

METHOD AND COMPOSITIONS FOR IMPROVING SCALP HEALTH

Field of the Invention

The present invention relates to the use of a composition for controlling scalp malodour by selectively modulating the growth of commensal and odour-causing scalp bacteria.

- 5 More particularly, the composition comprises luteolin, for example, obtained from or contained within an extract of *Citrus reticulata* (tangerine) peel. The present invention also provides methods of improving scalp health, especially for the population of Hijab wearers.

10 **Background Description**

Individuals who wear a hijab for cultural and/or religious reasons are often exposed to long hours of an enclosed scalp environment and may develop characteristic malodour which is exacerbated under hot and humid conditions. Currently, the introduction of anti-microbial compositions on the scalp for the treatment of odour are non-selective in

15 killing both odour causing bacteria and normal scalp commensal bacteria.

- Consequently, known anti-microbial compositions may disturb the scalp microbiome profile required for healthy scalp maintenance. In addition, scalp microbiota imbalance can trigger scalp inflammation and consequently impact hair cycle, which is commonly associated with chronic inflammatory scalp disorders. Current odour masking
- 20 technology does not address the root cause of the problem, and specific odour molecules are yet to be characterized for the hijab wearing population. Therefore, there is a need to identify a solution that can control the scalp microbiome balance to prevent the possible onset of scalp disorders (including malodour, dandruff, sensitive scalp) by modulating pathogenic strains while maintaining normal commensal for overall scalp
- 25 health.

Niacinamide and its derivatives have been disclosed (WO 2019/086327) to act as microbiome balancing agents when applied to the external surface of the human body by selectively reducing the harm-causing microbes whilst keeping the beneficial microbes at the same level or having minimal effect on them. However, WO

2019/086327 focuses on the reduction of at least one of harmful microorganisms such as *Staphylococcus hominis*, *Staphylococcus aureus* and *Staphylococcus capitis*, noting only small detrimental effects on non-harmful bacteria *Staphylococcus epidermidis*.

WO 2019/086327 refers to *S. epidermidis* as a beneficial microbe without recognising its significance in odour production.

Plant extracts are known to have beneficial effects in skin and scalp care. Several approaches have disclosed the use of herbal combinations for modulating various conditions. US 2007/0036742/A1 discloses a composition that can be used to stimulate increased hair growth and/or prevent or slow the loss of hair. It comprises numerous extracts e.g. *Undaria pinnatifida* (brown algae extract), *boswellia serrata* extract (frankincense) and extracts of *Camellia sinensis* leaf, and *Perilla ocymoides* leaf extract. US 2017/0049687/A1 concerns the use of Chinese herbal combinations for treating scalp conditions including psoriasis, eczema, dandruff and melanoma, comprising Da Huang, Sheng Di Huang and Jin Yin Hua and combinations thereof, as well as certain herbal extracts and/or certain molecular components of certain herbs. Both herbal combinations include extracts which may comprise luteolin as well as many other components.

Plants with high luteolin content have been used traditionally to treat inflammation-related disorders and is well-studied for its anti-cancer properties. Studies have also shown luteolin to possess *in vitro* and *in vivo* neuroprotective effects, together with antioxidant and neuromodulatory properties.

The present invention is predicated, at least in part, on the discovery that luteolin (2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-chromenone) acts as a microbiome-balancing agent that selectively promotes the growth of beneficial bacteria *Delftia. tsuruhatensis* whilst concomitantly suppressing odour causing *S. epidermidis* in the hijab wearing population.

Summary of Invention

In one aspect, the present invention provides a composition comprising luteolin or a derivative thereof, for use in promoting scalp microbiome balancing in a subject.

Figure 2

(A)

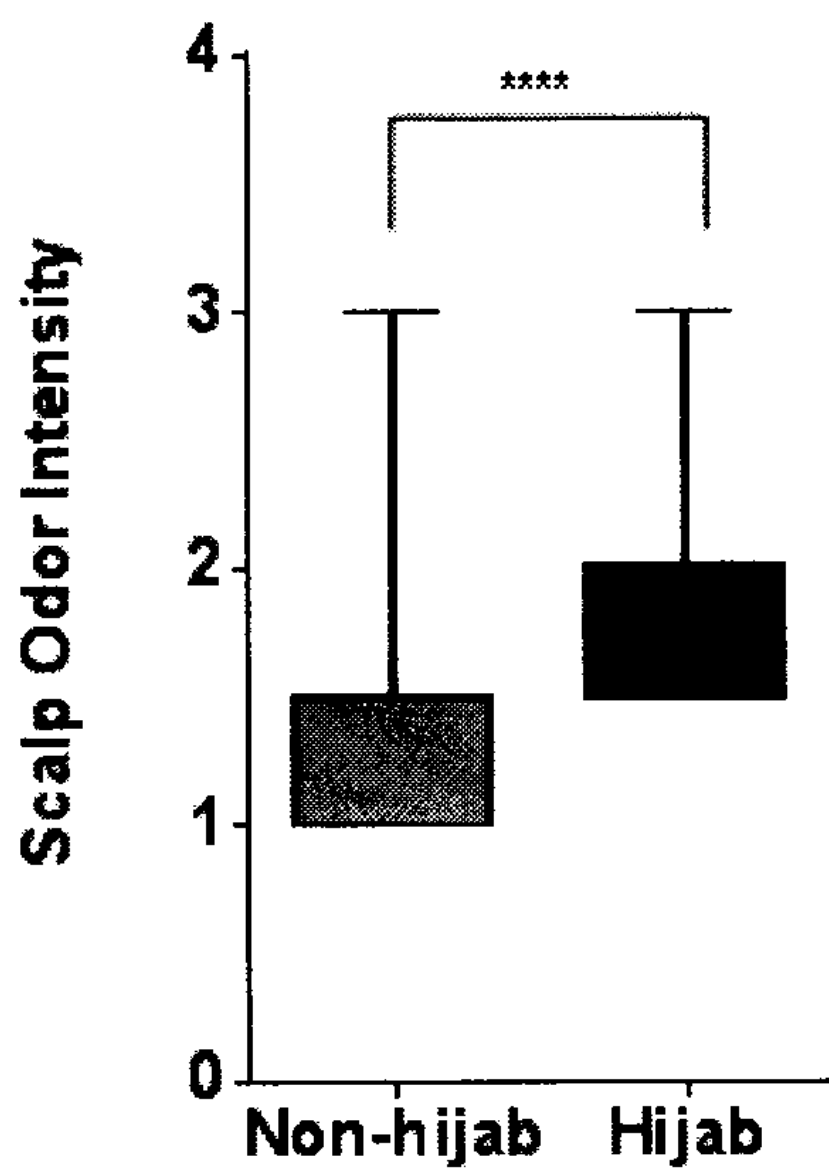


Figure 6 provides a graphical representation of the effects of luteolin on the growth rate of selected microbes. Data expressed in mean $\pm$ S.E.M. of two independent experiments (n=2). **p<0.01, ****p<0.001 is significantly different when compared with the respective control group.

- 5 **Figure 7** provides a graphical representation of the effects of 0.008% w/w luteolin on the growth rate of *D. tsuruhatensis* and *S. epidermidis* co-culture in terms of number of bacteria (A) and ratio of *D. tsuruhatensis* versus *S. epidermidis* (B). Data expressed in mean $\pm$ S.E.M. of three independent experiments (n=3). ****p<0.0001 significant compared with the control group in respective time point.

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Detailed Description of the Invention/Embodiments

Definitions

It is to be understood that this invention is not limited to any particular methodology or protocols described herein. Further, unless defined otherwise, all technical and
15 scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. It is also to be understood that the terminology used herein is for describing particular embodiments only and is not intended to limit the scope of the present invention, which will be limited only by the claims.

- 20 In the specification, except where the context requires otherwise due to express language or necessary implication, the word “comprise” or variations such as “comprises” or “comprising” is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention. In other words, the listed steps or options need
25 not be exhaustive.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Malaysia or any other country.

Unless otherwise indicated, the present invention employs conventional chemistry, molecular biology and microbiology techniques within the capacity of those skilled in the art. Such techniques are well known to the skilled worker and are explained fully in the literature.

- 5 Where a range of values are expressed, it will be clearly understood that this range encompasses the upper and lower limits of the range, and all values in between these limits.

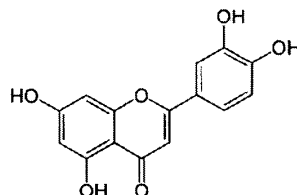
As used herein, the singular forms “a”, “an”, and “the” include the corresponding plural reference unless the context clearly dictates otherwise. Thus, for example, a reference
10 to “luteolin or a derivative thereof”, includes a mixture of more than one compound within this class.

Compositions

The present invention relates to compositions and methods for promoting microbiome balancing via modulation of the scalp microbiome profile. Specifically, the present
15 invention provides the use of luteolin or a derivative thereof, to promote scalp microbiome balancing via modulation of the scalp microbiome profile. In a first aspect, the present invention provides topical composition comprising luteolin or a derivative thereof, wherein the luteolin is present in an amount of from 0.0001% to 0.02% by weight of the composition. In some embodiments, the luteolin or derivative thereof is
20 luteolin. In some embodiments, the luteolin is substantially pure. In other embodiments, the luteolin is present in a natural extract, such as a *Citrus reticulata* (tangerine) extract. In particular embodiments, the luteolin modulates the growth of *D. tsuruhatensis*, a commensal or beneficial bacterium found on the scalp.

Luteolin has the structure as given below:

25



Luteolin is present in several plant products, for example, luteolin is available as extracts from *Citrus reticulata* (tangerine peel) and *Arachis hypogaea* L (peanut shell). Commercial sources are available, for example, from MMP, France (Trade name: Luteolin 98MM; extract: *Citrus reticulata*), Bio-India Biologicals Corporation, China (Trade name: Citrus reticulata; extract: *Citrus reticulata*), Kingherbs, China (Trade name: Luteolin; extract: *Arachis hypogaea* L), Shanxi Guanjie Biotechnology, China (Trade name: Luteolin; extract: *Arachis hypogaea* L). Alternatively, the luteolin or extracts containing luteolin used in the compositions of the present invention may be obtained using any known extraction methods. As used herein, and “extract” is a substance made by extracting a part of a raw material, such as a plant. Those of skill in the art will appreciate that there are many other extraction processes, both known in the art and described in various patents and publications that can be used to obtain the extracts to be used in practicing the present invention. For example, the extraction procedures described in the following references, which are incorporated herein by reference, could be used in practicing the present invention: Wang et al., “Extraction and Chromatography-Mass Spectromic Analysis of the Active Principles from Selected Chinese Herbs and Other Medicinal Plants. 2003. *Am. J. Chin. Med.* 31(6):927–44; Murga et al., “Extraction of natural complex phenols and tannins from grape seeds by using Supercritical mixtures of carbon dioxide and alcohol. *J. Agric Food Chem.* 2000 Aug:48(8):3408-12; Hong et al., “Microwave-assisted extraction of phenolic compounds from grape seed. *Nat Prod Lett.* 2001; 15(3):197-204; Ashraf-Khorassani et al., “Sequential fractionation of grape seeds into oils, polyphenols, and procyanidins via a single system employing CO-based fluids.” *J. Agric Food Chem.*, 2004 May 5: 52(9):2440-4.

The derivatives of luteolin encompassed by the present invention include but are not limited to derivatives of luteolin in which the double bond is saturated, and/or the compound is substituted such that one or more hydrogen atoms is replaced with one or more substituents selected from alkyl, alkenyl, alkynyl, aryl, halo, haloalkyl, haloalkenyl, haloalkynyl, haloaryl, hydroxy, alkoxy, alkenyloxy, aryloxy, benzyloxy, haloalkoxy, haloalkenyloxy, haloaryloxy, nitro, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroaryl, nitroheterocyclyl, amino, alkylamino, dialkylamino, alkenylamino,

alkynylamino, arylamino, diarylamino, benzylamino, dibenzylamino, acyl, alkenylacyl, alkynylacyl, arylacyl, acylamino, diacylamino, acyloxy, alkylsulphonyloxy, arylsulphenyloxy, heterocyclyl, heterocycloxy, heterocyclamino, haloheterocyclyl, alkylsulphenyl, arylsulphenyl, carboalkoxy, carboaryloxy, mercapto, alkylthio, benzylthio, acylthio, and phosphorus-containing groups. Other derivatives include but are not limited to glucoside variants in which sugars are appended (via a glycosidic linkage) to the phenolic oxygens. The derivative of luteolin may be a structural analogue of luteolin.

In some embodiments, the luteolin may be used in “substantially pure form”. This means that luteolin or a derivative thereof, is used in a form that is substantially free of the other principal components of the plant from which it is obtained, such as *Citrus reticulata*. In this context, substantial means that the luteolin used in the composition contains no more than 20% w/w of the non-luteolin components of said plant, or no more than 15% w/w, no more than 10 % w/w, no more than 5% w/w of the other principal components of said plant. In some embodiments, the substantially pure form means at least 80% w/w luteolin, at least 85% w/w luteolin, at least 90% w/w luteolin, at least 95% w/w luteolin, at least 96% w/w luteolin, at least 97% w/w luteolin, at least 98% w/w luteolin, at least 99% w/w luteolin, at least 100% w/w luteolin. Thus, this level covers pure luteolin extracts, and extracts retaining small levels of impurities from the plant from which it is obtained, such as *Citrus reticulata*. In other embodiments, the luteolin or derivative thereof may be produced synthetically (Zhang et al, J. Chemical Research, 2014: doi.org/10.3184%2F174751914X13867643876192), in which case it would not be expected that any of the non-luteolin components of a plant extract would be present.

In some embodiments, the composition comprises luteolin or a derivative thereof, in an amount in the range of from 0.0001-0.02 %, especially 0.0005-0.02 %, more especially 0.0005-0.016 %, and most especially 0.001 – 0.016% by weight of the composition.

The compositions of the present invention are compositions for topical use. Suitable compositions include leave-on scalp lotions, serums, liquids and creams in the forms of oil/silicone in water emulsion, water in oil/silicone emulsion and multiple emulsions,

shampoos, conditioners, hair tonics, ointments, oil-in-water emulsions, water-in-oil emulsions, sprays, pastes, mousses, foams, gels, liposomes, or other topically suitable forms, in particular embodiments, the composition may be a leave-on (scalp care) and rinse-off (hair shampoo, hair conditioner and hair tonic).

- 5 The compositions of the present invention additionally may contain various known and conventional additives so long as they do not detrimentally affect the microbiome-balancing (malodour reduction) capabilities provided by the compositions of the present invention. The CTFA Cosmetic Ingredient Handbook, Second Edition, 1992 describes a wide variety of non-limiting cosmetic and pharmaceutical ingredients commonly used
- 10 in the skin care industry, which are suitable for use in the compositions of the present invention. Examples of suitable formulation ingredients include: antioxidants, binders, biological additives, buffering agents, colorants, thickeners, polymers, astringents, fragrance, humectants, opacifying agents, conditioners, exfoliating agents, pH adjusters, preservatives, rheological modifiers, foaming agents, surfactants, emollients, natural
- 15 extracts, essential oils, skin sensates, scalp soothing agents, and scalp healing agents.

Exemplary preservatives include ethylhexylglycerine and phenoxyethanol. Exemplary emollients include C₁₂₋₁₅ alkyl benzoate, PPG-3 myristyl ether, isopropyl lauroyl sarcosinate. An exemplary humectant is 1,2-hexane diol. An exemplary rheological modifier includes hydroxyethyl acrylate, sodium acryloyldimethyl taurate copolymer,

20 or mixtures thereof.

In the case where the composition is in the form of a spray, the carrier is suitably water, and the composition may further comprise preservative such as benzyl alcohol (for example, in an amount of from 0.1 – 5 wt%), cosolvents such as propylene glycol (for example, in an amount of from 1 – 20 wt%), and surfactants such as PEG-40

25 hydrogenated castor oil (for example, in an amount of from 1 – 20 wt%).

In the case where the composition is in the form of a lotion, it may further comprise water in the presence of a suitable emulsifying agent. Examples of suitable emulsifying agents are cetareth-20, PEG-100 stearate, glyceryl stearate, sodium cetearyl sulfate. The composition may further comprise emollients in the form of esters and/or

30 hydrocarbons and/or silicones and/or plant oils and fragrances, preservatives (such as

benzyl alcohol at levels of, for instance, 0.1 – 10%) and humectant and/or excipients (such as glycerol, for instance at levels of 0.5 – 10% and cyclomethicone at levels of between 0.1 – 20%).

When the composition is in the form of an ointment, it may further comprise a
5 conventional ointment base to which the active ingredient is added. The ointment base may be a paraffin, such as soft paraffin, or a combination of soft and liquid paraffin. Other ointment bases may also be used such as polyalkylene glycol (such as polyethylene or polypropylene glycol) base. Other possible components of an ointment composition include emulsifying wax (for example, in an amount of between 1 – 40%,
10 preferably 5 – 40%), and optionally preservative.

When the composition is in the form of a liquid soap, any known liquid soap may be used. A preferred liquid soap with key composition saponified fatty acids derived from palm kernel oil or coconut oil. The pH of the liquid soap base may be adjusted to that which is suitable for topical use.

15 When the composition is an anhydrous cream, any suitable cream base may be used. A preferred cream base is petrolatum cream and/or natural plant oils and waxes with introduction of consistency factors.

When the composition is a shampoo or conditioner, it may further comprise one or more of a surfactant, a thickening agent, a pH adjuster/buffer, an aesthetic additive,
20 water, a conditioner, a preservative and moisturizers/vitamins in addition to the active ingredients. Shampoos may also include other active components known to those skilled in the art as having some advantage when included in a shampoo formula or in a medicinal combination for treating a skin condition such as psoriasis, eczema, dermatitis or hair loss or another head or scalp disorder or ailment, such as scalp
25 malodour.

A shampoo may comprise a primary surfactant to provide flash foam for cleaning the hair by removing dirt and other impurities. A secondary surfactant may be included to provide stable foam and to reduce the harshness of the primary surfactant. A surfactant may be used that includes a charged, hydrophilic head group and a long, hydrophobic

alkyl chain tail. Surfactants are configured to reduce surface tension of an interphase between dirt and hair and allowing the dirt to be transported into an aqueous medium to be rinsed free from the hair and scalp. Examples of surfactants that may be contained in a shampoo in accordance with certain embodiments include sodium laureth sulphate, ammonium laureth sulfate, and sodium cocoyl isethionate. Examples of co-surfactants include cocamide MEA and cocoamidopropyl betaine. Suitable thickening or suspending agents include carbomer and PEG 150 distearate. The thickening agent may be included to stabilize the shampoo during storage and/or to prevent the settling or dumping of pigments and silicone. Suitable pH adjusters or buffers include citric acid, tartaric and sodium hydroxide. The pH adjuster or buffer is configured to cause the shampoo to be gentle to the skin. A lower pH may cause hair to be compact and to shine and to protect the surfactant from hydrolysis, and as such, the pH adjuster or buffer may serve to lower the pH of the shampoo. However, alternative embodiments include pH adjusters that serve to raise the pH of a shampoo that contains an herbal formula that exhibits an exceptionally low pH. Suitable aesthetic additives include colorants, opacifiers, UV absorbers, perfumes and natural and artificial fragrances. Suitable conditioners include cationic polymers such as guar hydroxypropyltrimonium chloride, or silicone and/or a silicone emulsion such as dimethiconol, dimethicone, or amodimethicone. The silicone and/or silicone emulsion may serve to coat the hair and cause the hair to become soft, smooth and shiny. Examples of suitable moisturizers and/or vitamins include D-panthenol, vitamin E acetate, Sodium PCA, glycerine and one or more amino acids or combinations thereof.

In some embodiments, the shampoo or conditioner may be in the form of a hair serum and may be configured as a concentrated product that is typically left on the hair for a more extended duration than an ordinary shampoo with a typical shower routine. The hair serum may be configured to be light and non-sticky on the scalp and as a non-irritant, to be light and non-sticky on the hair, to have little or no effect on hair volume, to strengthen scalp, to provide keratinization and collagen synthesis, to promote hair growth or to control hair fall, or combinations thereof. A hair serum in accordance with certain embodiments may include water, alcohol, humectant, solubilizer, water-based

polymer, scalp conditioner, caffeine and panthenol. The ratio of alcohol, water and solubilizer may be adjusted depending on the solubilization.

Methods

- 5 In one aspect, the present invention provides a composition comprising luteolin or a derivative thereof, for use in promoting scalp microbiome balancing in a subject. In another aspect there is provided a method of promoting scalp microbiome balancing in a subject comprising applying to the scalp of the subject luteolin or a derivative thereof. In yet a further aspect, the present invention provides a use of luteolin or a derivative
10 thereof in the manufacture of a medicament for promoting scalp microbiome balancing.

The present invention provides methods of microbiome balancing and/or malodour reduction, by preventing, inhibiting and/or promoting the growth of microorganisms, specifically, odour-causing and commensal microorganisms. As used herein “preventing” means to kill the microorganism. In contrast, “inhibiting” means to
15 decrease, or limit the growth of the microorganism. Further, “promoting” means to increase or encourage the growth of the microorganism. As used herein, “growth” means an increase in the size and/or numbers of a microorganism. The growth of a microorganism may be determined by any method known in the art, including both direct and indirect methods.

- 20 A “microorganism” or “microbe” is any organism of microscopic size. Microorganisms include bacteria, viruses, fungi such as yeasts and moulds, algae, and the like.

“Bacteria” are single-celled organisms which are enclosed by a cell wall and whose genetic material is not enclosed in a nuclear membrane. Bacteria may be Gram-positive or Gram-negative, aerobic, anaerobic, or facultative anaerobes, and may have one of
25 several shapes, such as rod-like (bacillus), spherical or ovoid (coccus), corkscrew (spiral), star shaped or square shaped. Individual bacteria may form pairs, chains, clusters or other groupings. Examples of bacteria whose growth may be prevented or inhibited according to the invention include, but are not limited to: *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus hominis*, *Staphylococcus capitis*,
30 *Micrococcus luteus*, *Micrococcus tetragenus* *verstiles*, *Micrococcus sedentarius*,

Corynebacterium reigelii, *Corynebacterium striatum*, *Corynebacterium jeikeium*, *Corynebacterium kroppenstedtii* and *Corynebacterium xerosis*. Examples of bacteria whose growth may be promoted include but are not limited to *Delftia* species such as *Delftia tsuruhatensis*, *Delftia acidovarans*, *Delftia lacustris* and *Delftia litopenaei*.

- 5 The composition used in the methods and uses of the present invention is a composition as described above.

The phrase “microbiome balancing” used herein means selectively eliminating/killing/reducing the harm causing microbes existing on the scalp whereas keeping the beneficial microbes substantially at the same level or has minimal/less
10 effect on them.

In some embodiments, the methods and uses of the invention balance scalp microbiome for malodour reduction. In other embodiments, balancing the scalp microbiome treats or prevents dandruff, hair loss, hair thinning, sensitive scalp, irritated scalp, atopic dermatitis or seborrheic dermatitis. In particular embodiments, balancing the scalp
15 microbiome reduces malodour.

The composition is a topical composition and is applied on an external surface of the subject. In particular embodiments, the external surface is the scalp of a subject.

Generally, the compositions of the present invention are topically administered at least on a daily, and preferably a twice daily, basis for a period of time sufficient to bring
20 about the desired level of improvement in the scalp, such as malodour reduction. Alternatively, the compositions may be applied, once, twice, three or four times a week. For example, a user may topically administer a composition of the present invention directly to the scalp where decreased malodour is desired by gently massaging the composition of the present invention into the desired area. This process may be repeated
25 later the same day. In some embodiments, the composition of the present invention is left on the scalp or other area where decreased malodour and/or increased hair growth is desired between applications occurring on the same day.

Topical application of the compositions of the invention may continue for any suitable period of time. For example, within a few days to a few weeks of the initial application,

a user may notice a reduction in malodour, improved hair growth and health, reduced irritation and the like. It should be appreciated that the frequency with which the compositions of the present invention should be applied will vary depending on the desired effect. In some embodiments, the degree of therapeutic and/or cosmetic
5 enhancement will vary directly with the total amount of composition used.

When topically applied, the compositions of the present invention may be applied to the area to be treated, for example, the scalp in humans, by spraying, dabbing, swabbing, rubbing or massaging. For example, in one embodiment, a composition of the present invention may be provided in a shampoo, conditioner, or other leave-on or rinse-off hair
10 product formulation, which preferably may be applied on at least a daily basis.

The “subject” may be a mammal. In particular embodiments, the subject is human.

In some embodiments of the present invention, the scalp of the subject may be enclosed for extended periods, for example where the subject uses a headdress, or head covering, including but not limited to hijabs, wigs, burkas or keffiyehs for medical, cultural
15 and/or religious reasons.

The subject may reside in a hot and/or humid climate. As used herein, “hot and/or humid climate” may refer to any tropical, dry or temperate climate. In particular embodiments, the subject resides in a tropical climate.

In particular embodiments, the methods and uses of the present invention are for scalp
20 malodour reduction, particularly in a subject that has their scalp enclosed in a headdress or head covering for an extended period of time. As used herein, “malodour” refers to any foul or unpleasant smell. Malodour on a human subject can be caused by the reaction of skin biproducts e.g. sebum and sweat with some harmful microorganisms like *S. epidermidis*. Without wishing to be bound by theory, *S. epidermidis*, has been
25 characterised by GC/MS to secrete the odour molecules trimethylamine and azomethane upon metabolism of sweat and sebum. Thus, *S. epidermidis* is considered to be an “odour-causing” microbe.

In some embodiments, the present invention balances the scalp microbiome and/or reduces malodour by application of luteolin or its derivatives, optionally in the form of

a *Citrus reticulata* extract or in substantially pure form, by selectively reducing prominent odour-causing bacterium via the growth promotion of commensal scalp bacteria, thereby maintaining a high level of beneficial microorganisms on the surface, consequently improving overall scalp health and odour.

- 5 In some embodiments, the microbiome balancing is obtained by selectively reducing at least one microorganism selected from the group consisting of *Staphylococcus epidermidis*, *Corynebacterium rigeilli*, *Micrococcus lutues*, especially *S. epidermidis*. In some embodiments, the microbiome balancing is obtained by selectively promoting the growth of the beneficial microorganisms such as *Delftia* species, such as *D.*
- 10 *tsuruhatensis*, *Delftia acidovarans*, *Delftia lacustris* and *Delftia litopenaei*, such as *D. tsuruhatensis*. Without wishing to be bound by theory, *D. tsuruhatensis* has been shown to inhibit the growth of the prominent odour-causing bacteria *S. epidermidis*.

Examples

- 15 The present invention is further illustrated by the following experimental investigations and examples, which should not be construed as limiting. The contents of all references, patents and published applications cited throughout this patent are hereby incorporated by reference herein.

Example 1 - Evaluation of the scalp odour and signature microbiome profile of Hijab Wearer

Methodology

Scalp malodour assessment and microbial sampling

In this study, 72 females aged from 20 to 60 years old were sub-divided into participants with Hijab practice (24 panellists) or without Hijab practice (48 panellists).

- 25 All panellists were instructed not to shampoo their hair for at least 12 hours (but not exceeding 24 hours) prior scalp odour evaluation and scalp microbial sampling. Subjects were excluded from participation if they had scalp disorders including psoriasis and seborrheic dermatitis; pregnant or breastfeeding; visited a doctor/dermatologist/scalp and hair therapist to receive any treatment or medication for

hair and scalp ailments within the last six months; visited a saloon for dyeing, perming, re-bonding or receiving other hair and scalp treatment within the last six months. The scalp odour of each subject was assessed by two independent assessors with malodour severity rated based on scale from 1 to 5 and results expressed in mean score obtain
5 from both assessors. Besides, the hair thickness was visualized and quantitatively measured by a Aramo SC scalp analyzer (Aramhuvis, South Korea). In addition, the scalp microbial sampling on each panellist was conducted simultaneously and total genomic DNA was extracted from the swabs by using DNeasy PowerSoil DNA Isolation Kit (Qiagen, USA) according to manufacturer's instructions.

10 *Next generation sequencing*

Primers 341F (5'- CCTAYGGGRBGCASCAG-3') and 806R (5'- GGACTACNNGGTATCTAAT-3') were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene from bacteria using PCR. Sequencing libraries were generated using NEB Next Ultra DNA Library Pre ® Kit for Illumina, following
15 manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific, USA) and Agilent Bioanalyzer 2100 system. Lastly, the library was sequenced on an Illumina platform and 250 bp paired-end reads were generated. Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer
20 sequence. Paired-end reads were merged using FLASH (V1.2.7) (Magoč & Salzberg, 2011) to produce raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich *et al.*, 2013) according to the QIIME (V1.7.0) quality controlled process (Caporaso *et al.*, 2010). The tags were compared with the reference database (Gold database) using UCHIME
25 algorithm (Edgar *et al.*, 2011) to detect chimera sequences, and then the chimera sequences were removed to obtain the effective tags (Haas *et al.*, 2011).

Taxonomic assignment

Bacterial taxonomic assignment was performed using Uparse software (v7.0.1001) (Edgar, 2013). Sequences with $\geq 97\%$ similarity were assigned to the same Operational
30 Taxonomical Units (OTUs) and representative sequence for each OTU was screened for

further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database (Wang, *et al.*, 2007) for species annotation at each taxonomic rank (Threshold:0.8~1).(kingdom, phylum, class, order, family, genus, species) (Quast *et al.*, 2013). In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignments were conducted using the MUSCLE software (Version 3.8.31) (Edgar, 2004). OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed basing on this output normalized data.

Next Generation Sequencing Analysis

Alpha diversity is applied in analyzing complexity of species diversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, ACE, Good-coverage. All indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity analysis was used to evaluate differences of samples in species complexity, Beta diversity on both weighted and unweighted UniFrac were calculated by QIIME software (Version 1.7.0). Cluster analysis was preceded by principal component analysis (PCA), which was applied to reduce the dimension of the original variables using the FactoMineR package and ggplot2 package in R software (Version 2.15.3). Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of weighted or unweighted UniFrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (Version 2.15.3). Unweighted Pair-group Method with Arithmetic Means (UPGMA) Clustering was performed as a type of hierarchical clustering method to interpret the distance matrix using average linkage and was conducted by QIIME software (Version 1.7.0). Linear discriminant analysis (LDA) effect size tool- LEfSe was used to identify OTUs with differential relative abundance comparing between Hijab wearers and non-

hijab wearers (Segata et al., 2011). LDA score was calculated for OTUs with $p \leq 0.05$ and LDA score threshold ≥ 4.00 .

Results and Discussion

5 *Unique microbiome profile in Hijab wearer in association with scalp malodour and hair thinning*

Alpha diversity of the samples was measured by observed species, Chao1 index and the Shannon diversity index. The observed species index measures the number of different species per sample while the Chao1 index considers the ratio of singletons to
10 doubletons, thus giving more weight to rare species. Based on the observed species and Chao1 index, it can be observed that Hijab wearer had a significantly higher number of different bacteria species observed than non-Hijab wearer (Figure 1A and 1B). In addition, Shannon diversity index in which factors in both OTU richness and evenness showed that Hijab wearer had a higher bacterial diversity than Non-hijab wearer (Figure
15 1C). In association with clinical measurement, it was observed that the scalp odour for Hijab wearer group is significantly stronger than non-Hijab wearer group (Figure 2). In addition, the average hair thickness is significant thinner found in Hijab wearer group when compared to non-Hijab wearer group. Taken together, the Hijab wearer was proven to have stronger scalp malodour and thinner hair which are associated with its
20 unique scalp microbiome profile and higher bacteria diversity.

Identification of odour causing bacteria

The analyses have unravelled the different bacterial population abundance with respect to scalp malodour. As shown in Figure 3, three bacteria were identified that are associated with the increase of scalp malodour, including *Micrococcus luteus*,
25 *Corynebacterium riegelii*, *Staphylococcus epidermidis*.

Identification of Delftia tsuruhatensis as an odour controlling bacteria

As shown in Figure 4A, low abundancy of *Delftia tsuruhatensis* in the panellist with a high scalp odour score. Furthermore, Figure 4B demonstrates that *D. tsuruhatensis* is negatively correlated with *S. epidermidis* which was identified as a prominent
30 malodour-causing bacteria strain. This behaviour is further supported by previous work

that *D. tsuruthansis* possesses anti-microbial activity via synthesis of antibiotic compound ‘amino(5-(4-methoxyphenyl)-2-methyl-2-(thiophen-2-yl)-2,3-dihydrofuran-3-yl)methanol’ (AMTM) and anti-quorum sensing compound (1,2-benzenedicarboxylic acid) to prevent biofilm formation of other bacteria (Han *et al.*, 2005, Prasannakumar *et al.*, 2015, Singh *et al.*, 2017). Therefore, it was hypothesized that *D. tsuruhatensis* may exhibit a bio-monitoring role to control the growth rate of *S. epidermidis* which is a prominent bacterium that generates scalp malodour.

Conclusion

The Hijab wearer group was proven to have stronger scalp malodour and thinner hair associated with its unique scalp microbiome profile when compared to the non-Hijab wearing group. Three bacteria were identified as potential scalp malodour bacteria strains, including *M. luteus*, *C. riegelii* and *S. epidermidis*. *D. tsuruhatensis* was identified as a potent scalp commensal to control other pathogenic strains, more specifically on *S. epidermidis*.

Example 2: Characterization of the scalp malodour molecule

Methodology

Preparation of artificial premix of sweat and/or sebum in TAE buffer

First, 0.8 mL of Tris-acetate-EDTA (TAE, SIGMA) buffer was added into 50 mL of artificial sweat (Pickering Laboratories Inc, USA) and mixed by vortex. This mixture was then topped to 100 mL with artificial sweat and mixed well. To sterilize sweat-TAE solution, the solution was filtered through a 0.22 µm membrane filter. In a sterile environment, 0.25 g of artificial sebum (Pickering Laboratories Inc, USA) was aliquoted into a sterile 50 mL falcon tube. For artificial sebum and sweat, 50 mL of sweat-TAE solution was added to 0.25 g of artificial sebum, and vortexed vigorously for 30 min to make a homogenous sebum emulsion containing artificial sweat, 0.8% v/v TAE and 0.5% w/v artificial sebum.

Odour molecule identification of identified microbes

Briefly, *S. epidermidis* (ATCC 12228) and *M. luteus* (ATCC 272) were cultured in the Mueller Hinton Broth (MHB) media and standardized at 2×10^8 cells/mL. On the other hand, *C. riegl*i (ATCC 700782) was cultured in peptone water (PW) and standardized at 2×10^8 cells/mL. Then, 1 mL of respective bacteria suspension or respective culture media (as control) mixed with 1ml of artificial sweat containing TAE buffer (0.8% v/v) and sebum (0.5% v/v). Then, the mixtures were cultured with shaking condition of 150 rpm for 48h at 37 °C. Lastly, the cultured were subjected to volatile organic compound (VOC) analysis and malodour molecule were detected by using headspace Shimadzu Gas Chromatography-Mass Spectrometry (Kyoto, Japan) that equipped with Teledyne tekma model versa headspace injector. Lastly, the detected molecule identity was matched in similarity to NIST (National Institute of Standards and Technology) mass library.

Results and Discussion

As shown in Table 1, trimethylamine, azomethane and 2-pentanone were the major malodour molecules identified from three cultured microbes after being normalized with the respective control of culture media. Specifically, trimethylamine and azomethane could be produced by all tested strains with different intensities, but 2-pentanone was produced by *M. luteus* only in the presence of sweat and sebum conditions.

Table 1 Odour characterization of volatile organic compounds (VOC) derived from the selected bacteria cultured in the presence of sweat and sebum by using Gas Chromatography-Mass Spectrometry (GC-MS). The results were expressed in peak area that are normalized by the respective culture media control.

No.	Sample ID	Condition	Peak Area		
			Trimethylamine	Azomethane	2-Penthanone
1	A1	<i>S. epidermidis</i> in MHB	858836	86553	-
2	A2	<i>M. lutes</i> in MHB	394185	491173	113258
3	A3	<i>C. riegalie</i> in PW	155312	74945	-
4	A4	Culture media control (MHB)	-	-	-
5	A5	Culture media control (PW)	-	-	-

5

Conclusion

Trimethylamine was identified as the prominent odour molecule derived from the sweat component that is metabolized by *S. epidermidis*. 2-Penthanone was identified as the prominent odour molecule that was derived from sebum component that is metabolized by *M. lutes*. Azomethane was identified as the prominent odour molecule that can be derived from both sweat or sebum component that being metabolized by *M. lutes*.

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Example 3: Validation of the inhibitory role of *D. tsuruhatensis* on *S. epidermidis*.**Methodology:**

Modified Broth microdilution test for determination of anti-microbial property of culture filtrate

- 5 Briefly, cell suspension stock of *D. tsuruhatensis* culture (ATCC BAA-2377) was standardized to 10^8 cells/mL in MHB in total volume of 10 mL. Then, the bacteria culture was grown and incubated at 37 °C for 72 hours with shaking at 200 rpm. On the other hand, cell suspension stock of *S. epidermidis* (ATCC 12228) was standardized to 10^8 cells/mL in MHB media and adjusted to cell concentration at 10^9 cells/mL in total
- 10 volume of 100 μ L/well in 96 wells plates. Then, 100 μ L culture filtrate of *D. tsuruhatensis* was added into 96-well plate containing *S. epidermis* culture and incubated at 37 °C for 24 hours. Lastly, the growth of *S. epidermidis* is assessed by reading the suspended culture in 96-well plate at absorbance of 600 nm by using Microplate reader (Infinite M200, Tecan).

15 **Results and Discussion**

As shown in Figure 5, the growth rate of *S. epidermidis* was suppressed significantly by the conditioned media of *D. tsuruhatensis* culture when compared with the control media without *D. tsuruhatensis*.

Conclusion

- 20 *D. tsuruhatensis* as a scalp commensal bacteria strain was shown to significantly inhibit the growth of the scalp odour causing bacteria, specifically *S. epidermis*.

Example 4: Selective inhibitory role of Luteolin on odour-causing bacteria but not scalp commensal strain

25 **Methodology:**

Broth microdilution test for determination of anti-microbial property of Luteolin

S. epidermidis (ATCC 12228), *C. riebelli* (ATCC 700782) and *M. luteus* (ATCC 272) were cultured in the Cation-adjusted Mueller Hinton Broth (CAMHB) media at 37 °C under aerobic conditions. Meanwhile, Luteolin 98MM (MMP, France) with a purity of

≥ 98%, was pre-dissolved in ethanol and then in CAMHB media, and serially diluted at various concentrations in a 96-well plate at 100 µL/well. Then, 100 µL of bacterial cells (2×10^5 /well) were seeded into a 96-well plate containing the test compound and further incubated at 37 °C for 24 hours. Then, the plate was read at 600 nm to determine the growth inhibition rate of each bacteria in response to the treated compound.

Results and Discussion

As shown in Figure 6, quantitative evaluation of growth rate based on OD 600 revealed that Luteolin (*Citrus reticulata* peel extract) at dosage from 0.001% to 0.016%, selectively inhibited the growth of odor causing microbes (*Micoccus luteus*, *Staphylococcus epidermidis* and *corynebacterium reigeli*) but not scalp odor regulator strain (*Delftia tsuruhanensis*). This demonstrated the potential use of luteolin to control scalp odor by selectively modulating the odor causing bacteria and maintaining the scalp normal commensal for overall scalp healthiness of the Hijab wearer.

Conclusion

Luteolin (*Citrus reticulata* (tangerine peel)) extract demonstrates its potential modulatory effects on scalp microbial growth, in which selectively promotes the growth of *D. tsuruhanensis* as scalp normal commensal and concomitantly suppresses odour a causing microbe, specifically *S. epidermidis*.

Example 5: Modulating role of Luteolin on co-culture of odour causing bacteria and scalp commensal strain

Methodology

Cell suspension stock of *D. tsuruhanensis* (ATCC BAA-2377) and *S. epidermidis* (ATCC 12228) culture were standardized to 10^4 and 10^6 cells/mL in MHB. One mL of each culture at same cell concentration were mixed and added with 2 mL of peptone water (control group) or 2 mL of peptone water that pre-dissolved with 0.016% w/w Luteolin 98MM (MMP, France) with purity of luteolin ≥ 98% (treatment group). The mix cultures were incubated at 37 °C in a shaking incubator (150 rpm) for intervals of 8 hours, 16 hours and 24 hours. During the interval, the culture was harvested for cell

count by using hemocytometer and further differentiated based on cell morphology by microscopy observation. Morphologically, *D. tsuruhatensis* characterized as clumps of 2 to 3 of slightly elongated cells and *S. epidermidis* characterized as relatively small singular cells.

5 **Results and Discussion:**

To further understand the effects of luteolin on the competition growth of both microbes, a co-culture of *D. tsuruhatensis* and *S. epidermidis* was conducted at starting concentration of 10^6 bacteria. As shown in Figure 7, *D. tsuruhatensis* demonstrated trend of succession growth over *S. epidermidis* after 24 h of the co-culture incubation.

- 10 The treatment of 0.008 % w/w luteolin further promoted the growth of *D. tsuruhatensis* and concomitantly suppressed the growth of *S. epidermidis*. This observation further strengthened the hypothesis for modulation effects of *D. tsuruhatensis* in controlling the growth rate of *S. epidermidis*, whereby the modulating effects were significantly demonstrated after 16 h and 24 h of luteolin post-treatment.

15 **Conclusion**

- D. tsuruhatensis* as scalp commensal bacteria strain inhibits the growth of scalp odour causing bacteria, specifically *S. epidermis*. Luteolin (*Citrus reticulata* (tangerine) peel extract at 0.008% w/w demonstrates its potential modulatory effects on the scalp microbiome profile by promoting the growth of *D. tsuruhatensis* and concomitantly
- 20 suppressing the growth of odour causing microbe, specifically *S. epidermidis*.

Example 6: Application of luteolin in formulation composition

- Luteolin may be incorporated into formulation for hair topical application in both leave-on (scalp care) and rinsed-off (hair shampoo and hair conditioner), more specifically for hair tonic application. According to Table 2, the aqueous phase was prepared by
- 25 dissolving ethylhexylglycerin and phenoxyethanol into water. Then, the oil phase was prepared by dissolving 0.01 % w/w *Citrus reticulata* (tangerine) peel extract with isopropyl lauroyl sarcosinate and 1,2-hexanediol, followed by swelling of the oil mixture by using a hydroxyethyl acrylate/sodium acryloyldimethyl taurate copolymer. Lastly, addition of the oil phase into aqueous phase followed by homogenization at

2500 rpm for 15 minutes. Then, the bulk was transferred to an overhead stirrer and stirred at 600 rpm for 30 minutes.

Table 2 - Example of a formula composition containing luteolin

Phase	No	Ingredients	Function	%
A	1	Deionized water		Qs 100
	2	Ethyhexylglycerine	Preservative	0.06
	3	Phenoxyethanol	Preservative	0.54
B	4	Isopropyl lauroyl sarcosinate	Emollient	0.2
	5	1,2-Hexanediol	Humectant	0.1
	6	<i>Citrus reticulata</i> (tangerine) peel extract	Active ingredient	0.01
	7	Hydroxyethyl Acrylate/sodium Acryloyldimethyl Taurate copolymer	Rheological modifier	3
		Total		100

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