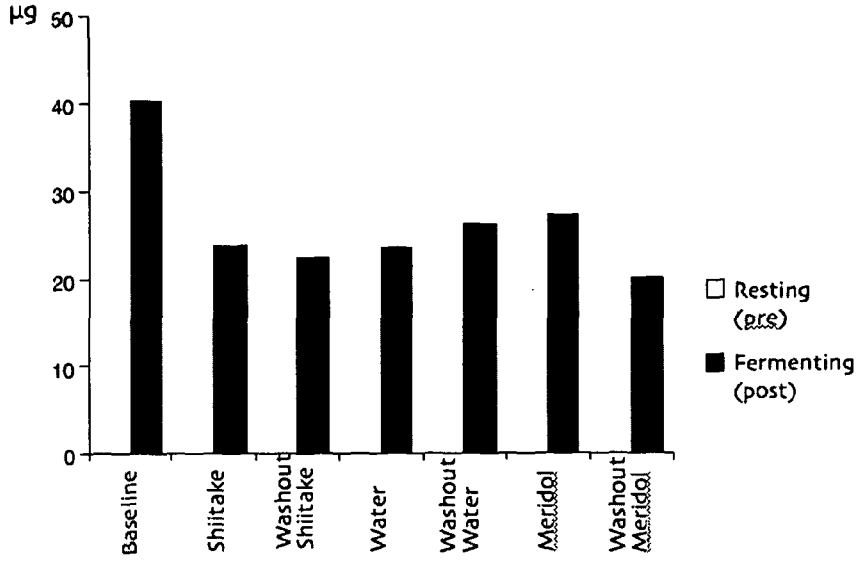

µg
50
40
30
20
10
0
Baseline
Shiitake
Washout Shiitake
Water
Washout Water
Meridol
Washout Meridol
Resting (pre)
Fermenting (post)

Fig. 18

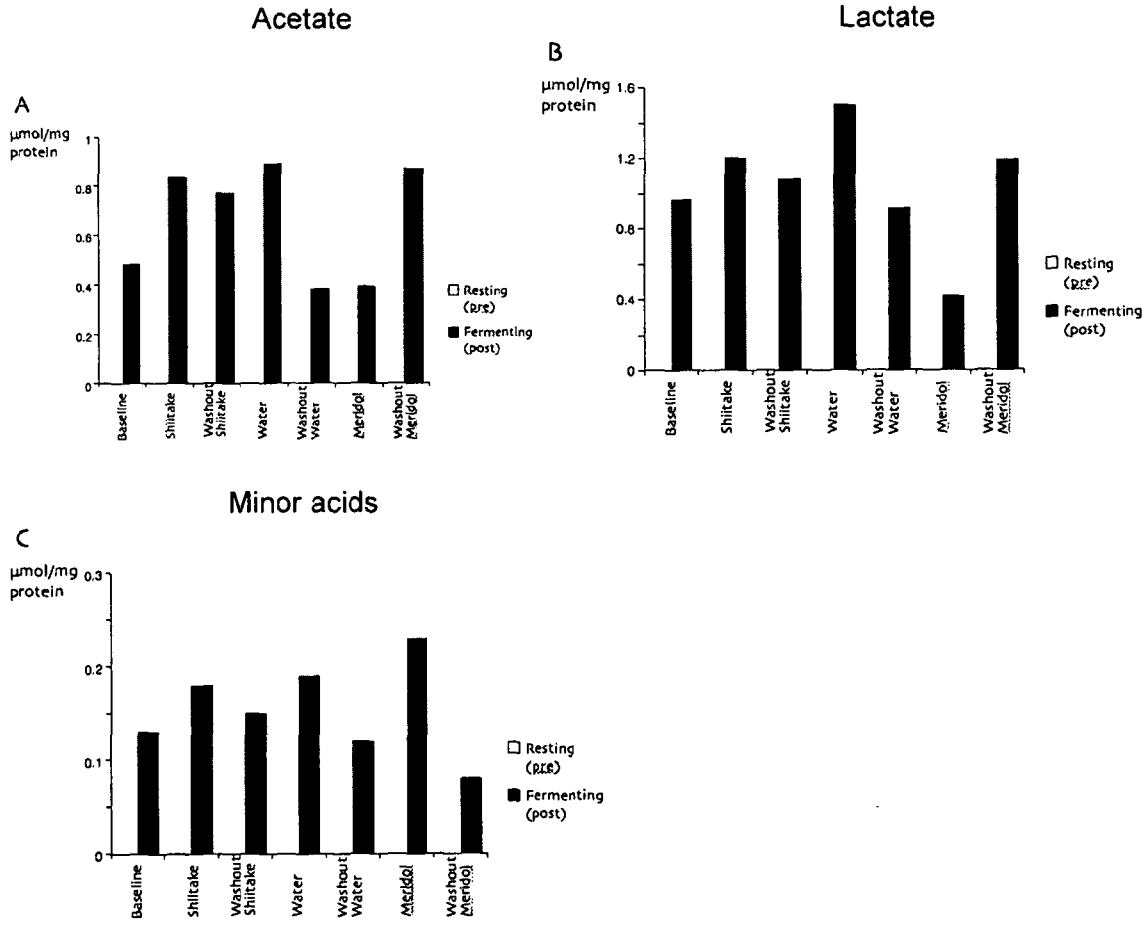

Acetate
A
µmol/mg protein
Lactate
B
µmol/mg protein
Minor acids
C
µmol/mg protein
Baseline
Shiitake
Washout Shiitake
Water
Washout Water
Meridol
Washout Meridol
Resting (pre)
Fermenting (post)

Fig. 19

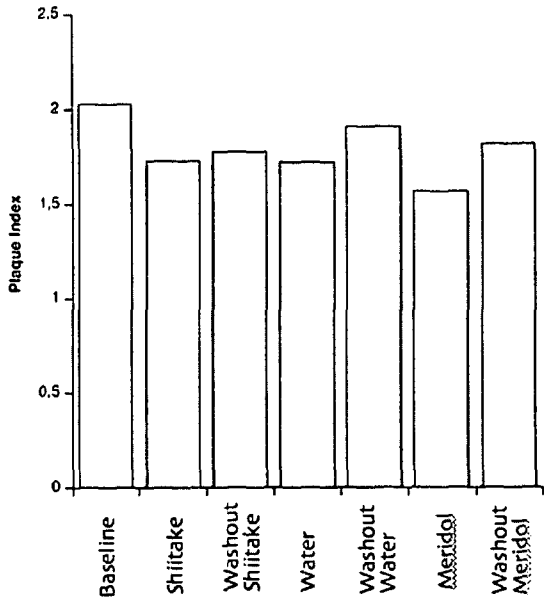

Plaque Index
2,5
2
1,5
1
0,5
0
Baseline
Shiitake
Washout Shiitake
Water
Washout Water
Meridol
Washout Meridol

Fig. 20

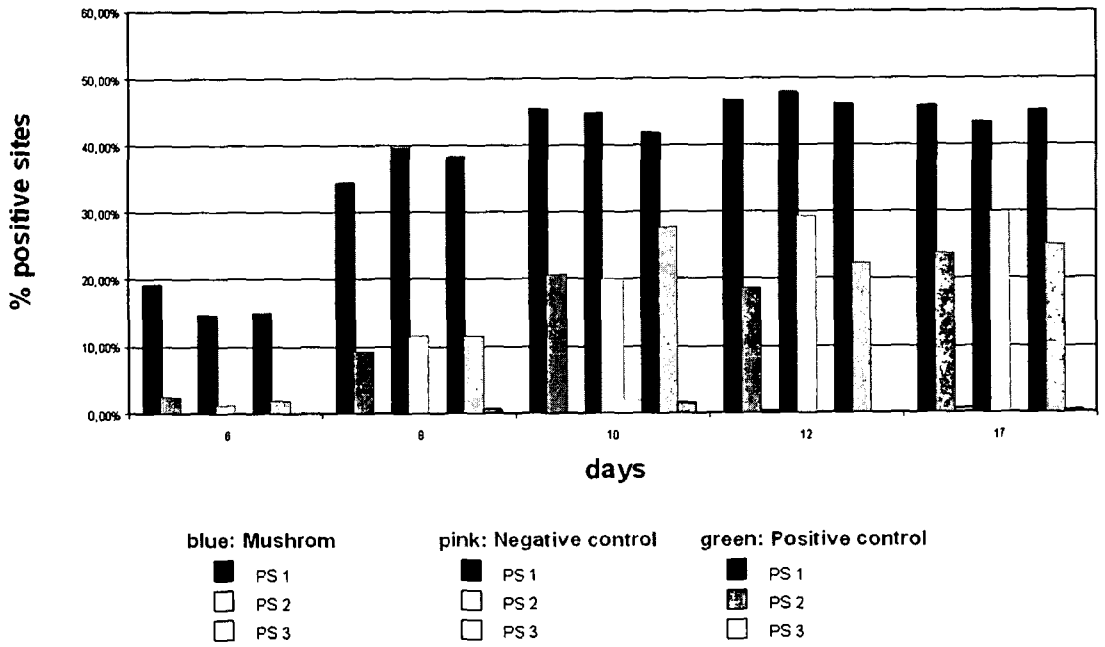

% positive sites
60,00%
50,00%
40,00%
30,00%
20,00%
10,00%
0,00%
6
8
10
12
17
days
blue: Mushrom
PS 1
PS 2
PS 3
pink: Negative control
PS 1
PS 2
PS 3
green: Positive control
PS 1
PS 2
PS 3

Fig. 21

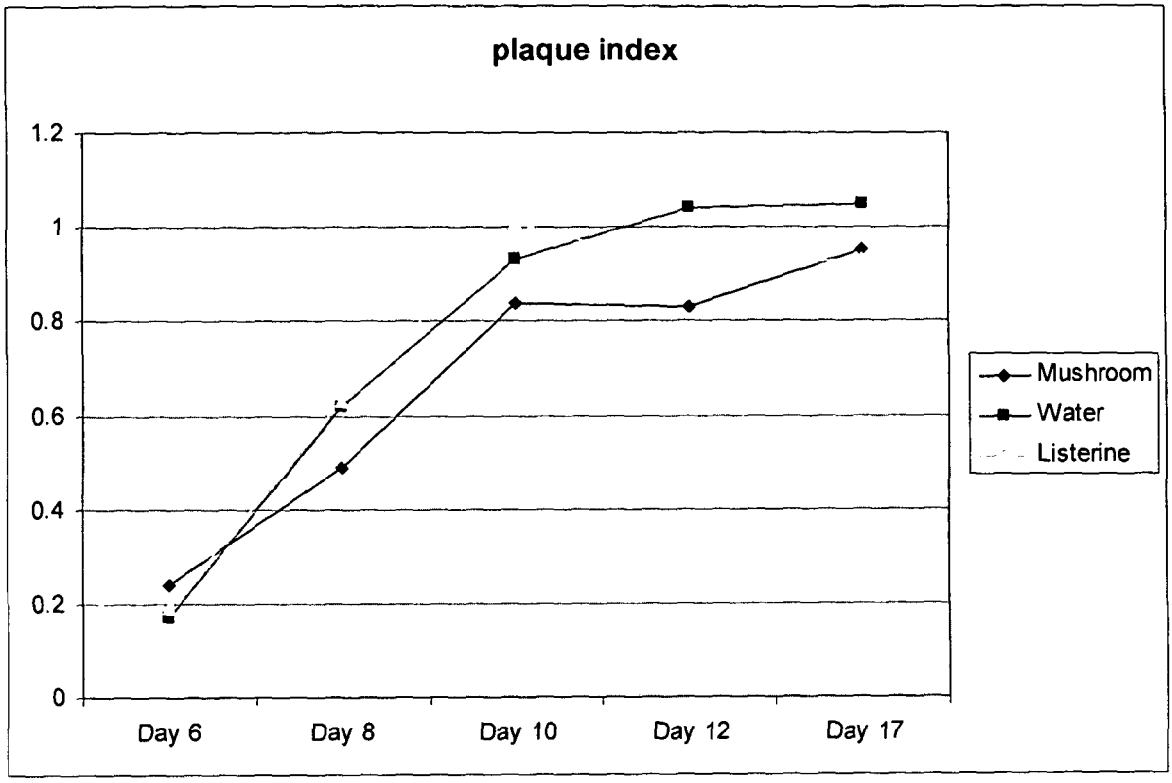

plaque index
1.2
1
0.8
0.6
0.4
0.2
0
Day 6
Day 8
Day 10
Day 12
Day 17
Mushroom
Water
Listerine

Fig. 22

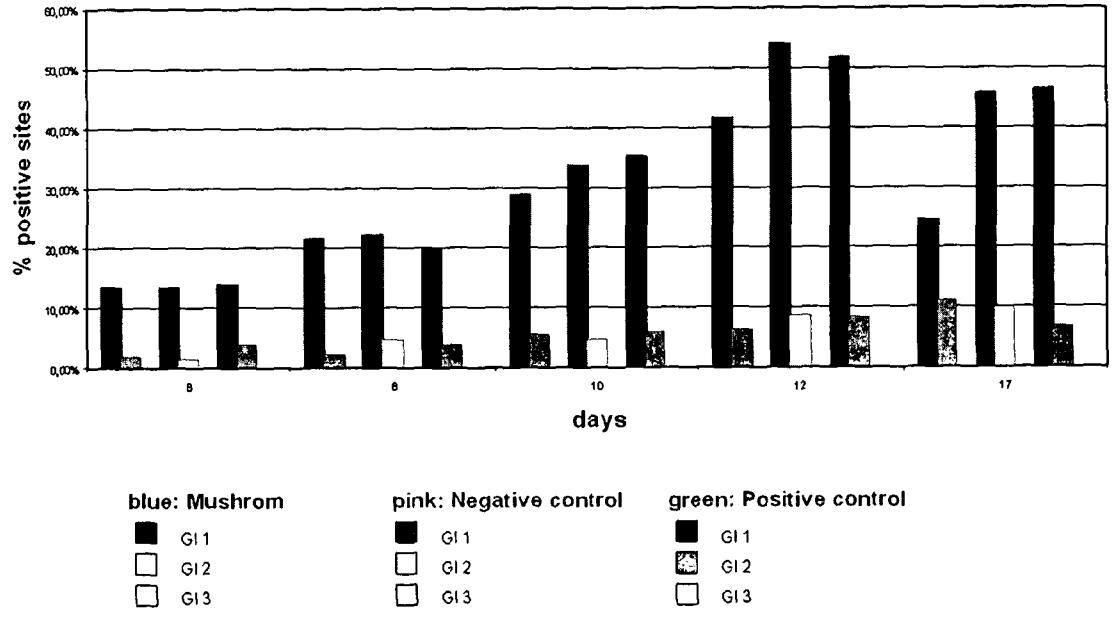

% positive sites
days
blue: Mushrom
pink: Negative control
green: Positive control
GI 1
GI 2
GI 3

Fig. 23

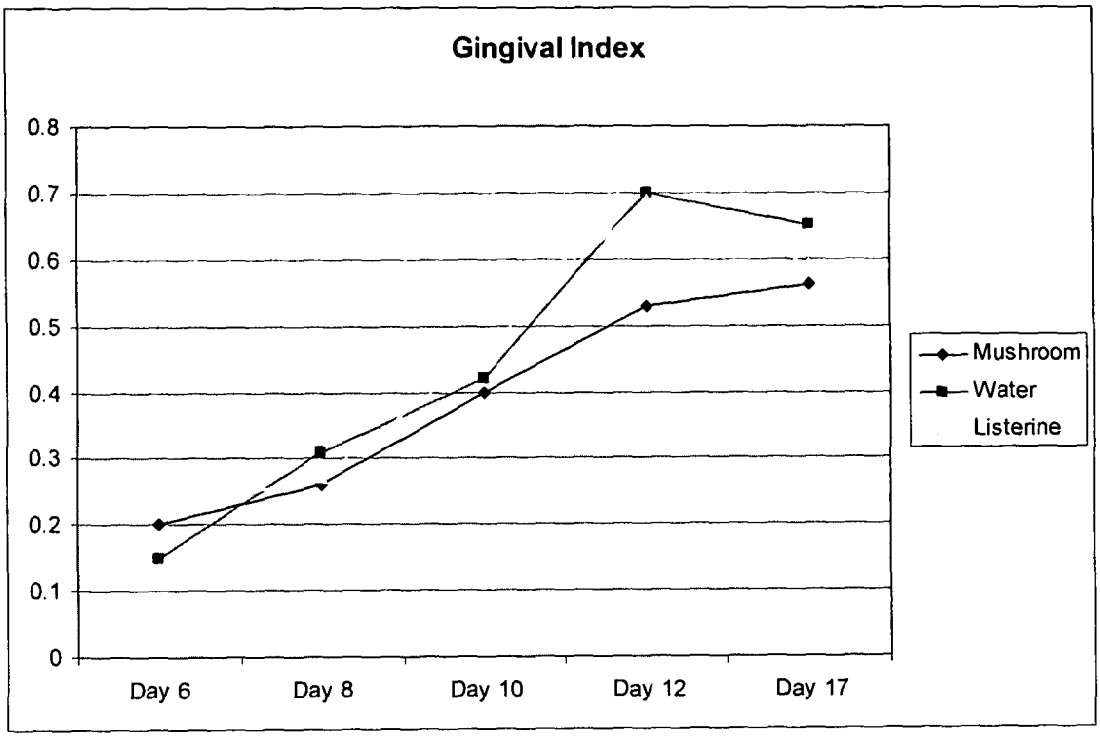

Gingival Index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 6
Day 8
Day 10
Day 12
Day 17
Mushroom
Water
Listerine

Fig. 24

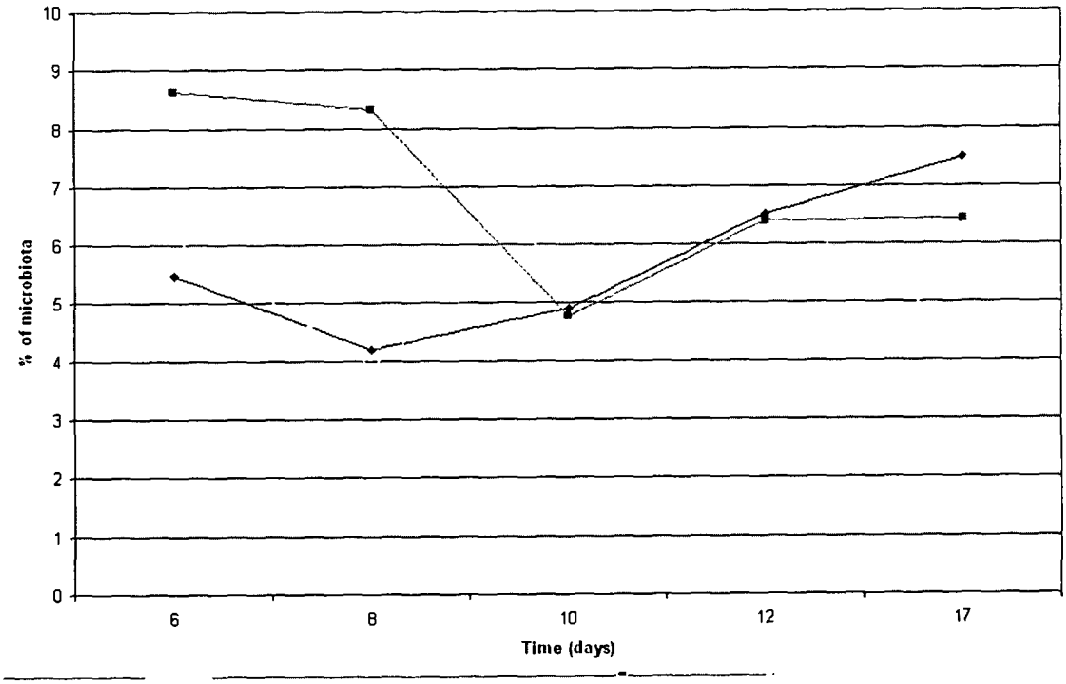

% of microbiota
10
9
8
7
6
5
4
3
2
1
0
6
8
10
12
17
Time (days)

Fig. 25

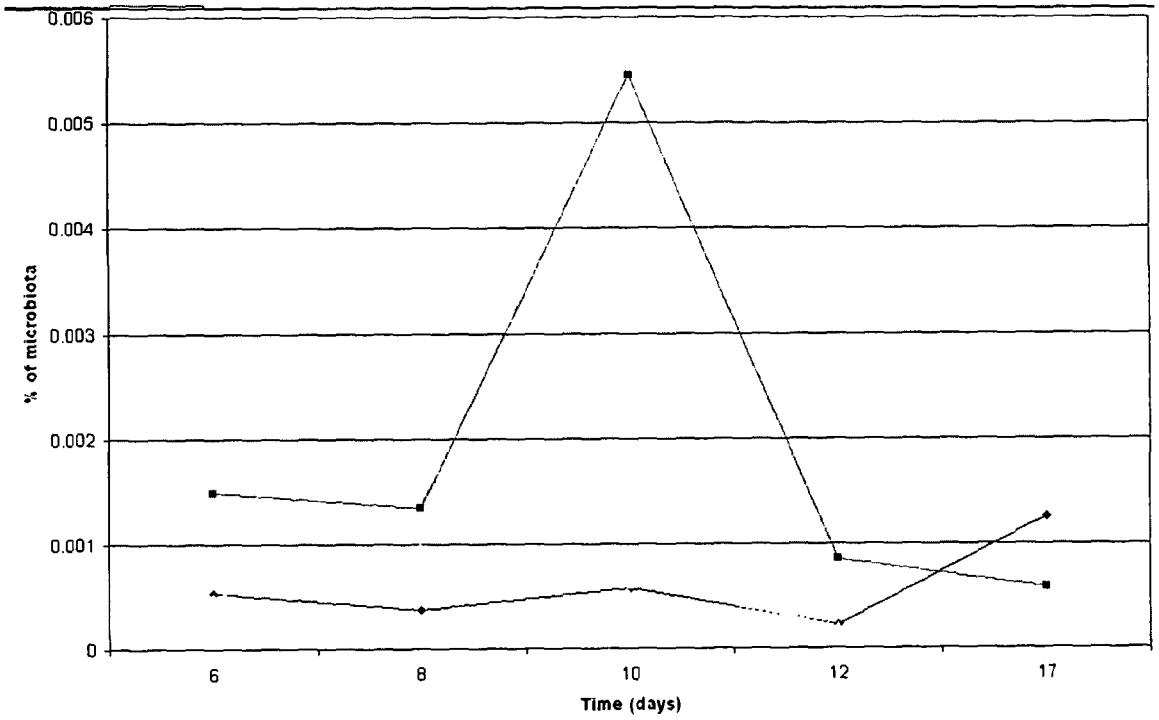

0.006
0.005
0.004
0.003
0.002
0.001
0
% of microbiota
6
8
10
12
17
Time (days)

Fig. 26.

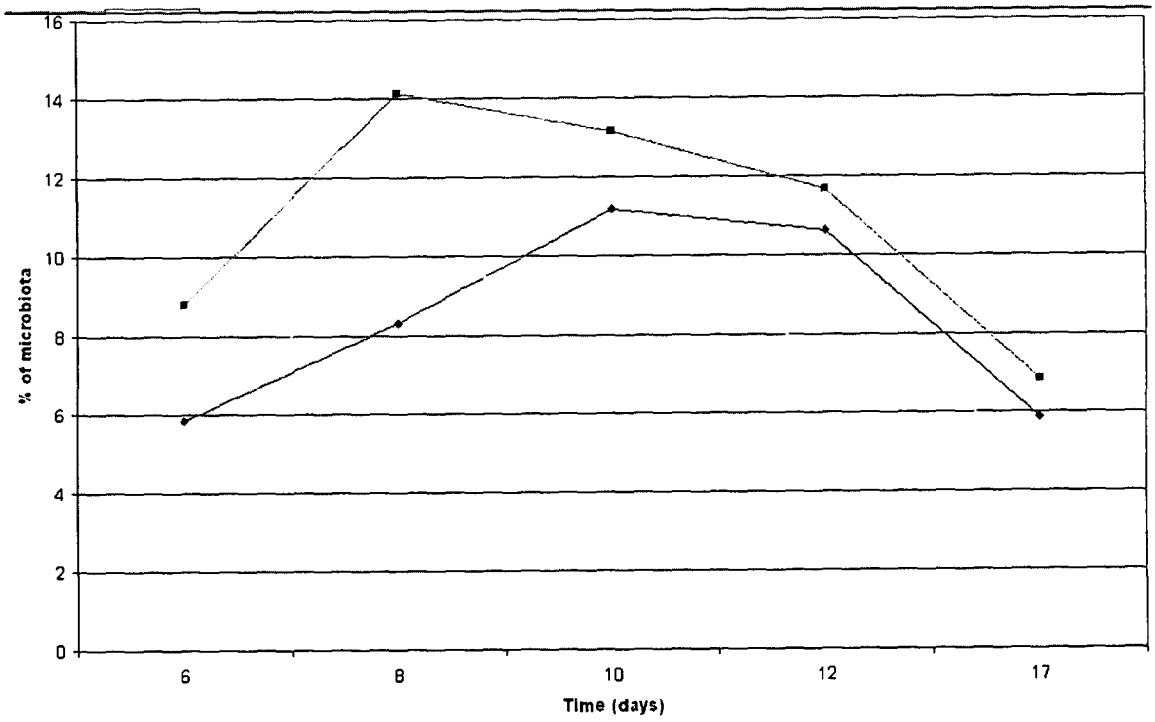

16
14
12
10
8
6
4
2
0
% of microbiota
6
8
10
12
17
Time (days)

Fig. 27

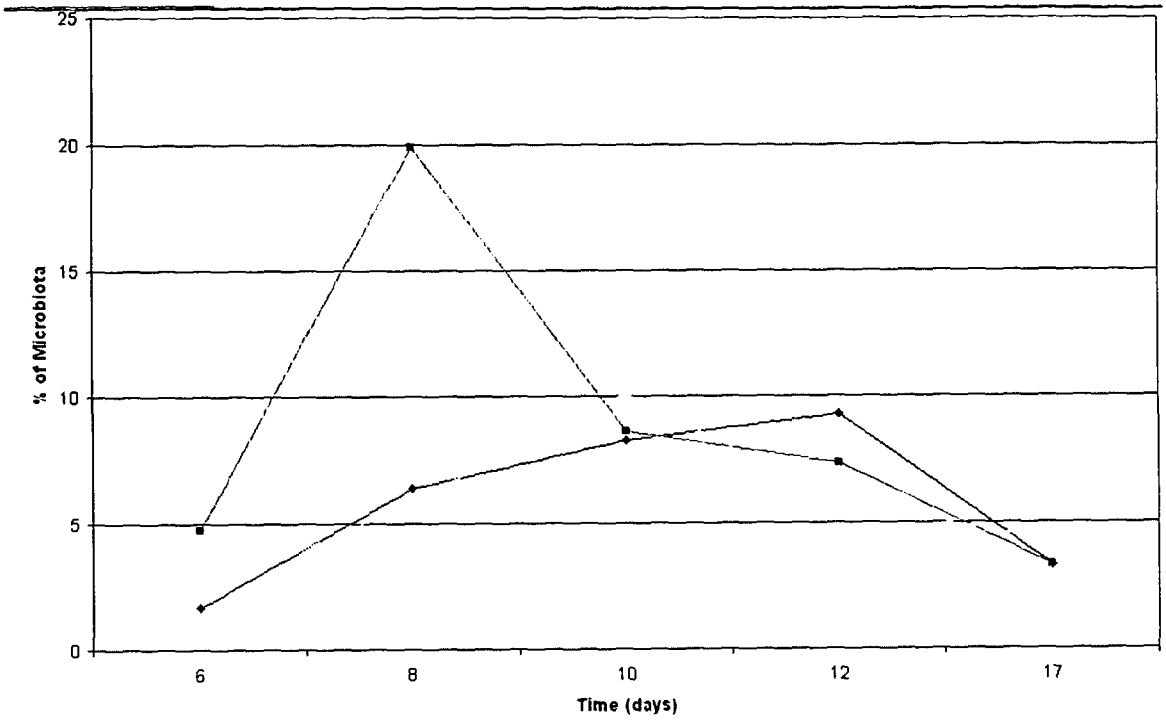

25
20
15
10
5
0
% of Microbiota
6
8
10
12
17
Time (days)

Fig. 28

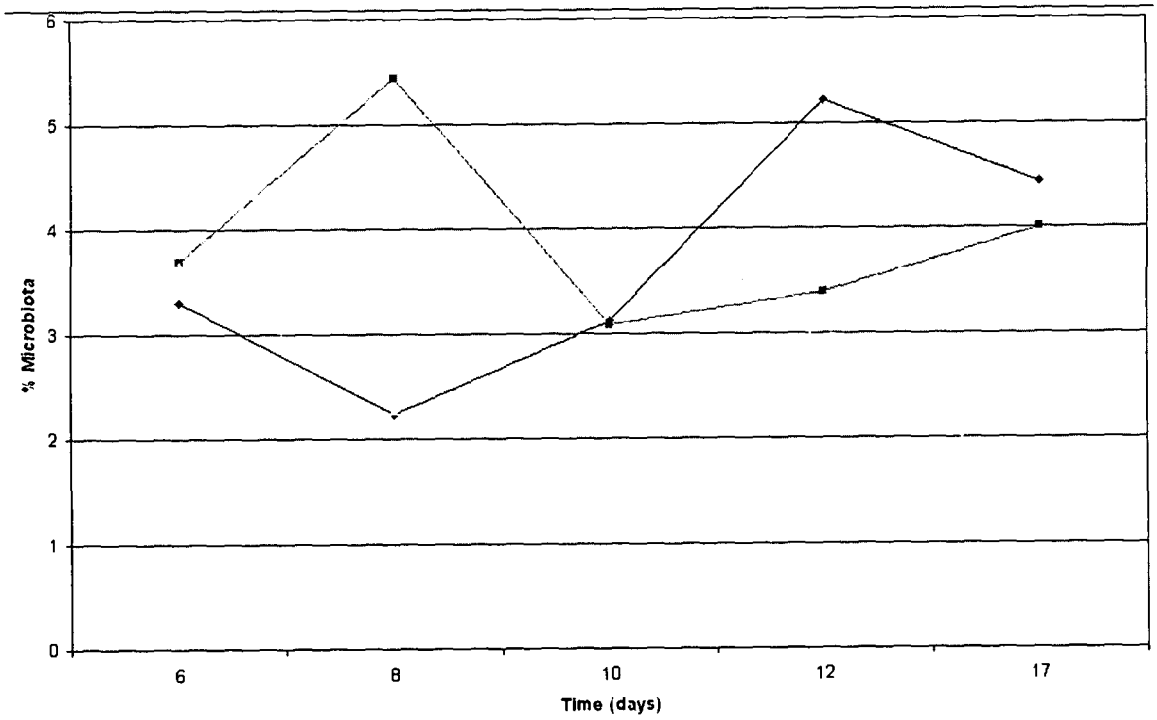

6
5
4
3
2
1
0
% Microbiota
6
8
10
12
17
Time (days)

Fig. 29

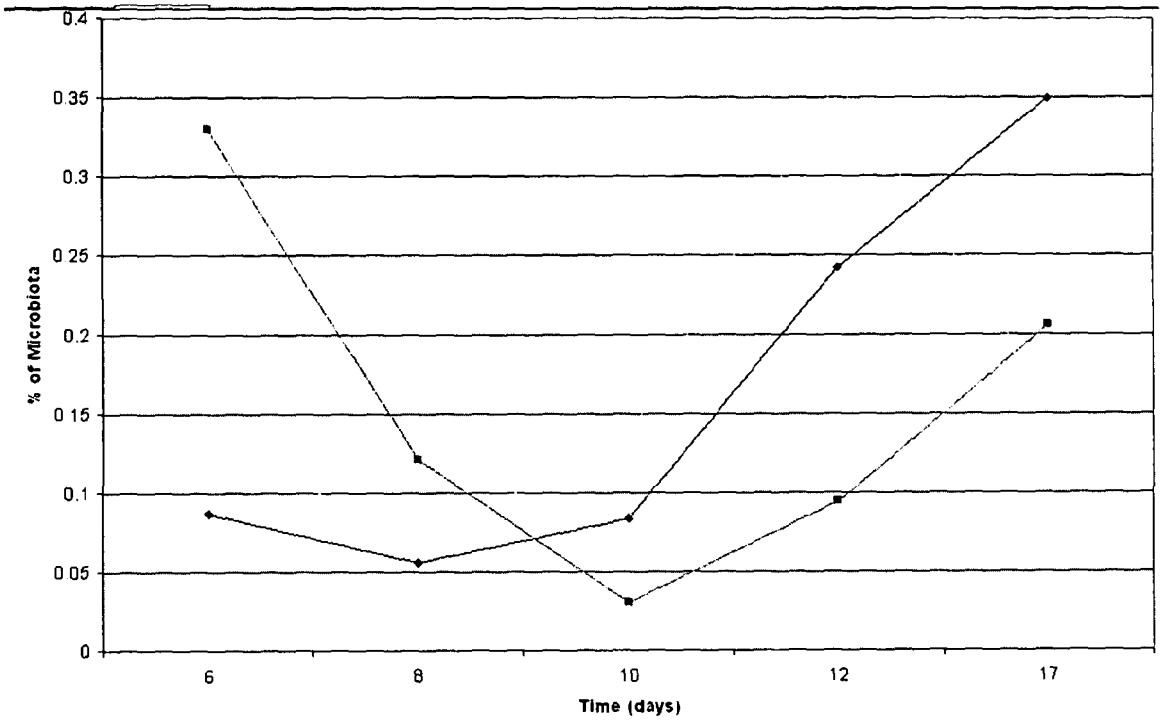

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
% of Microbiota
6
8
10
12
17
Time (days)

Fig. 30

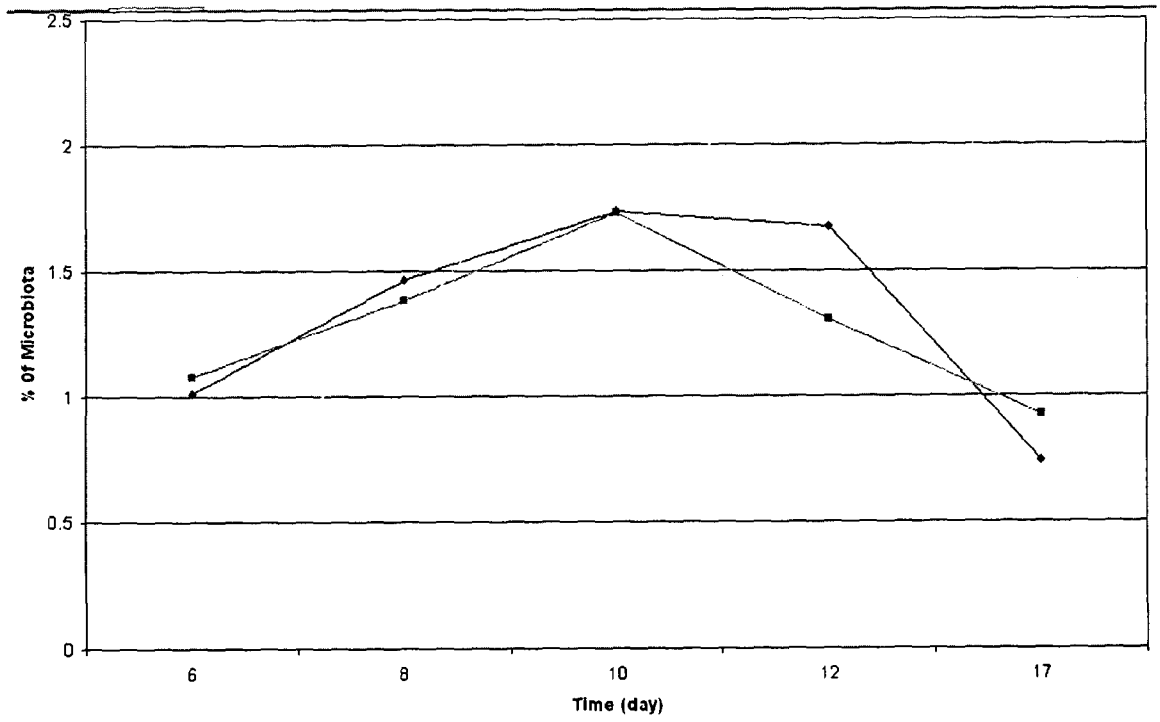

2.5
2
1.5
1
0.5
0
% of Microbiota
6
8
10
12
17
Time (day)

Fig. 31

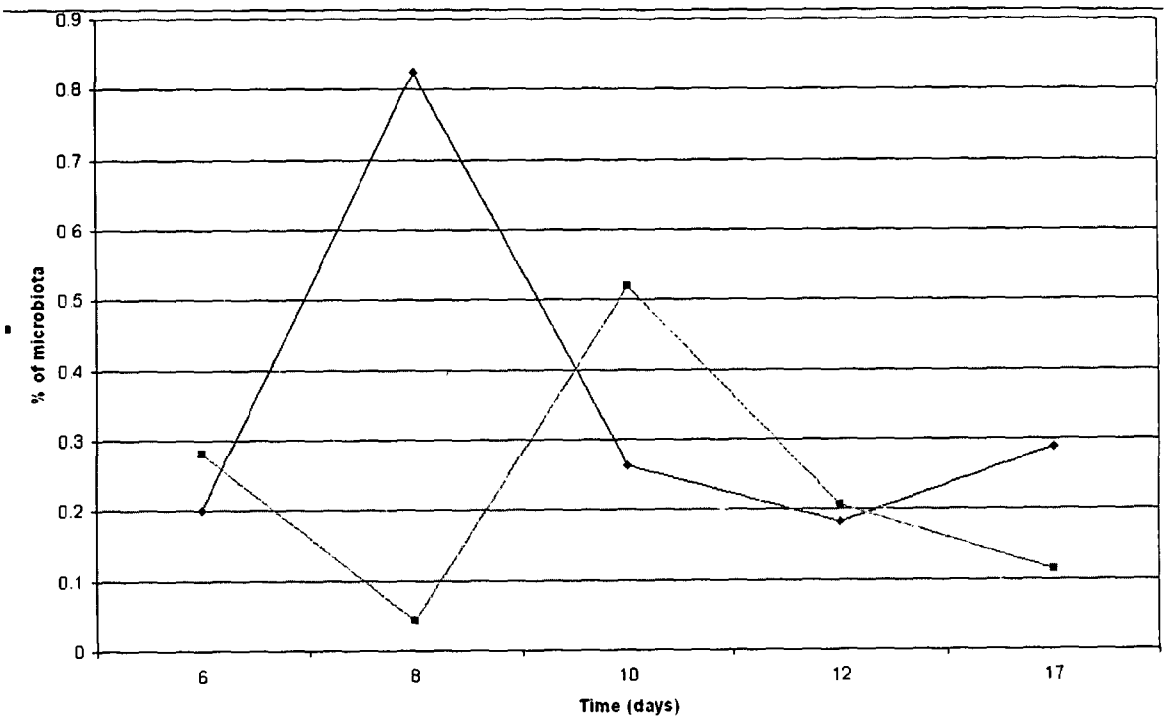

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% of microbiota
6
8
10
12
17
Time (days)

Fig. 32

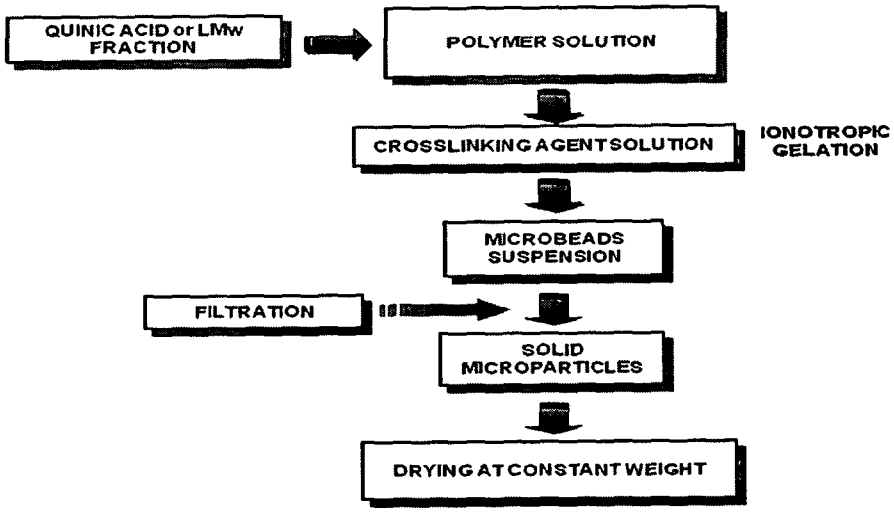

QUINIC ACID or LMw FRACTION
POLYMER SOLUTION
CROSSLINKING AGENT SOLUTION
IONOTROPIC GELATION
MICROBEADS SUSPENSION
FILTRATION
SOLID MICROPARTICLES
DRYING AT CONSTANT WEIGHT

Fig 33

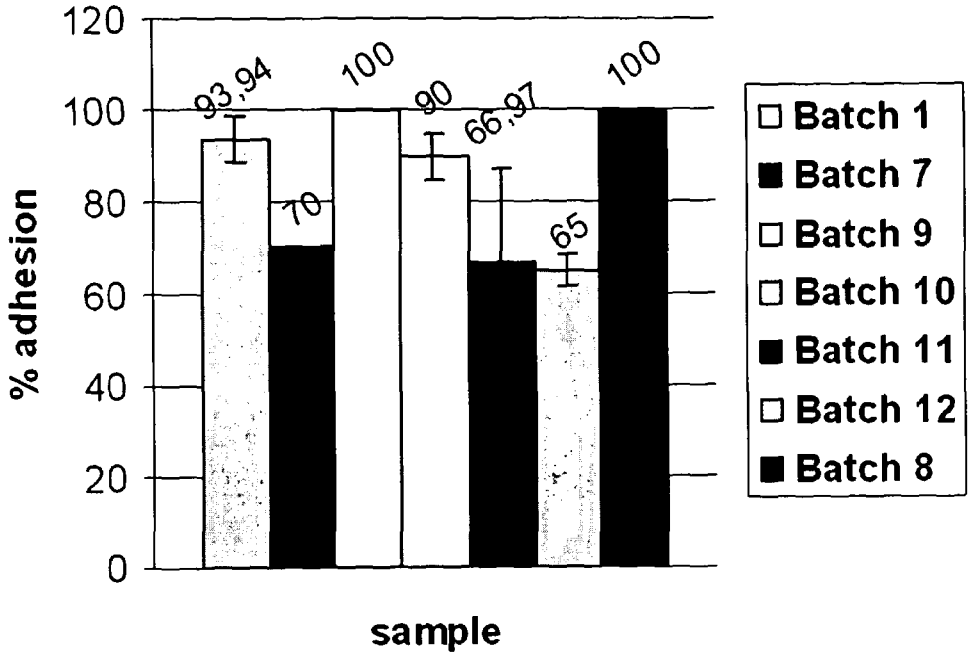

120
100
80
60
40
20
0
% adhesion
93,94
70
100
90
66,97
65
100
sample
Batch 1
Batch 7
Batch 9
Batch 10
Batch 11
Batch 12
Batch 8

Fig. 34

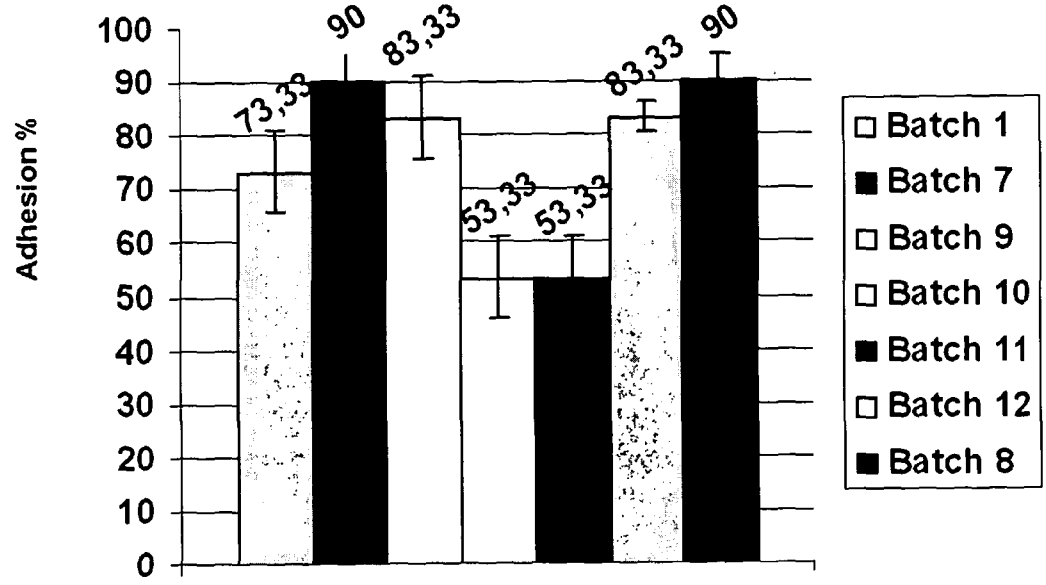

100
90
80
70
60
50
40
30
20
10
0
Adhesion %
73,33
90
83,33
53,33
53,33
83,33
90
Batch 1
Batch 7
Batch 9
Batch 10
Batch 11
Batch 12
Batch 8

Fig. 35

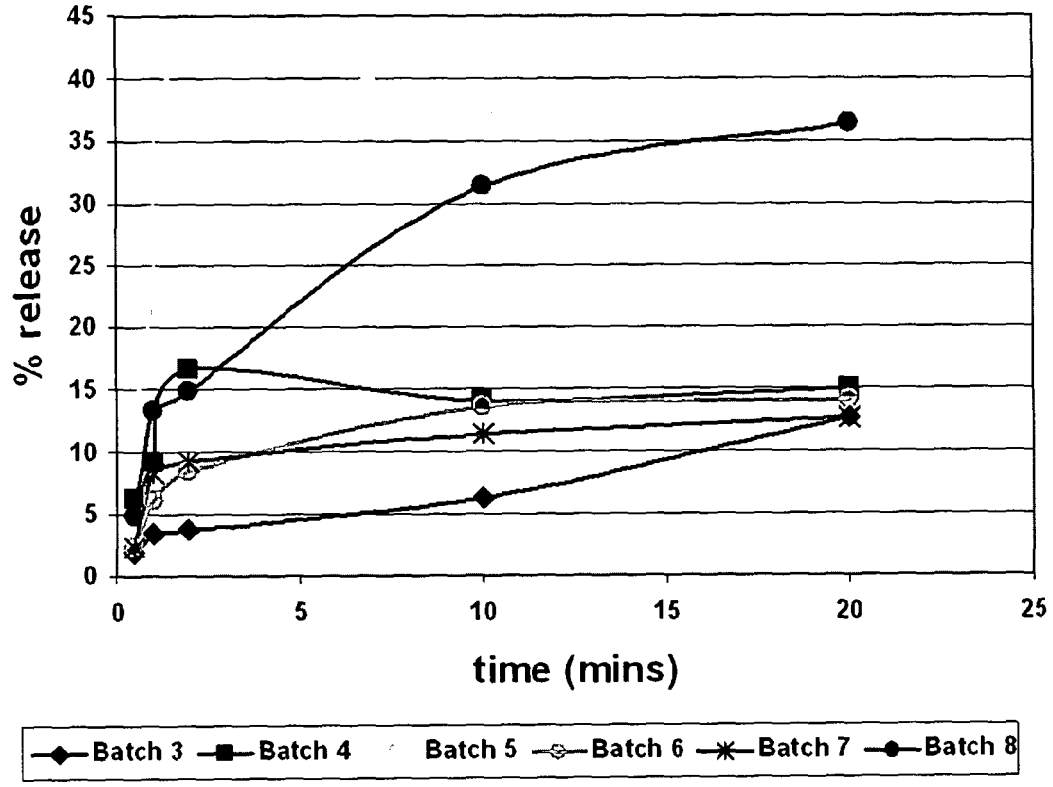

45
40
35
30
25
20
15
10
5
0
% release
0
5
10
15
20
25
time (mins)
Batch 3
Batch 4
Batch 5
Batch 6
Batch 7
Batch 8

Fig. 36

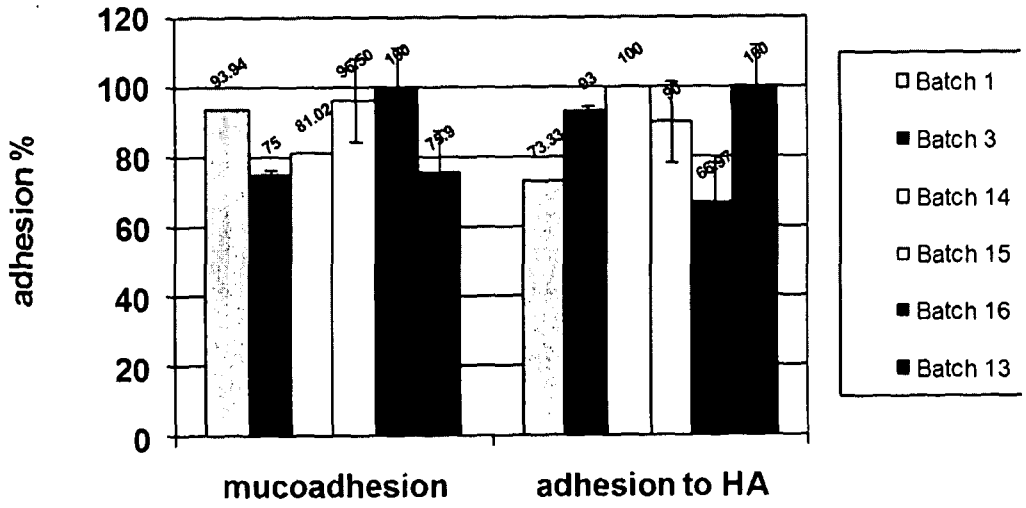

120
100
80
60
40
20
0
adhesion %
93.94
75
81.02
73.33
93
100
mucoadhesion
adhesion to HA
Batch 1
Batch 3
Batch 14
Batch 15
Batch 16
Batch 13

Fig. 37

Fig. 38.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/000969

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/28 A23L1/30 A61K8/97 A61Q11/00 A61K36/07
A61K36/28 A61K36/73
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23L A61K A61Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , WPI Data, FSTA, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHOUJI N ET AL: "Anti cari es Effect of a Component from Shi i take (an Edi bl e Mushroom) ", CARI ES RESEARCH , S. KARGER AG, BASEL, CH , vol . 34, no. 1, 1 January 2000 (2000-01-01) , pages 94-98, XP008142728, ISSN : 0008-6568, D0I : 10. 1159/000016559 ci ted i n the appl i cati on the whol e document -----	1-4, 15-17 , 19-27 ,29

-/- ·

X Further documents are listed in the continuation of Box C.

X See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu¬ments, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 2011	13/12/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fi scher, J

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/000969

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 198848 Thomson Scientific, London, GB; AN 1988-341490 XP002658826, & JP 63 253019 A (SUNSTAR KK) 20 October 1988 (1988-10-20) cited in the application abstract	1-3, 15-17, 19,21-27
X	DATABASE WPI Week 201025 Thomson Scientific, London, GB; AN 2010-E05617 XP002658827, & JP 2010 077028 A (IWATE KEN) 8 April 2010 (2010-04-08) cited in the application abstract	1-4, 15-17, 19-27,29
X	HIRASAWA ET AL.: "Three kinds of antibacterial substances from Lentinus edodes (Berk.) Sing. (Shiitake, an edible mushroom)", INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS, vol. 11, 1999, pages 151-157, XP002658828, the whole document	1-3, 15-17, 19,20, 23-27
X	DATABASE BIOSIS [Online] BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 2000 (2000-01), SHOUJI N ET AL: "Anti caries effect of a component from shiitake (an Edible Mushroom)", XP002658829, Database accession no. PREV200000090057 abstract & CARIES RESEARCH, vol. 34, no. 1, January 2000 (2000-01), pages 94-98, ISSN: 0008-6568	1-3, 15-17, 19-27
X	DATABASE FSTA [Online] INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANkFURT-MAIN, DE; YANO A ET AL: "The inhibitory effects of mushroom extracts on sucrose-dependent oral biofilm formation.", XP002658830, Database accession no. FS-2010-05-Jq2279 abstract & APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 86, no. 2, 2010, page 615,	1-3, 15-17, 19-27

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/000969

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE [Online] January 2000 (2000-01) , SHOUJI N ET AL: "Anti caries effect of a component from shiitake (an edible mushroom) .", XP002658831 , Database accession no. NLM10601791 abstract & CARIES RESEARCH 2000 JAN-FEB LNKD-PUBMED: 10601791 , vol . 34, no. 1, January 2000 (2000-01) , pages 94-98, ISSN: 0008-6568	1-3 , 15-17 , 19-27

X	DATABASE WPI Week 200273 Thomson Scientific, London , GB; AN 2002-679383 XP002658832 , & KR 2002 0034259 A (Y00N J W) 9 May 2002 (2002-05-09)	1-4, 15-17 , 19-27 ,30
A	abstract	8,9

X	WO 2006/027248 A2 (HENKEL KGAA [DE] ; GERKE THOMAS [DE] ; JANSSEN FRANK [DE] ; WEIDENHAUPT R) 16 March 2006 (2006-03-16)	1-3 , 15-17 , 19-27
A		10, 18,31

A	EP 2 017 290 Al (CASTER [FR]) 21 January 2009 (2009-01-21) the whole document	1-7 , 15-27 ,29

A	EP 1 393 734 Al (AJIN0M0T0 KK [JP]) 3 March 2004 (2004-03-03) the whole document	1-7 , 15-27 ,29

A	DATABASE CAPLUS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; Aoyagi : "Studies on low-molecular peptides of dried shiitake (Lentinus edodes) ", XP002658833 , retrieved from STN Database accession no. 1984:422195 abstract	1-7 ,29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2011/000969

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

5-7, 29 (completely); 1-4, 15-27 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5-7, 29(completely) ; 1-4, 15-27(partially)

   A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of Shiitake mushroom, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of an extract of Shiitake mushroom in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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2. claims: 8, 9, 30(completely) ; 1-4, 15-27(partially)

   A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of chicory, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of an extract of chicory in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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3. claims: 10, 31(completely) ; 1-4, 15-27(partially)

   A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of raspberry, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of an extract of raspberry in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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4. claims: 5-9, 29, 30(completely) ; 1-4, 15-27(partially)

   A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of Shiitake mushroom and one or more extract of chicory, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of a combination of an extract of Shiitake mushroom and an extract of chicory in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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5. claims: 5-7, 10, 29, 31(completely) ; 1-4, 15-27(partially)

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A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of Shiitake mushroom and one or more extract of raspberry, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of a combination of an extract of Shiitake mushroom and an extract of raspberyy in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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6. claims: 5-10, 29-31 (completely) ; 1-4, 15-27 (partially)

A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of Shiitake mushroom, one or more extract of chicory and one or more extract of raspberry, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of a combination of an extract of Shiitake mushroom, an extract of chicory and an extract of raspberry in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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7. claims: 8-10, 30, 31(completely) ; 1-4, 15-27 (partially)

A composition for promoting oral health, which composition is a functional food or oral preparation which comprises one or more extracts of raspberry and one or more extract of chicory, activities of the composition relevant to the promotion of oral care, a method of promoting oral health with the composition, use of a combination of an extract of raspberry and an extract of chicory in the manufacture of a composition to promote oral health, a method of promoting oral health and a process for the manufacture of the composition.

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8. claims: 11, 13 (completely) ; 14-22, 26, 27 (partially)

A composition for promoting oral health, which composition is a functional food or oral preparation which has been supplemented with a compound selected from the list consisting of: quinic acid; adenosine; inosine; trans-aconitic acid; oxalic acid; adenosine; cis-aconitic acid and succinic acid, or a salt thereof.

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9. claims: 12 (completely) ; 14-22, 26, 27 (partially)

A composition for promoting oral health, which composition

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is a functional food or oral preparation which has been supplemented with a compound selected from the list consisting of: shikimic acid; uridine; epicatechin, or a salt thereof.

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10. claim: 28

A method for assessing the potential oral health benefits of a plant or fungus, the method comprising assessing the level of a compound in the plant or fungus, wherein the compound is selected from the list consisting of: quinic acid; adenosine; inosine; transaconitic acid; oxalic acid; adenosine; cis-aconitic acid; succinic acid; shikimic acid; uridine; epicatechin.

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Information on patent family members

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